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Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
INVENTARIO

PCT

DELEGATURA DE PROPIEDAD INDUSTRIAL
DIVISIÓN DE NUEVAS CREACIONES

09-35922

SOLICITUD DE PATENTE DE
INVENCION

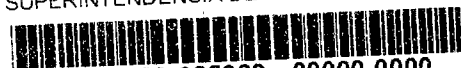
TITULO: PARTÍCULAS DE NÚCLEO DIVIDIDO PARA LA PRESENTACIÓN DE
MOLÉCULAS EXTRAÑAS, ESPECIALMENTE PARA APLICACIONES EN
VACUNAS, Y MÉTODOS PARA PRODUCIRLAS

SOLICITANTE: UNIVERSITÄTSKLINIKUM FREIBURG

DIRECCIÓN: Hugstetter Strs. 49, 79106 Freiburg

APODERADO: J. IAN RAISBECK

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SOLICITUD DE:

1

☒

Patente de Invención.

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Patente de Modelo de Utilidad.

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Capítulo I.

(Folio 266)

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Capítulo II.

2

SOLICITANTE
(71)

Nombre: UNIVERSITÄTSKLINIKUM
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Dirección: Hugstetter Str. 49, 79106 Freiburg

Nacionalidad o Domicilio: Alemana

Lugar de Constitución: Alemania

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Cual _____

Número _____

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Número 79.376.996 Btá
TP 135.799

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9

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10

ANEXOS

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Comprobante de pago de la tasa de presentación de la solicitud.

☐

Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica.

☐

Poderes, si fuere el caso. Adjunto copia simple. Ver Expediente No.

☐

Documento de cesión del inventor al solicitante o a su causante.

☐

Declaraciones.

☒

Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos).

☒

Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos).

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Copia del examen preliminar internacional efectuado por la Autoridad Internacional de Examen Preliminar (IPEA). (IPRP)

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Traducción del examen preliminar internacional efectuado por la IPEA.

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De ser el caso, copia del contrato de acceso.

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De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas.

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De ser el caso, certificado de depósito del material biológico.

☐

Arte final 12 x 12.

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De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.

☐

De ser el caso, modificaciones efectuadas a la solicitud internacional.

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Otros. Búsqueda Internacional.

11

FIGURA CARACTERÍSTICA:

12

Solicito la concesión de la patente,

NOMBRE: J. IAN RAISBECK

FIRMA:

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***** CONSIGNACION *****

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1		TRAMITES DE SOL. DE PATENTE DE IN	540,000.00
		TOTAL :	\$ 540,000.00

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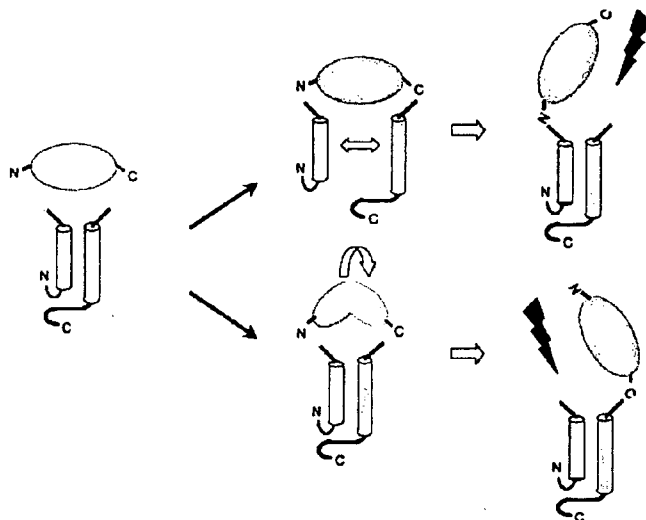
(81) Bestimmungsstaaten (soweit nicht anders angegeben, für
jede verfügbare nationale Schutzrechtsart): AE, AG, AL,
AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA,
CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE,
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[Fortsetzung auf der nächsten Seite]

(54) Title: SPLIT-CORE-PARTICLES FOR THE PRESENTATION OF FOREIGN MOLECULES, ESPECIALLY FOR VAC-
CINE APPLICATIONS, AND METHOD FOR THEIR PRODUCTION

(54) Bezeichnung: SPLIT-CORE-PARTIKEL FÜR DIE PRÄSENTATION VON FREMDMOLEKÜLEN, INSBESONDERE
FÜR IMPFSTOFFARTWENDUNGEN UND VERFAHREN ZU DEREN HERSTELLUNG



Prinzip des split-Core-Systems

Principle of the split-core system

(57) Abstract: Disclosed are split-core carrier substances which, as separate polypeptides, have the core N domain and the core C domain of the core protein of a hepatitis B virus and at least one foreign molecule against which an immune response is to be induced. According to the invention, the foreign molecule, especially the heterologous foreign amino acid sequence, is fused to the C terminus of the core N domain or to the N terminus of the core C domain and the core protein can form capsid-like particles. The invention also relates to the associated production method.

[Fortsetzung auf der nächsten Seite]

WO 2008/028535 A1



ZM, ZW), eurasisches (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht:

— mit internationalem Recherchenbericht

(57) Zusammenfassung: Offenbart werden Split-Core-Trägerstoffe, die als getrennte Polypeptide die Core N- und die Core C-Domäne des Core-Proteins eines Hepatitis B Virus und wenigstens ein Fremdmolekül, gegen das eine Immunantwort induziert werden soll, aufweisen, wobei das Fremdmolekül, insbesondere die heterologe Fremdaminosäuresequenz an den C-Terminus der Core N-Domäne oder an den N-Terminus der Core C-Domäne fusioniert sind und das Core-Protein capsid-like-particles bilden kann, sowie Verfahren zu deren Herstellung.

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**Split-Core-Partikel für die Präsentation von Fremdmolekülen, insbesondere für
Impfstoffanwendungen und Verfahren zu deren Herstellung**

Gegenstand der vorliegenden Erfindung sind neue Träger für Impfstoffe, aber auch für andere Moleküle, auf der Grundlage des Core-Antigens des Hepatitis B-Virus. In das Core-Antigen des Hepatitis-Virus werden fremde Aminosäuresequenzen eingebaut, die von Krankheitserregern stammen können. Gegen diese Proteinsequenzen sollen durch den Impfstoff Antikörper gebildet werden, wobei schützende oder neutralisierende Antikörper bevorzugt sind. Durch die erfindungsgemäßen Partikel kann auch die zelluläre Immunantwort (T-Zellen) stimuliert werden. Das Core-Protein von Hepatitis B hat die Eigenschaft, dass sich mehrere Kopien zu sogenannten capsid-like particles zusammenfügen können. Diese capsid-like particles (CLP) eignen sich besonders für die Herstellung von Impfstoffen, da sie das Immunsystem stimulieren und so zu einer gesteigerten Antikörperproduktion führen. Der Ausdruck "Impfstoff" ist in der vorliegenden Anmeldung bevorzugt als Trägersystem zu verstehen, das sowohl humorale wie auch zelluläre Immunantworten hervorrufen kann.

Kapside von Hepatitis B Viren sind ikosahedral symmetrische Nanopartikel (Durchmesser ca. 30 nm), die aus 180 bzw. 240 Kopien des viralen Core-Proteins bestehen. Das Core-Protein wird als HBcAg bezeichnet. Sie können als partikulärer Träger für Fremdmoleküle

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dienen; die bevorzugte Anwendung ist die als immunverstärkender Antigen-Träger für Impfstoffe. Durch geeignete Modifikation des Core-Proteins können die Fremdmoleküle auf der Partikel-Oberfläche (bei Bedarf auch im Inneren) präsentiert werden. Für die Oberflächenexposition ist die Verknüpfung des Fremdmoleküls mit zentral in der AS-Sequenz des Core-Proteins gelegenen AS-Resten (Bereich der AS ca. 73-94) optimal, welche das immundominante B-Zellepitop "c/e1" enthalten und in der 3D-Struktur am stärksten auf der Partikeloberfläche exponiert sind (AS bedeutet Aminosäure).

Die WO 01/77158 beschreibt Hepatitis B-Core-Antigen-Fusionsproteine, wobei heterologe Epitope bevorzugt in den Bereich zwischen den Positionen 61 und 90 eingesetzt werden. Die US 2003/0198649 beschreibt Hepatitis B-Virus Core-Antigen-Partikel, bei der die Immunogene über Ligandenstrukturen mit dem Hepatitis B-Virus (HBV) Core-Protein verbunden sind. Kratz et al. [PNAS (1999), 1915-1920] beschreiben die Präsentation von GFP (Green Fluorescent Protein) auf der Oberfläche des Coreproteins von HBV. Dabei wurden die Aminosäuren 79 und 80 des Core-Proteins durch die 238 Aminosäure lange Sequenz von GFP ersetzt.

Nassal et al. [Eur.J.Immunol. (2005), S. 655-665] beschreiben ein Fusionsprodukt des gesamten OspA-Proteins von *Borrelia burgdorferi* und des Hepatitis B-Virus-Kapsidproteins. Auch hier wurden die Aminosäuren 79 und 80 durch die Aminosäuren 18 bis 273 von OspA ersetzt, wobei allerdings zwischen Core-Protein und OspA Linkersequenzen eingebaut wurden. Skamel et al. (Journal of Biological Chemistry, 2006, Seiten 17474-17481) beschreiben die Präsentation des kompletten OspC-Proteins von *Borrelia burgdorferi* mit Hilfe von Hepatitis B-Virus-Kapsid-ähnlichen Partikeln. Bei den aus dem Stand der Technik bekannten Lösungen hat sich aber herausgestellt, dass die veränderten Coreproteine nicht mehr in gewünschter Weise zur Bildung von Kapsid-ähnlichen Partikeln (CLPs) neigen und, dass daher die Immunantwort nicht mehr zufriedenstellend stimuliert wird.

Bei einer genetischen Insertionen von Fremdmolekülen auf Peptid- bzw. Proteinbasis in das Core-Protein bedeutet dies, dass die Fremdsequenz sowohl über ihren N- wie ihren C-Terminus mit dem Core-Protein verbunden sind. Es wurde nun gefunden, dass die beidseitige Verknüpfung die Art und Auswahl geeigneter Fremdsequenzen drastisch einschränkt.

Das hier beschriebene "split-Core-System" hebt diese Einschränkungen auf, indem das Core-Protein in zwei separaten Teilen erzeugt wird, die sich überraschenderweise spontan zusammenfinden und vergleichbar der kontinuierlichen Proteinkette Kapsidpartikel bilden.

Fremdmoleküle können entweder an das N-terminale ("coreN") oder das C-terminale ("coreC") Fragment fusioniert werden und sind dadurch nur noch über *ein* Ende mit dem Trägerprotein verknüpft. Die strukturellen Einschränkungen der beidseitigen Verknüpfung bei der Insertion von Fremdsequenzen in die kontinuierliche Peptidkette des Core-Proteins fallen damit weitestgehend weg.

Das erfindungsgemäße Split-Core-System erlaubt daher:

- (i) Die Präsentation von Fremdmolekülen, die aufgrund ihrer Größe und/oder Struktur im konventionellen kontinuierlichen Core-Protein-Kontext nicht, oder nur sehr schlecht, präsentiert werden können;
- (ii) Die Präsentation von heterodimeren Fremdproteinen;
- (iii) Die Präsentation von interaktionsfähigen Fremdmolekülen in einer flexiblen, gut zugänglichen Form, welche die Interaktion mit den gewünschten Partnermolekülen wesentlich erleichtert;
- (iv) Die Präsentation zusätzlicher Fremdmoleküle über die im split-Core, nicht aber im herkömmlichen kontinuierlichen Core-System, vorhandenen zusätzlichen oberflächenexponierten N- und C-Termini.

Hepatitis B Viren (HBVs) sind umhüllte Viren. Das innere Nukleokapsid wird auch als Corepartikel ("core") bezeichnet; serologisch ist es als Hepatitis B core antigen (HBcAg) definiert. Cores bestehen aus 180 bzw. 240 Kopien des 183-185 AS langen Core-Proteins (abhängig vom HBV Subtyp). Das Core-Protein kann heterolog (in Bakterien, Hefe, eukaryonten Zellen) exprimiert werden; dabei bilden sich spontan "capsid-like particles" (=CLPs). Da sie weder das Virusgenom noch die äußere Hülle enthalten, sind sie nicht infektiös. Solche CLPs lassen sich auch von den näher verwandten Säuger-HBVs (z.B. Walddarmmeltier = woodchuck; Erdhörnchen = grounds squirrel; und weitere) sowie den entfernter verwandten Vogel-HBVs (z.B. Ente = duck; Reiher = heron; und weitere) erhalten. Grundsätzlich können erfindungsgemäß alle HBV Sequenzen eingesetzt werden, bevorzugt sind allerdings die Sequenzen von HBVs, die Menschen infizieren können.

Die Aminosäuresequenz bzw. Nucleotidsequenz des HBV-Core-Proteins ist bekannt [Galibert et al., Nature (1979), S. 646-650; Nassal, Gene (1988), Seiten 279-294 oder WO 01/77158], auf diese Veröffentlichungen wird Bezug genommen. Bevorzugt eingesetzt wird die Core-Protein-Sequenz des Subtyps ayw, es können aber auch Varianten, Modifikationen der HBV-Sequenz ebenso verwendet werden, wie die Sequenz von anderen Säuger-HBVs oder Vogel-HBVs. Die Sequenzen sind in den öffentlich zugänglichen Genbanken gespeichert.

Neben dem humanpathogenen Hepatitis B Virus (HBV im engen Sinn) gibt es eine Reihe verwandter, tier-spezifischer Hepatitis B Viren, z.B. das HBV des Nordamerikanischen Waldmurmeltiers (Englisch: woodchuck; zool. *Marmota monax*; woodchuck hepatitis B virus = WHV), das HBV des kalifornischen Erdhörnchens (Englisch: California ground squirrel; zool. *Spermophilus beecheyi*; ground squirrel hepatitis B virus = GSHV), sowie weitere.

Diese Viren besitzen eine ähnliche genetische Struktur wie das humane HBV, weisen aber distinkte Unterschiede in ihrer Nukleotid- und damit Aminosäure-Sequenz ihrer Proteine auf.

Die Verwendung von humanem HBV Core als Impfstoff-Träger unterliegt zwei möglichen Einschränkungen:

1. Personen mit einer chronischen HBV-Infektion sind partiell tolerant gegen HBV-Antigene (diese fehlende Immunantwort ist mitverantwortlich für die Persistenz der Infektion). Core wirkt auch als T-Zell-abhängiges Antigen. T-Zell-Epitope in der Sequenz des Coreproteins sind daher an der starken Immunogenität von Impfstoffen auf HBV Core-CLP-Basis beteiligt. In chronisch HBV-Infizierten würde dies möglicherweise aufgrund ihrer T-Zell-Toleranz gegen HBV Core nicht zum Tragen kommen. Eine solche Toleranz besteht jedoch nicht gegen Core von WHV und anderen Tier-HBVs (vgl. Billaud JN, et al. *Advantages to the use of rodent hepadnavirus core proteins as vaccine platforms*. Vaccine. 2007 Feb 19;25(9):1593-606). Für diesen speziellen Kreis von potentiellen Impfungen könnte daher die Verwendung eines Tier-HBV Coreproteins als Träger von Vorteil sein.

2. Aufgrund seiner außergewöhnlich starken Immunogenität ruft Core bei allen HBV-Infizierten, ob akut oder chronisch, eine starke anti-Core B-Zellantwort hervor (anti-HBcAg); ausgeheilte Infektionen sind zusätzlich durch das Auftreten von Antikörpern gegen die HBV Hüllproteine (anti-HBsAg) charakterisiert. Derzeitige prophylaktische Impfstoffe gegen die HBV-Infektion basieren ausschließlich auf dem HBV Hüllprotein (HBsAg). Erfolgreiche

Impfung bewirkt daher - so wie überstandene Infektion - das Auftreten von anti-HBsAg. In einigen Fällen ist es von diagnostischem Interesse, ob eine Person mit anti-HBsAg dies durch Infektion, oder durch HBsAg-Impfung, erhalten hat - dies lässt sich durch den Nachweis von anti-HBcAg unterscheiden, welches nur nach Infektion auftritt.

Ein Impfstoff auf Basis von humanem HBV-Core würde neben der erwünschten Antwort gegen das inserierte Fremdprotein auch eine gewisse anti-Core (anti-HBcAg)-Antwort hervorrufen. Dies würde bei gleichzeitiger Anwesenheit von anti-HBsAg die Unterscheidung HBV-Infektion vs. HBsAg-Impfung erschweren.

Im split-Core-System ist die Entstehung von anti-HBcAg bereits minimiert, da das Hauptepitop, nämlich c/e1, physikalisch getrennt vorliegt und somit von der überwiegenden Anzahl von anti-HBcAg Antikörpern nicht mehr erkennbar ist. Eine Rest-anti-HBcAg Antwort gegen andere Sequenzbereiche des Coreproteins ist jedoch vorhanden. Dies lässt sich durch Verwendung eines Nicht-human-HBV-Coreproteins, bevorzugt des WHV-Coreproteins, als Trägerbasis verhindern bzw weiter vermindern.

Der wesentliche Bestandteil des erfindungsgemäßen Split-Core-Trägersystems ist einerseits das Core-Protein eines Hepatitis B-Virus und andererseits das Fremdmolekül, gegen das eine Immunantwort hervorgerufen werden soll.

Das Core-Protein wird in der breitesten Ausführungsform von einem beliebigen Hepatitis B-Virus abgeleitet. Es sind verschiedene Hepatitis B-Viren bekannt. Bevorzugt eingesetzt werden erfindungsgemäß solche Sequenzen, die von Hepatitis B-Viren abgeleitet sind, die aus Säugetieren isoliert wurden und für diese spezifisch sind. Besonders bevorzugt wird humanes HBV eingesetzt. Daneben können aber auch solche Hepatitis B-Viren die Sequenz des Core-Proteins liefern, die von anderen Tieren, beispielsweise Vögeln, wie Enten oder Reihern, herkommen.

Die andere Komponente, nämlich das Fremdmolekül, gegen das eine Immunantwort hervorgerufen werden soll, kann prinzipiell jedes beliebige Molekül sein. Bevorzugt handelt es sich hierbei um Proteinsequenzen und besonders bevorzugt um solche Proteinsequenzen, die auf den Oberflächen von Krankheitserregern gefunden werden und, die mit dem Immunsystem in Kontakt treten. Wenn das Immunsystem Antikörper gegen derartige Oberflächenstrukturen von Krankheitserregern ausbilden kann, werden die

Krankheitserreger nach Kontakt mit den einzelnen Komponenten des Immunsystems des geimpften Patienten inaktiviert, wobei der eingedrungene Krankheitserreger in der Regel zerstört wird.

Wenn man die in Datenbanken verfügbaren Sequenzen des woodchuck Hepatitis B-Virus (WHV) mit der Aminosäuresequenz des Coreproteins von HBV (Subtyp ayw) vergleicht, stellt man fest, dass eine Identität von etwa 60 % vorliegt. Die Sequenzunterschiede sind im Bereich der Aminosäurepositionen zwischen 66 und 94 besonders ausgeprägt, wobei diese Sequenz das c/e1-Epitop und im erfindungsgemäßen HBV-Split-Core-System die Trennstelle zwischen N-terminalem Bereich und C-terminalem Bereich des Coreproteins enthält. Diese Trennstelle liegt in einer besonders bevorzugten Ausführungsform zwischen Aminosäure 79 und 81.

Für die Herstellung von erfindungsgemäßen WHV-Split-Core-Trägersystemen wurde die Coreprotein-Sequenz des WHV-Coreproteins zwischen der Position 79 (kodiert für Aminosäure Glu) und der Position 80 (kodiert für die Aminosäure Glutamin) auf Nucleinsäure-Ebene getrennt. Auf Nucleotidebene wurde an den Carboxyterminus des N-terminalen Coreprotein-Segments von WHV (kurz als WcoreN bezeichnet) eine Sequenz kodierende BamHI-Schnittstelle angeführt. Daran schloß sich eine zweite Ribosomenbindungsstelle sowie eine Schnittstelle für die Restriktionsendonuclease NdeI an, welche wiederum mit dem Startkodon des WHV coreC (WcoreC)-Segmentes überlappt. Diese Anordnung ist identisch mit der bevorzugten Anordnung in den entsprechenden HBV-Split-Core-Konstrukten. Somit können Insertionen direkt mit Hilfe eines BamHI-NdeI-Verdauers von einem in das andere System transferiert werden. Im vorliegend beschriebenen Fall enthält WcoreN durch die eingeführte BamHI-Schnittstelle einen konservativen Austausch, nämlich E79D, und zusätzlich ein P als C-terminale Aminosäure. Dies ist unschädlich für die Partikelbildungsfähigkeit und das C-terminale Prolin, erhöht aufgrund seiner Protease-Resistenz die Stabilität des Split-Proteins. Analog HBV Split-Core enthält WcoreC ein zusätzliches Start-Methionin, in diesem Fall vor Q80, bei HBV vor S81. Dieses WHV-Split-Core-Konstrukt bildete ebenso gut Partikel wie das entsprechende HBV-Split-Core-Konstrukt.

In einer weiteren Ausführungsform der vorliegenden Erfindung können auch der C-terminale Bereich und der N-terminale Bereich des Coreproteins von unterschiedlichen Hepatitis B-Viren herkommen. Das erfindungsgemäße Hybrid-Split-Core-Trägersystem weist in diesem Fall im wesentlichen drei Komponenten auf, den N-terminalen Bereich des Coreproteins von

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einem Hepatitis B-Virus, beispielsweise einem humanen Hepatitis B-Virus, das Fremdmolekül, gegen das eine Immunantwort hervorgerufen werden kann und den C-terminalen Bereich des Coreproteins, das von einem anderen Hepatitis B-Virus her stammt, beispielsweise von dem WHV. Auch von einem solchen Hybrid-Split-Core-Konstrukt wurden problemlos Partikel gebildet. Da sich die Aminosäure-Sequenzen der Coreproteine aus unterschiedlichen Hepatitis B-Viren unterscheiden, unterscheiden sich auch die B- und T-Zellepitope. Somit kann durch das Hybrid-Split-Core-System die Immunantwort gegen den partikulären Träger gezielt beeinflusst werden.

Für die Herstellung von bevorzugten erfindungsgemäßen Hybrid-Split-Core-Trägersystemen wurde die Coreprotein-Sequenz des WHV-Coreproteins zwischen der Position 79 (kodiert für Aminosäure Glu) und der Position 80 (kodiert für die Aminosäure Glutamin) auf Nucleinsäure-Ebene getrennt. Auf Nucleotidebene wurde an den Carboxyterminus des N-terminalen Coreproteins von WHV (kurz als W-Core-N bezeichnet) eine Sequenz kodierende BamHI-Schnittstelle angeführt. Daran schloß sich eine zweite Ribosomenbindungsstelle sowie eine Schnittstelle für die Restriktionsendonuclease NdeI an, welche wiederum mit dem Startkodon des WHV coreC (WcoreC)-Segmentes überlappt. Diese Anordnung ist identisch mit der bevorzugten Anordnung in den entsprechenden HBV-Split-Core-Konstrukten. Somit können Insertionen direkt mit Hilfe eines BamHI-NdeI-Verdau von einem in das andere System transferiert werden. Im vorliegend beschriebenen Fall enthält WcoreN durch die eingeführte BamHI-Schnittstelle einen konservativen Austausch, nämlich E79D, und zusätzlich ein P als C-terminale Aminosäure. Dies ist unschädlich für die Partikelbildungsfähigkeit und das C-terminale Prolin, erhöht aufgrund seiner Protease-Resistenz die Stabilität des Split-Proteins. Analog HBV Split-Core enthält WcoreC ein zusätzliches Start-Methionin, in diesem Fall vor Q80, bei HBV vor S81. Bei diesem Konstrukt konnte Partikel problemlos gebildet werden.

Natürliches HBcAg wie auch rekombinante HBV CLPs sind hochimmunogen (T-Zell-unabhängig und T-Zell-abhängiges Antigen). Wesentlich dafür ist die symmetrische, multimere partikuläre Struktur des Corepartikels. Diese lässt sich in konventioneller "negative stain" Elektronenmikroskopie und auch Kryo-Elektronenmikroskopie darstellen. Biochemisch lässt sich die partikuläre Natur nachweisen durch Sedimentation in Sucrosegradienten (Sedimentationskoeffizient je nach Variante 60 - 80 S; zum Vergleich: lösliche monomere Proteine ca. 1-5 S; eukaryote ribosomale Untereinheiten 40S+ 60S). Außerdem haben die Partikel ein distinktes Laufverhalten bei der nativen Gelelektrophorese in Agarosegelen, das sich von den nicht-partikulären Formen unterscheidet. Dadurch ist feststellbar, ob sich CLP

bilden können, oder ob die Proteine ohne die CLP-Struktur vorliegen.

Das Core-Protein von HBV weist 183 oder 185 Aminosäuren (das von WHV 187 Aminosäuren) auf und besteht aus zwei Domänen: die ersten ca. 140 AS sind notwendig und hinreichend für das Assembly zu CLPs ("Assemblydomäne"); die C-terminale Region ist reich an basischen AS und bindet Nukleinsäuren. Eine C-terminal verkürzte Core-Protein-Variante (AS1-149) wurde in den ursprünglichen E. coli Expressionssystemen wesentlich besser exprimiert als das Volllängenprotein. CLPs aus Core-Protein 1-149, d.h. ohne Nukleinsäure-Bindungsdomäne wurden daher für mittel- und hochauflösende EM-Strukturuntersuchungen benutzt. So konnte die Kristallstruktur der CLPs und des Proteins bestimmt werden. Wie biochemisch vorhergesagt, bildet das Core-Protein sehr stabile, symmetrische Dimere, die sich zu CLPs aus 90 oder 120 Dimeren zusammenlagern.

Die Corepartikel besitzen auffällige "spikes"; jedes Dimer bildet einen spike, der strukturell aus einem 4-Helixbündel besteht, zu dem jedes Monomer 2 lange, zentral in der AS-Sequenz gelegene α -Helices beiträgt. Die spike-Spitzen umfassen die Aminosäuren (AS) in der Region von AS 74-85. Diese Region trägt auch das immundominante B-Zell-Epitop des HBcAg (c/e1-Epitop), seine besonders hohe Immunogenität erklärt sich aus der Oberflächen-Exposition dieser Reste.

Figur 1 zeigt die Core-Protein-Struktur schematisch. Auf der linken Seite der Figur 1 ist die Primärsequenz des HBV-Core-Proteins dargestellt. Das Gesamtprotein besteht aus 183 AS (in einigen HBV Subtypen 185 AS); nur die N-terminalen ca. 140 AS werden zur CLP-Bildung benötigt (Assembly-Domäne); eine bevorzugte C-terminale AS-Position ist 149; an diese und jede folgende AS, bis AS183, kann ein His-tag fusioniert werden, ohne die CLP zu stören. Das c/e1-Epitop liegt im Bereich der AS 78 - 83, und bildet einen Teil des Loops zwischen den zentralen α -Helices (links unten). Die Helices selbst können verkürzt werden; minimal benötigt werden wahrscheinlich in coreN die AS-Reste bis Pos. Gly73, in coreC die Reste ab AS Gly94. Die rechte Seite der Fig. 1 zeigt eine schematische Darstellung eines Core-Protein-Dimers. Nur die beiden zentralen Helices pro Monomer sind gezeigt. Das c/e1 Epitop ist auf der Partikeloberfläche.

Für eine erfolgreiche Partikelbildung muss die Core AS-Sequenz mindestens Pos. 140 enthalten, vorzugsweise mindestens Pos. 149, kann sich aber auch weiter erstrecken bis zur C-terminalen AS 183 bzw. 185, je nach HBV Subtyp. Die AS-Sequenz nach Pos. 140, besser 149, kann durch Fremdsequenzen ersetzt werden, diese Fremdsequenzen befinden sich dann üblicherweise im Inneren der CLPs. Gegen solche Fremdaminosäuren werden

nicht in erster Linie Antikörper gebildet, sie können aber andere Funktionen erfüllen.

Aufgrund der außergewöhnlich hohen Immunogenität des HBcAg sind rekombinante HBV CLPs attraktiv für die Verwendung als Immunogenitäts-steigernder Träger ("carrier") für Fremdanigene.

Ein alternativer Lösungsweg ist die *chemische* Kopplung von Fremdmolekülen an vorgeformte CLPs. Bei der vorliegenden Erfindung liegt eine *genetische Fusion* von Fremd-AS-Sequenzen an das Core-Protein vor. Hierbei stellte sich die Region des c/e1-Epitops noch vor der röntgenkristallographischen Strukturaufklärung anhand empirischer Versuche als bestgeeignet für die Induktion potenter B-Zellantworten heraus. Anhand der Struktur ist heute klar, dass solche Fusionen Insertionen in die Schleife ("loop") zwischen den zentralen Helices darstellen. Da dieser *loop* per se keine strukturgebende Funktion hat, kann die 3D-Struktur des Core-Proteins bei Insertion von Fremdsequenzen zumindest grundsätzlich erhalten bleiben. Abhängig von der inserierten Fremdsequenz können einige derartige Fusionsproteine weiterhin reguläre CLPs bilden, jedoch ist dies nicht immer gewährleistet, weil die Fremdaminosäuresequenz die Formung der Struktur des Fusionsproteins negativ beeinträchtigen kann. Die partikuläre Struktur ihrerseits ist essentiell für hohe Immunogenität.

Es wurden schon eine Vielzahl von Fremdsequenzen in das Core-Protein inseriert, z.T. auch die erhöhte Immunogenität experimentell nachgewiesen [Ulrich et al., Adv. Virus Res. (1998), S. 141-182]. Da viele Versuche zur Insertion von längeren Fremdsequenzen mit mehr als ca. 40 AS aber scheiterten (keine Partikelbildung mehr), nahm man an, es gebe eine natürlich begrenzte Aufnahme-Kapazität für Fremdsequenzen für bis zu 40 AS bzw. in einem speziellen Fall bis zu 120 AS.

Es wurde gefunden, daß Fremdaminosäuresequenzen, die am C- und N-Terminus mit dem Core-Protein fusioniert sind, nur dann Partikel ausbilden, wenn mindestens zwei essentielle Kriterien erfüllt sind:

- (i) Die 3D-Struktur des Fremdproteins muss so geartet sein, dass sein N- und C-Terminus zur räumlichen Lage der Verknüpfungspunkte im Core-Protein (C-Terminus des N-terminalen Coreanteils, d.h. Core-AS ca. 1- bis ca. 78 [coreN]; N-Terminus des C-terminalen Coreanteils, d.h. Core-AS ca. 80 bis 149 oder 183 [coreC]) passen
- (ii) falls das Fremdprotein selbst homomere Interaktionen eingeht (Dimere, Trimere, etc) muss die Struktur dieser Homo-Oligomere ebenfalls zur Struktur

der beiden Core-Protein-Anteile passen

Gut geeignet für die Präsentation in vorbekannten Fusionsproteinen sind daher Fremdproteine, deren N- und C-Terminus in der nativen 3D-Struktur nahe beieinander liegen. GFP erfüllt beide dieser Voraussetzungen, viele andere Proteine aber nicht; ein Beispiel hierfür ist das outer surface protein A (OspA) des Lyme-Borreliose Erreger *Borrelia burgdorferi*, dessen N- und C-Terminus auf entgegengesetzten Seiten der langgestreckten 3D-Struktur liegen.

Die Insertion von OspA oder ähnlich ungünstig strukturierten anderen Proteinen führt zu Spannungen im Fusionsprotein. Entweder bleibt das Fremdprotein korrekt gefaltet und verhindert die Annäherung der beiden Core-Proteinanteile, so dass deren Faltung und nachfolgende Dimerisierung und Partikelbildung verhindert wird; oder die Faltung des Core-Protein-Anteils beeinträchtigt die Faltung des Fremdproteins, so dass dieses entweder nicht mehr nativ vorliegt (> veränderte Antigenität), oder - falls massiv missgefaltet - zur Aggregation führt.

Im Fall von OspA konnte dieses Problem nur partiell durch Benutzung sehr langer Verbindungssequenzen ("Linker") umgangen werden. Gerade für Vakzine-Anwendungen stellen diese Linkersequenzen ein potentiell Problem dar, da sie unerwünschte eigene Antigenität besitzen könnten, die zu schädlichen Kreuzreaktionen, bspw. mit körpereigenen Antigenen, führen können. Außerdem waren entsprechende Protein-Präparationen nur sehr begrenzt zur Bildung regulärer CLPs in der Lage.

Aufgrund der dimeren Struktur des Coreprotein-Trägers und der geometrisch begrenzten Oberfläche der Trägerpartikel könnte bei sehr großen oder stark von einer globulären Struktur abweichenden Fremdproteinen zusätzlich *generelle* sterische Hinderung ein Problem darstellen (der auf der "Kugeloberfläche" der CLPs verfügbare Raum ist begrenzt). Die Maximalgröße des Fremdproteins hängt von mehreren Faktoren ab. Mit dem erfindungsgemäßen Verfahren gelang schon die Präsentation eines ca. 320 AS umfassenden Fremdproteins (CSP).

Gegenstand der vorliegenden Erfindung ist daher ein Split-Core-Impfstoff, der als getrennte Polypeptide die Core N- und die Core C-Domäne des Core-Proteins eines Hepatitis B Virus und wenigstens eine Fremdaminosäuresequenz, gegen die eine humorale sowie gegebenenfalls zelluläre Immunantwort hervorgerufen werden soll, aufweist, wobei die

Fremdaminosäuresequenz an den C-Terminus der Core N-Domäne oder an den N-Terminus der Core C-Domäne fusioniert ist und das Core-Protein capsid-like-particles binden kann. Bevorzugt werden durch das Trägersystem neutralisierende Antikörper hervorgerufen.

Wesentlich bei dem erfindungsgemäßen Split-Core-Impfstoff ist, dass das Core-Protein am c/e1 Epitop, also etwa zwischen den Aminosäuren 73 und 94 unterbrochen, also getrennt ist. Die Fremdaminosäuresequenz kann nun an das C-terminale Ende des Core N-Bereichs anfusioniert sein. In diesem Falle beginnt das N-terminale Ende des Core C-Teiles mit derjenigen Aminosäure, bei der das Core N-Protein getrennt wurde. Es ist auch möglich, dass ein Teil des c/e1 Epitops deletiert ist, dass also eine oder mehrere Aminosäuren aus dem Bereich zwischen Aminosäure 73 und 94 entfernt wurden. Alternativ hierzu ist möglich, dass die Fremdaminosäuresequenz, gegen die Antikörper gebildet werden sollen, an das N-terminale Ende des Core C-Bereichs anfusioniert ist. In diesem Falle endet der C-terminale Bereich von Core N an der Aminosäure, bei der das Core-Protein unterbrochen wurde. Ein wesentlicher Effekt, den der Split-Core-Impfstoff beibehalten muß, ist, dass die Split-Core-Impfstoffpartikel, die anfusionierte Fremdaminosäuresequenzen tragen, dennoch die core-capsid-like particles bilden können. Dies wird entweder im Saccharose-Gradienten oder in der nativen Agarose-Gelelektrophorese überprüft. Auch eine Überprüfung mit Hilfe des Elektronenmikroskops ist möglich.

Bei der Fremdaminosäuresequenz, gegen die Antikörper gebildet werden sollen, handelt es sich bevorzugt um Sequenzen, die von Oberflächenstrukturen von Mikroorganismen herkommen. Derartige Mikroorganismen rufen üblicherweise Erkrankungen beim Menschen hervor. Wenn gegen diese Strukturen neutralisierende Antikörper gebildet werden können, führt dies dazu, dass die Immunabwehr eingedrungene Mikroorganismen sehr schnell beseitigen kann. Ein Bakterium, bei dem die Impfstoffentwicklung schon relativ weit fortgeschritten ist, ist *Borrelia burgdorferi*, der Erreger der Lyme Disease. Bevorzugt eingesetzt werden im Rahmen der Erfindung als Fremdaminosäuresequenzen die Oberflächenproteine OspA und OspC von *Borrelia burgdorferi*. Bei den Fremdaminosäuresequenzen kann es sich aber auch um andere Sequenzen handeln, die von pathogenen Organismen herkommen. Ein derartiges Beispiel ist der Malaria-Erreger *Plasmodium falciparum*.

Bei weiteren Fremdaminosäuresequenzen, gegen die Antikörper gebildet werden können, handelt es sich um Sequenzen, die von Viren herkommen. Bevorzugt werden solche Aminosäuresequenzen eingesetzt, die von Proteinen von Viren herkommen, die mit dem

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Immunsystem des Wirtsorganismus in Kontakt kommen. In erster Linie handelt es sich hierbei um Oberflächenproteine, da diese in der Regel zuerst mit dem Immunsystem des Wirtsorganismus in Kontakt treten. Alternativ hierzu kann es sich aber auch um Proteine handeln, die im Verlauf des viralen Lebenszyklusses freigesetzt werden. Dabei kann es sich, je nach Virustyp, beispielsweise um Core-Proteine oder Nucleocapsidproteine eines Virus handeln.

Mit dem vorliegenden Split-Core-Impfstoff können aber nicht nur Antikörper gegen körperfremde Aminosäuresequenzen erzeugt werden, sondern auch Antikörper gegen unerwünschte körpereigene Strukturen, wie beispielsweise Tumormarker. Tumorzellen exprimieren häufig andere Oberflächenmarker als gesunde Zellen. Wenn also derartige körpereigene Aminosäuresequenzen im Split-Core-Impfstoff präsentiert werden, wird die Bildung einer Immunantwort angeregt, so dass der Körper in verstärktem Ausmaß Antikörper gegen die Tumorzellen bildet. Durch diese Antikörper können die Tumorzellen leicht von der Immunabwehr erkannt und schließlich eliminiert werden. Durch das erfindungsgemäße Trägersystem kann aber auch die zelluläre Immunantwort angeregt werden, was zu einer verbesserten Wirksamkeit führen kann.

Gegenstand der vorliegenden Anmeldung ist auch ein Verfahren zur Herstellung eines Impfstoffes gegen eine heterologe Proteinsequenz. Der Split-Core-Impfstoff besteht aus zwei Teilen des Core-Antigens eines Hepatitis B-Virus, wobei entweder an die Core N-Region die heterologe Proteinsequenz anfusioniert ist, oder an das C-terminale Ende der heterologen Proteinsequenz das Core C-Protein anfusioniert ist. Diejenige Position in der Aminosäurekette, an der die beiden Domänen voneinander getrennt sind, befindet sich zwischen Position 73 und Position 94. Einzelne Aminosäuren dieser Aminosäureregion, die das c/e1 Epitop darstellen, können deletiert sein.

Das erfindungsgemäße Verfahren kann grundsätzlich auf zwei verschiedene Arten durchgeführt werden. Wenn die beiden Teile des Split-Core-Antigens als nur eine Polypeptidkette exprimiert werden, wird erfindungsgemäß eine Erkennungssequenz für eine Protease an der vorgesehenen Position eingebaut. Dadurch kann das Polypeptid nach Expression durch die Protease in zwei definierte Teile gespalten werden. Bei dieser Ausführungsform ist es auch möglich, diejenige Protease, die an der vorgesehenen Schnittstelle schneidet, mitzuexprimieren. Die hierfür kodierende Gensequenz kann sich entweder auf demselben Vektor oder auf einem separaten Vektor befinden.

In einer anderen, bevorzugten Ausführungsform werden die beiden oben erwähnten Polypeptide aber mit einem speziellen Vektor exprimiert. Bevorzugt eingesetzt wird ein sogenannter bicistronischer Vektor. Dies bedeutet, dass der eine Teil des Split-Core-Impfstoffes als Polypeptidkette exprimiert wird und, dass sich am C-Terminus ein Stopkodon befindet. Kurz danach können aber die Ribosomen wieder ansetzen und das zweite Polypeptid exprimieren. Dadurch wird gewährleistet, dass sich die beiden Teile des Split-Core-Impfstoffes in etwa gleicher Menge bilden und es müssen keine Vorsichtsmaßnahmen getroffen werden, damit der Wirtsorganismus nicht einen der Vektoren verliert, was dazu führen würde, dass nur noch ein Teil des Split-Core-Impfstoffes bereitgestellt wird.

Der Ausdruck "Fusionsprotein" oder "anfusionsiert" bedeutet, dass zwei unterschiedliche Proteine oder Polypeptide über eine Peptidbindung miteinander verbunden sind. Solche Fusionsproteine entstehen durch Expression der dafür kodierenden, hintereinander geschalteten Nucleinsäuresequenzen.

Es wurde im Rahmen der Erfindung gefunden, daß sich als proteinchemisches Hauptproblem für eine breitere Verwendbarkeit des HBV-CLP Trägersystems (spezifisch als Impfstoff-Carrier, generell für die Präsentation von Fremdmolekülen) die beidseitige Verknüpfung des inserierten Fremdproteins über N- und C-Terminus darstellt. Läßt sich *eine* der beiden kovalenten Bindungen (auf der N-oder der C-terminalen Seite des Inserts) lösen, und kommt es trotzdem zur Partikelbildung, weil sich die getrennten Core-Protein-Anteile ("split-core") finden und korrekt falten, erhöht sich die Anzahl und Art der inserierbaren Fremdproteine drastisch. Dies ist ein wesentlicher Aspekt der vorliegenden Erfindung.

Figur 2 zeigt schematisch, dass die Insertion eines Fremdproteins mit ungünstiger 3D-Struktur (z.B. OspA) die Bildung der Partikel-bildungskompetenten Struktur des Core-Protein-Trägers verhindern kann (oberer Weg); alternativ kann die Ausbildung der korrekten 3D-Struktur des Core-Protein-Trägers die Formation der nativen 3D-Struktur des Fremdproteins verhindern (unterer Weg). Dies würde die Bindung der gewünschten Antikörper ungünstig beeinflussen. Diese sterische Problematik wird aufgehoben, wenn eine der beiden kovalenten Verknüpfungen zwischen Fremdprotein-Insert und Träger (auf der C-terminalen oder der N-terminalen Insert-Seite) gelöst wird (Pfeil). Eine Möglichkeit der Realisierung ist die nachträgliche Spaltung eines kontinuierlichen Fusionsproteins; dies erfordert die zusätzliche Einführung einer Schnittstelle für eine spezifische Protease. Bevorzugt werden daher die beiden Fragmente von vornherein als separate Entitäten

exprimiert.

Durch die erfindungsgemäße Lösung werden noch weitere Konsequenzen bewirkt:

- (i) da das c/e1 Epitop auf der Partikeloberfläche liegt, entstehen auf ebendieser Oberfläche neue N- und C-Termini, die weiter derivatisiert werden können. Z.B. kann an das coreN-Fragment ein Fremdmolekül X, und an das coreC-Fragment ein Fremdmolekül Y fusioniert werden. Eine mögliche Anwendung ist die Präsentation von heterodimeren Fremdmolekülen. Hierbei muß allerdings beachtet werden, dass keine sterische Hinderung der beiden anfusionierten Teile auftreten darf.
- (ii) spezifische Teilregionen einer inserierten Fremdsequenz, z.B. ein besonders wichtiges Epitop können je nach Verknüpfung über N- oder C-Terminus zur Partikeloberfläche hin, oder von ihr weg, orientiert werden. Ein Beispiel ist das sog. LA2-Epitop von B. burgdorferi OspA, das nachgewiesenermaßen im C-terminalen Bereich liegt. Antikörper, die dieses Epitop erkennen, sind neutralisierend.

Dies ist auch wichtig für die Insertion von Fremdsequenzen, die ihrerseits weitere Interaktions-Oberflächen darbieten (für die Anheftung dritter Moleküle; siehe Beispiele). Da nur einseitig verknüpft, wird der interaktionsfähigen Fremdsequenz keine artifizielle 3D-Struktur aufgezwungen; stattdessen ist sie, je nach Art, flexibel oder kann ihre korrekte 3D-Struktur ungestört einnehmen. Damit werden Interaktionen mit dem Interaktionspartner wesentlich erleichtert.

Gegenstand der vorliegenden Erfindung sind auch Arzneimittel, insbesondere Impfstoffe, die capsid-ähnliche Partikel enthalten, welche auf dem Split-Core-System basieren.

Die erfindungsgemäßen Arzneimittel beinhalten die erfindungsgemäßen Capsid-ähnlichen Partikel, wobei die Arzneimittel bevorzugt einen positiven Einfluß auf das Immunsystem aufweisen. Üblicherweise wird durch das erfindungsgemäße Split-Core-Trägersystem dem Immunsystem eine Fremdaminosäuresequenz dargeboten, gegen die das Immunsystem Antikörper, bevorzugt neutralisierende Antikörper, produziert. Diese Antikörper wirken dann maßgeblich bei der Bekämpfung des in den Körper eingedrungenen Mikroorganismus oder des Virus mit, oder sie helfen bei der spezifischen Zerstörung von unerwünschten Tumorzellen mit.

In einer weiteren Ausführungsform kann das erfindungsgemäße Split-Core-Trägersystem als

diagnostisches und/oder analytisches Mittel eingesetzt werden. In den Beispielen 12 und 13 sind derartige Anwendungen näher erläutert. Bei derartigen Mitteln können beispielsweise selbstfluoreszierende, Antikörper bindende Partikel zugänglich gemacht werden, die ihrerseits Antigen binden und dieses per Fluoreszenz sichtbar machen. In einer weiteren Ausführungsform können die erfindungsgemäßen Split-Core-Trägersysteme Peptidsequenzen beinhalten, die spezifisch bestimmte Metallionen/Lantanide binden. Wenn derartige Peptide in das Split-Core-System inkorporiert werden, werden Partikel bereitgestellt, die pro individuellem Partikel viele derartige Reporteratome/Ionen enthalten und besser nachweisbar sind.

Beispiel 1

Wildtyp-Core-Protein mit inserierter TEV-Protease Erkennungssequenz

In das c/e1 Epitop von wt-Coreprotein 1-149 wurde eine Erkennungssequenz für die tobacco-etchvirus (TEV) Protease eingeführt (Ersatz der AS P79+A80 durch GGGGT-ENLYFQGT-GGGG; G-Reste als Linker, um Zugänglichkeit der Erkennungssequenz für die Protease zu gewährleisten). Das rekombinant exprimierte Protein formte Partikel. Diese wurden mit TEV Protease inkubiert, der Erfolg der Spaltungsreaktion durch SDS-PAGE überprüft. Der Protease-Verdau-Ansatz wurde anschließend durch einen Sucrosegradienten sedimentiert. Es kam zur praktisch vollständigen Spaltung an der erwarteten Position, da 2 nahezu gleich große Fragmente entstanden. Trotz Spaltung sedimentierte das Protein im Gradienten etwa an dieselbe Position wie ungespaltenes wt-Core-Protein 1-149. Vergleichbare Ergebnisse wurden mit einem Fusionsprotein auf Basis des Volllängen-Core-Proteins 1-183 erhalten.

Beispiel 2

Core-Protein-Varianten mit größeren inserierten Fremdsequenzen

Als Beispiel für ein Fusionsprotein mit moderat großer Insertion wurde in das c/e1-Epitop die Sequenz für ein artifizielles Peptid "ACID" (flankiert von Gly-reichen Linkern; gesamt 65 AS) inseriert, welches mit einem komplementären Peptid "BASE" interagieren kann [O'Shea et al., Curr.Biol. (1993), 658-667]; daran anschließend die TEV-protease-Erkennungssequenz

GGGSGGGVEDGGGSGGGGT-AQLEKELQALEKENAQLEWELQALEKELAQTG-ENLYFQGTGGGG.

Das Fusionsprotein bildete CLPs. Diese wurden isoliert und wie oben mit TEV-Protease inkubiert. Auch hier kam es zu spezifischer Spaltung, die Partikel blieben aber intakt. In diesem Fall sind die TEV-Spaltfragmente unterschiedlich groß und daher im SDS-Gel direkt zu unterscheiden. Trotz Spaltung ko-sedimentieren die Fragmente in die CLP-spezifischen Gradienten-Fractionen.

Dieses Beispiel zeigt, dass sich auch CLPs aus Core-Protein mit einem inserierten 65 AS Peptid (Linker + ACID + TEV-Schnittstelle) spezifisch spalten lassen, die partikuläre Struktur bleibt trotzdem erhalten.

Beispiel 3

Als weiteres Beispiel ist ein entsprechender Versuch mit einem Core-Protein gezeigt, das die AS Sequenz 18-273 des *Borrelia burgdorferi* OspA Proteins enthält. Das entsprechende Protein ohne TEV-Spaltstelle ist in Nassal et al., 2005 beschrieben; nur ein Bruchteil davon bildete reguläre Partikel, was sich auch in einer breiten Verteilung im Sucrosegradienten gezeigt hatte. Der nicht geschnittene Impfstoff ist also als solcher nur bedingt zu gebrauchen. Da zudem sehr lange Extra-Linkersequenzen mit eigenem, unerwünschten Antigenpotential notwendig waren, um wenigstens einen kleinen Anteil der Präparation in CLP-Form zu erhalten, wäre dieser nicht geschnittene Impfstoff nicht im Menschen/medizinisch anwendbar.

Nach Einführung einer TEV-Spaltstelle wurde das Fusionsprotein wie in Beispiel 2 mit TEV-Protease behandelt, dann im Sucrosegradienten sedimentiert. Auch hier kam es zur spezifischen Spaltung, allerdings nicht vollständig (vermutlich durch sterische Hinderung via OspA schlechtere Zugänglichkeit der TEV-Schnittstelle); Zuordnung der Fragmente mittels monoklonaler Antikörper, die spezifisch den N-terminalen bzw. C-terminalen Coreanteil erkennen; der a-coreN Antikörper reagiert demzufolge mit dem ungespaltenen Fusionsprotein sowie mit dem N-terminalen Spaltfragment. Trotzdem zeigte das partiell gespaltene Material ein Partikel-typisches Laufverhalten. Diese Daten deuteten darauf hin, dass durch Öffnen einer der beiden Verbindungen zwischen inseriertem Fremdprotein und Coreträger die Partikelbildung deutlich verbessert werden konnte.

Das Beispiel belegt, dass sich auch ein Corefusionsprotein mit einer *großen* Fremdprotein-Insertion (255 AS OspA + Linker + TEV-Schnittstelle) spezifisch spalten lässt (wenn auch weniger effizient). Während das zuvor beschriebene kontinuierliche coreOspA

Fusionsprotein sich breit über den Gradienten verteilt (Nassal et al., 2005), reichert sich das (partiell) gespaltene Fusionsprotein distinkt in partikeltypischen Gradientenfraktionen an. Dies ist ein Indiz für verbesserte Partikelbildungseffizienz durch die Spaltung.

In einem weiteren Versuch konnte gezeigt werden, dass (von einem kompatiblen Plasmid) ko-exprimierte TEV-Protease zur Spaltung von TEV-Schnittstellen tragenden Corefusions-Proteinen führt, teilweise effizienter als durch nachträgliche in vitro Spaltung möglich. Auch für die schon in den Bakterien gespaltenen Fusionsproteine konnte Partikelbildung via Sucrosegradienten-Sedimentation nachgewiesen werden.

Die Beispiele 1 bis 3 zeigten, dass *vorgeformte* CLPs nach Spaltung im Bereich des c/e1Epitops intakt bleiben. Dies ist strukturell damit zu erklären, dass die Verbindungsschleife zwischen den spike-bildenden Helices keine *per se* strukturgebende Rolle hat, konnte so aber nicht erwartet werden. Dies führte zu der bevorzugteren Ausführungsform, bei der, statt nachträglicher Spaltung der kontinuierlichen Proteinkette die coreN und coreC-Teile direkt als separate Proteinfragmente exprimiert werden, wobei sie sich offensichtlich spontan zusammenfinden und Partikel bilden. Ein solcher Ansatz hat mindestens drei wesentliche Vorteile:

- (i) Vereinfachung: der zusätzliche Spaltungsschritt mit TEV-(oder einer anderen) Protease ist nicht mehr notwendig
- (ii) keine zusätzliche Peptidsequenz im Fusionsprotein für die spezifische Protease-Erkennung notwendig
- (iii) eine - wenn auch nur partielle - Partikelbildung mit sterisch ungünstigem Fremdprotein wie OspA erforderte sehr lange Linkersequenzen; diese sind verzichtbar, wenn sterische Hinderungen durch die nicht mehr beidseitig notwendige Verknüpfung mit dem Core-Trägerprotein vermieden würden.

Die Punkte (ii) und (iii) sind besonders für Vakzineanwendungen bedeutsam, da jegliche zusätzliche Sequenz zu unvorhersehbaren immunologischen Konsequenzen führen kann (neue Epitope durch die Zusatzsequenzen, möglicherweise Kreuzreaktionen mit körpereigenen Epitopen)

Beispiel 4

Expressionkonstrukte zur annähernd äquimolaren Expression von coreN und coreC Fragmenten

In der kontinuierlichen Peptidkette liegen die späteren Spaltprodukte zwangsläufig in äquimolaren Mengen vor. Bei separater Expression sollten die beiden Teile daher für eine effiziente Zusammenlagerung ebenfalls in annähernd äquimolaren Mengen entstehen. In ersten Versuchen wurden zwei separate, kompatible Plasmide zur Expression der beiden Trägerprotein-Teile eingesetzt, allerdings mit mäßigem Erfolg.

In bevorzugter Ausführungsform werden bicistronische Vektoren eingesetzt, die zudem den Vorteil bieten, dass auf Selektion mit einem weiteren Antibiotikum (für den Erhalt des ansonsten notwendigen zweiten Plasmids) verzichtet werden kann.

Typ1 Konstrukte enthalten hinter einem gemeinsamen Promotor (hier T7 Phagen RNA Polymerase Promotor) zwei Expressionskassetten mit jeweils einer vorgeschalteten Ribosomen-Bindungsstelle (ribosome binding site, RBS; "Shine-Dalgarno-Sequenz"). In den hier realisierten Vektoren wird die Translation des coreN Fragments durch ein artifizielles Stop-Codon nach AS Prolin 79 (P79) beendet, die Translationsinitiation des 2. Fragmentes durch ein artifizielles Start-Codon vor AS Serin81 (S81) ermöglicht. Eine solche Operonstruktur ist in Bakterien häufig zu finden ("polycistronische mRNAs"; Ribosomen können relativ unabhängig an jede RBS auf der mRNA binden und die Translation des 3' gelegenen Gens initiieren). In den realisierten Konstrukten ist die zweite RBS eine exakte Kopie der ersten RBS, die aus dem originalen pET-Vektoren stammt; andere RBS-Sequenzen sind mit Sicherheit ebenfalls möglich. Das ATG Startcodon des 2. Cistrons ist Teil einer Nde-Schnittstelle (CATATG) für das leichte Einklonieren von Fremdsequenzen.

Typ2 Konstrukte ("stop/start") enthalten keine separate zweite RBS; stattdessen überlappt mit dem Stopcodon des *upstream* Gens (NNN TGA) ein Initiationscodon für das *downstream* Gen (NNA TGA) in einem um 1 verschobenen Leseraster. In diesem Fall können Ribosomen nach Ablesen des ersten Gens direkt am ATG des zweiten Gens re-initiieren; eine solche Anordnung erlaubt die Koexpression zweier Gene; ähnliche Stop-Start Arrangements finden sich in einigen Bakteriophagen. Sie ist auch in einigen eukaryonten non-LTR Retrotransposons verwirklicht (Verbindung ORF1/ORF2). Anhand der bekannten Sequenzen z.B. im E. coli Trp Operon sollten andere Kombinationen von nahe benachbarten, oder überlappenden Stop-/Startcodons (z.B. TAA TG, TGA TG, etc.) ähnlich geeignet sein.

Bevorzugte Expressionsvektoren sind schematisch in Fig. 3 dargestellt.

Oben: Typ 1 Konstrukte. Ein Promotor, z.B. für T7 RNA Polymerase, führt zur Transkription einer bi-cistronischen mRNA (für effiziente Termination kann 3'-seitig eine Terminatorsequenz eingefügt sein). Das erste Cistron kodiert die HBV Coreprotein-AS-Sequenz 1-79. Eine vorgeschaltete Ribosomen-bindungsstelle (RBS1) ermöglicht die Translationsinitiation (kleine und große Ribosomen-Untereinheit als Ovale dargestellt). Am 3'-Ende des Codons für P79 folgt ein Stop-Codon. Am 5'-Ende des zweiten Cistrons liegt eine weitere RBS (RBS2) für die Translationsinitiation des coreC-Fragments. Dieses verfügt dazu über ein eigenes Initiationscodon (ATG). Im gezeigten Beispiel beginnt die

coreC-Sequenz mit Ala80; sie kann sich bis AS-Position 140, besser 149, oder bis zum authentischen Ende bei Pos. 183 erstrecken. Die AS-Sequenz ab Pos. 140, besser 149, kann auch durch Fremdsequenzen ersetzt werden.

Unten: Typ2 Konstrukte. Diese enthalten nur eine RBS vor dem ersten Cistron. Die Translation des coreC-Fragments erfolgt durch Re-Initiation, indem das artifizielle Startcodon von coreC (ATG) mit dem Stop-Codon von coreN (TGA) wie gezeigt überlappt. Im Beispiel beginnt die coreC-Sequenz mit Ser81.

Die AS 77, 78 und 79 des Core-Proteins sind Glutamat (E; Codons: GAA, GAG), Aspartat (D; Codons: GAC oder GAT), AS 79 Prolin (P; Codons: CCN). In den realisierten Typ 1 Konstrukten wurde für die AS-Sequenz EDP die Nukleotid-Sequenz GAG GAT CCN gewählt, wodurch eine Schnittstelle für BamHI entsteht (GGATCC); durch diesen singulären BamHI Schnitt ist eine simple Einklonierung von unterschiedlichen coreN- und coreC-kodierenden DNA-Fragmenten möglich. Das Nucleotid N im Prolin Codon CCN ist nicht festgelegt; das Stop-Codon kann TAA, TAG, oder TGA sein.

In Typ 2 Konstrukten lautet die Sequenz bis zum Ende des ersten Gens G GAT CCa TGA. Auch hier ist eine BamHI Schnittstelle (unterstrichen) für das Einklonieren unterschiedlicher coreN bzw. coreC kodierender DNA-Fragmente vorhanden. Für P79 muss in den realisierten Typ2-Konstrukten das Codon CCa sein, das Stop-Codon muss TGA (> CCa TGA) sein, um das Entstehen eines überlappenden ATG Initiationscodons für das zweite Gen zu erlauben. Das Codon für die erste AS des zweiten Gens nach dem ATG-Startcodon muss mit A beginnen. Alternative Stop/Start Nt-Folgen wären TGA TG oder TAA TG wie im E. coli Trp Operon zwischen TrpE und TrpD oder auch TrpB und TrpA [Das and Yanofsky (1989), Nucl. Acids Res., S. 9333-9340; Oppenheim and Yanofsky (1980), Genetics, S. 785-795].

In den realisierten Vektoren (pET28a2-xxx) beruht das Grundgerüst auf dem kommerziellen

Vektor pET-28, bei dem jedoch das Kanamycin-Resistenzgen gegen dasjenige für Ampicillin-Resistenz ausgetauscht wurde. Mit Sicherheit sind jedoch andere Vektor-Grundgerüste ebenfalls anwendbar (verschiedene replication oris, verschiedene Resistenzgene, verschiedene Promotoren, verschiedene RBS). Essentiell ist die bicistronische Anordnung der Gene für coreN und coreC, entweder durch 2 separate RBS, oder durch Stop/Start Anordnung. In eigenen Versuchen liessen sich mehrere split-Core Proteine von Typ1 wie von Typ 2-Konstrukten exprimieren. Für Typ 2 (Stop/Start) Konstrukte war dies jedoch in

einer geringeren Anzahl von Konstrukten der Fall. Ausserdem ist die mögliche Sequenzvariabilität bei Typ2-Konstrukten geringer (s.o.). Typ1 Konstrukte sind daher breiter anwendbar.

Als C-terminale AS des coreN-Anteils wurde P79 gewählt, da Prolin besonders Protease-resistent ist und somit eine Degradation des Fragments minimiert wird. Aufgrund der bekannten 3D-Struktur sowie beschriebener Deletionsvarianten in dieser zentralen Region lässt sich aber annehmen, dass

- (i) das Ende des coreN Fragments im Bereich der AS ca. ab Pos. 72 - 74, besonders Gly73
- (ii) der Beginn des coreC Fragments im Bereich der AS ca. ab Pos. 78-86, möglicherweise bis Gly94 liegen könnte, ohne dass wesentlich andere Ergebnisse als mit den hier gewählten Grenzen (coreN Ende bei P79, coreC Start bei S81, mit vorgeschaltetem Start-ATG) erhalten würden.

Für die Expression in eukaryonten Zellen sind die beschriebenen Konstrukte aufgrund des unterschiedlichen Transkriptions- und Translations-Mechanismus so nicht anwendbar; statt des prokaryonten Promotors muß ein eukaryonter Promoter verwendet werden (z.B. CMV-IE und viele andere), sowie ein Poly-Adenylierungssignal (z.B. aus SV40 Virus, oder viele andere) nach dem Ende des/der offenen Leseraster(s) eingeführt werden.

Eine annähernd gleiche Expression von coreN und coreC in Eukaryonten kann erreicht werden durch:

- (i) Ko-Transfektion zweier Plasmide (je eines für coreN, eines für coreC);

- (ii) Transfektion eines Plasmids mit 2 unabhängigen Expressions-Kassetten;
- (iii) Transfektion eines Plasmids mit funktionell bicistronischer mRNA; z.B. könnte das zweite Gen durch eine vorgeschaltete "internal ribosome entry site" (IRES) exprimiert werden;
- (iv) Möglicherweise ist auch ein Typ2-Vektor-artige Stop-/Start Anordnung in Eukaryonten funktionell.

Beispiel 5

Separate Expression von wt-coreN und coreC Fragmenten führt zur Bildung intakter CLPs

Mittels Expressionsvektoren auf Typ1-Basis (Fig. 3) wurde Wildtyp-Core-Protein in zwei Teilen in E. coli BL21 Zellen exprimiert, einmal auf Basis der AS 1-149, einmal AS 1-183. Beide erwarteten Fragmente wurden gebildet und lagerten sich zu intakten CLPs zusammen. Dies wurde mit Sucrose-Gradienten sowie nativ-Agarosegel-Elektrophorese nachgewiesen. Als direkter Beweis für die Bildung von CLPs wurde ein elektronenmikroskopischer Vergleich (negative staining) von Partikeln aus kontinuierlicher AS 1-183 Kette mit C-terminalem His6-tag vs. Partikeln aus dem entsprechenden split-Core-System durchgeführt. Bei dieser Auflösung waren keine Unterschiede detektierbar.

Wt-HBV-CLPs sind sehr robust und beständig gegen Harnstoff in molaren Konzentrationen; CLPs aus C-terminal trunkiertem Core-Protein 1-149 lassen sich durch 3-5 M Harnstoff in Dimere dissoziieren und durch Änderung der Pufferbedingungen (Entfernen des Harnstoffs, neutraler pH, erhöhte Salzkonzentration) in vitro wieder zu CLPs reassoziieren. Eine vergleichende Untersuchung zur Stabilität der CLPs aus split-Core zeigte eine ähnlich hohe Stabilität der split-Core CLPs. Dies zeigt die hohe Affinität der coreN und coreC-Anteile zueinander, und ist wichtig für die Verwendung als Trägersystem für Fremdmoleküle.

Beispiel 6

Separate Expression von Fremdprotein-fusionierten-coreN und wt-coreC Fragmenten, sowie *vice versa*, führt zur Bildung intakter CLPs

Zum Nachweis der grundsätzlichen Eignung des split-core-Systems für die CLP-Bildung und Präsentation von Fremdproteinen wurde als Modell GFP entweder an coreN oder an coreC fusioniert; für das coreN-Konstrukt wurde entweder ein Typ1 (zweite RBS) oder Typ2 (Stop/Start) Vektor benutzt. Alle Konstrukte führten zur Expression grün fluoreszierender Proteine, die laut Sedimentation in Sucrosegredienten Partikel bildeten.

Zum weiteren Nachweis der physikalischen Assoziation der jeweiligen coreN und coreC Fragmente wurden Aliquots der Gradientenfraktionen der Elektrophorese in nativen Agarosegelen unterzogen. Dabei bleiben Partikel erhalten, ebenso der GFP-Chromophor. In den Fraktionen fand sich sowohl bei GFP an coreN, wie GFP an coreC, eine distinkte, grün fluoreszierende Bande; nicht assembliertes GFP Fusionsprotein verblieb in den oberen Gradientenfraktionen, und zeigte ein anderes Laufverhalten sowie eine diffusere Verteilung (schnellere Diffusion im Gel aufgrund der wesentlich kleineren Struktur im Vergleich zum Partikel aus 180 oder 240 Untereinheiten). Sowohl für GFP an coreN wie an coreC ließ sich mit dem α -coreC Antikörper in der grün fluoreszierenden (dito GFP enthaltenden) Bande auch coreC nachweisen.

Dieses Beispiel zeigt zweierlei, nämlich

- (i) Im split-core-System kann ein Fremdprotein von ca. 240 AS, hier GFP, sowohl an coreN wie an coreC fusioniert werden, ohne die CLP-Bildung zu stören.
- (ii) Sowohl Typ1 wie Typ2 split-Core-Vektoren sind für die Ko-Expression von coreN und coreC mit anfusionierter Fremdsequenz geeignet.

Beispiel 7

CLP-Präsentation von medizinisch relevanten Fremdproteinen im splitcore-System, die konventionell nicht, oder nur sehr eingeschränkt, möglich ist

Borrelia burgdorferi OspA

Wie eingangs dargelegt, führt die im bisherigen System erforderliche beidseitige Verknüpfung des inserierten Fremdproteins mit dem Trägerprotein zu starken topologischen Einschränkungen. Während GFP eine natürliche "Passform" hat, trifft dies für OspA nicht zu. Als Präzedenz-Fall für ein ungünstig strukturiertes Fremdprotein wurde daher OspA im split-Core-System eingesetzt, und entweder an coreN oder coreC fusioniert. Im Gegensatz zum

früheren kontinuierlichen Konstrukt [beschrieben in Nassal et al., 2005] mit breiter Verteilung im Gradienten reicherten sich beide Fusionsproteine distinkt in den Partikel-typischen Fraktionen an. Eine Elektronenmikroskopie-Analyse zeigte eine drastisch verbesserte CLP-Bildungseffizienz verglichen zum alten, kontinuierlichen Konstrukt, sowohl bei Fusion von OspA an coreN wie an coreC.

Dieses Beispiel belegt, daß

- (i) das split-core-System die CLP-Präsentation von Fremdproteinen erlaubt, deren Struktur im herkömmlichen kontinuierlichen Core-System mit der CLP-Bildung interferiert,
- (ii) im split-Core-System effiziente CLP-Bildung durch Fusion des Fremdproteins sowohl an coreN wie auch coreC möglich ist.

In analoger Weise ließ sich auch *Borrelia burgdorferi* OspC erfolgreich im erfindungsgemäßen Split-Core-System präsentieren.

Beispiel 8

Circumsporozoiten-Protein des Malaria-Erregers *Plasmodium falciparum*

Ein weiteres unter Vakzine-Gesichtspunkten hochrelevantes Pathogenprotein ist das Circumsporozoiten-Protein (CSP) des Malaria-Erregers *Plasmodium falciparum*. CSP (hier verwendete Form: 319 AS Gesamtlänge) enthält eine ca. 110 AS lange Wiederholungssequenz der Tetrapeptid-Motive NANP/NVDP. Die Struktur von CSP ist nicht bekannt; die C-terminale ca. 50 AS Domäne mit 4 Cys-Resten hat wahrscheinlich (aufgrund Sequenz-Homologie) eine ähnliche Faltung wie Thrombospondin Typ1 repeats. Die repeat-Sequenz ist immunogen, möglicherweise enthalten aber auch die anderen Regionen von CSP wichtige Epitope. Im konventionellen kontinuierlichen Coresystem konnte das Vollängen-CSP nicht in CLP-Form präpariert werden. Dagegen war dies sehr wohl mit dem split-Core-System möglich. Eindeutige Daten liegen derzeit für die Fusion von CSP an coreN vor; bei Koexpression mit coreC (sowohl als 149 wie als 183-Konstrukt) bilden sich effizient partikuläre Strukturen.

Es wurde eine Serie von Konstrukten (sowohl auf Basis von core 1-149 wie 1-183) hergestellt, die entweder nur die repeat-Sequenz, oder trunkiertes CSP ohne Cys-reiche Domäne jeweils im kontinuierlichen oder split-Core-System enthalten; dazu komplettes CSP fusioniert an coreN. Die CLP-Präparationen wurden in einer vergleichenden Immunogenitäts-Studie in Mäusen eingesetzt, die aber noch nicht abgeschlossen werden konnte. Die bisherigen Daten zeigen, dass der im Split-Core-System hergestellte Impfstoff überlegene Eigenschaften aufweist. Dies ist darauf zurückzuführen, dass das komplette CSP ausschließlich bei Anwendung des erfindungsgemäßen Trägersystems die Fähigkeit zur CLP-Bildung aufweist. Bei anderen Systemen entstehen dagegen keine CLPs. Daher dürften mit Hilfe des erfindungsgemäßen Trägersystems hergestellte CSP-Impfstoffe eine überlegene Immunogenität aufweisen und insbesondere neutralisierende Antikörper induzieren.

Beispiel 9

Split-Core-CLPs als Träger für Fremdproteine verstärken die B-Zell-Antwort gegen das Fremdprotein

In einer Immunogenitätsstudie in Mäusen werden 5 core-OspA-Konstrukte aus dem split-Core-System vergleichend untersucht mit nicht an Core fusioniertem, lipidiertem OspA (LipOspA); dieses ist die Basis der kommerziellen Lymerix-Vakzine, daher der derzeitige "Goldstandard" für einen Lyme-Krankheits-Impfstoff. Der Lipidanteil in LipOspA (Tris-Palmitoyl-Cystein-(Pam3-Cys)) ist essentiell für dessen relativ hohe Immunogenität, nicht lipidiertes OspA ist nur sehr schwach immunogen (vgl. Nassal et al, 2005). Drei der core-OspA-Konstrukte enthalten Volllängen-OspA (AS 18-273), zwei weitere verkürztes OspA (beginnend bei AS 185, bis AS 273).

6 Gruppen von je 5 BALB/c Mäusen wurden dazu mit je 10 µg Antigen 4-mal immunisiert (Tag 0, 14, 29, 49):

- Gruppe 1: LipOspA
- Gruppe 2: Volllängen-OspA (AS 18-273) an coreN, coreC von Core-AS 81 bis 183
- Gruppe 3: Volllängen-OspA (AS 18-273) an coreN, coreC von Core-AS 81 bis 149
- Gruppe 4: Verkürztes OspA (AS 185-273) an coreN, coreC von Core-AS 81 bis 183

Gruppe 5: Verkürztes OspA (AS 185-273) an coreN, coreC von Core-AS 81 bis 149

Gruppe 6: Volllängen-OspA (AS 18-273) an coreC bis Core-AS 183, coreN von AS 1-79

Zur Bestimmung der induzierten Antikörper wurde Blut entnommen an Tag -1 (= 1 Tag vor der ersten Immunisierung; = Präimmunserum), sowie an den Tagen 8, 26, 36 und 57. Mittels ELISA wurden bestimmt: die Kinetik der Bildung OspA-spezifischer Antikörper; der jeweilige Anteil LA2-äquivalenter Antikörper, wobei LA2 ein monoklonaler, bekannt neutralisierender Antikörper ist, der ein komplexes konformationelles Epitop aus diskontinuierlichen AS-Sequenzen *downstream* von AS 185 von OspA erkennt.

Die Ergebnisse dieses Versuches sind in Fig. 4 detailliert dargestellt.

Fig. 4A zeigt die Kinetik der Induktion von Gesamt-anti-OspA Antikörper (in µg spezifischen Ak pro ml Serum). Bereits nach der zweiten Immunisierung enthalten die Seren aus Mäusen immunisiert mit den Volllängen OspA split-Core Konstrukten nachweisbare und mit den durch LipOspA hervorgerufenen vergleichbare anti-OspA Titer. Nach der 3. und 4. Immunisierung übersteigen die anti-OspA Titer in den split-Core Volllängen-OspA immunisierten Mäusen deutlich diejenigen der mit LipOspA immunisierten Mäuse; besonders hoch sind die Titer nach Immunisierung mit OspA18-273 an coreC (Gruppe 6). Verkürztes OspA185-273 ruft im Kontext von core149 (Gruppe 5) und core183 (Gruppe 4) nur eine wesentlich geringere OspA-spezifische Antwort hervor.

Fig. 4B vergleicht direkt die Gesamt-anti-OspA Ak-Titer mit denjenigen, die gegen das bekannt neutralisierende LA2-Epitop im C-terminalen Bereich von OspA gerichtet sind. Volllängen-OspA an coreN, sowohl im Kontext von core149 wie core183 (Gruppe 2 bzw. 3) induzieren höhere anti-LA2-äquivalente Ak-Titer als LipOspA, Volllängen-OspA an coreC deutlich niedrigere. Eine Untersuchung zum jeweiligen Gehalt an *neutralisierenden* Ak (Protektion gegen challenge mit dem *B.burgdorferi* Erreger) wird weiterhin durchgeführt.

Das Beispiel zeigt, dass split-Core CLPs spezifische Antikörper gegen das präsentierte Volllängen-OspA Fremdprotein induzieren, wobei die Titer höher sind als mit dem etablierten lipidierten LipOspA, bei dem der Lipidanteil wesentlich für die Immunogenität ist. Der unterschiedliche Anteil LA2-äquivalenter Ak nach Immunisierung mit OspA an coreN (ca. 30 %) vs. OspA an coreC (< 5 %) zeigt, dass die Art der Verknüpfung die Art der entstehenden Ak beeinflusst; siehe dazu Beispiel 10.

Beispiel 10

Gezielte Induktion regionsspezifischer Antikörper gegen ein Fremdprotein durch alternative Fusion entweder an coreN oder an coreC

Bei Proteinen, deren N- und C-Terminus nicht unmittelbar benachbart sind wie in GFP, führt die Fusion an coreN zu einer anderen Orientierung relativ zur Partikeloberfläche als die Fusion an coreC (coreN: C-Terminus des Fremdprotein "out"; coreC: N-Terminus des Fremdproteins "out"). Es ist zu erwarten, dass bei Verwendung als B-Zell-Vakzine die jeweils am weitesten ins Lösungsmittel ragenden Proteinanteile die stärkste Antikörperantwort hervorrufen. Somit lassen sich unterschiedliche regionsspezifische Antikörper induzieren, indem das Fremdprotein entweder an coreN (C-terminale Seite des Fremdproteins "out") oder an coreC fusioniert wird (N-terminale Seite des Fremdproteins "out").

Figur 5 zeigt schematisch, wie bei einem Fremdprotein mit gestreckter Struktur, wie hier OspA, über die Fusion an entweder coreN, oder coreC, bestimmt werden kann, welcher Teil des Moleküls am meisten von der CLP-Oberfläche weg zeigt. Ein gut kartiertes Epitop in OspA ist LA2 im C-terminalen Bereich, das von einem neutralisierenden monoklonalen Antikörper, LA2, erkannt wird. Wie erwartet, induzieren split-Core-OspA CLPs mit OspA an coreN (daher OspA C-Terminus exponiert) eine starke LA2-äquivalente Antwort, CLPs mit OspA an coreC nur eine schwache LA2-äquivalente Antwort. Dafür ist die Antwort gegen andere Bereiche von OspA verstärkt.

Für OspA als Fremdprotein war zu erwarten, dass eine Fusion an coreN das LA2-Epitop im C-terminalen OspA Bereich gut zugänglich macht, eine Fusion an coreC dagegen die bislang immunologisch nicht gut charakterisierte N-terminale OspA Region. Die oben gezeigten Daten bestätigen eindrücklich dieses Konzept (mit OspA an coreC sehr hohe anti-Gesamt-OspA Titer, aber nur geringe LA2-äquivalente Ak-Titer).

Das split-core-System besitzt also eine hohe immunverstärkende Aktivität, sowohl bei Fusion des Fremdproteins an coreN wie an coreC.

Durch Wahl des Verknüpfungsortes (coreN vs. coreC) kann die Art/Regionsspezifität der induzierten Antikörper gesteuert werden. Für optimale Impfergebnisse können Mischungen von Split-Core-Impfstoffen eingesetzt werden, bei denen die heterologe Fremdaminosäuresequenz einmal am Core N und einmal am Core C Teil anfusioniert sind,

so dass verschiedene Epitope optimal präsentiert werden.

Beispiel 11

Fusionen mit Bedeutung jenseits von Vakzine-Anwendungen

Neben der direkten Bedeutung als immunverstärkender partikulärer Träger für Peptid- und hier besonders Protein-Antigen-Vakzine sind eine Vielzahl weiterer Anwendungen für eine solche Träger-Plattform vorstellbar. Immer wird eine große Anzahl (180 oder 240) präsentierter Moleküle in eine nahe, symmetrische Nachbarschaft gebracht. Handelt es sich um interaktionsfähige Fremdmoleküle (Vakzine stellt einen Spezialfall dar: Interaktion mit Antikörpern), so hat der Partikel mit vielen Kopien des Fremdmoleküls eine drastisch erhöhte Avidität verglichen zum monomeren Fremdmolekül (vgl. natürliche IgM Pentamere; auch MHC-Tetramere in der Immundiagnostik). Damit solche interaktionsfähigen Fremdmoleküle mit ihren Interaktionspartnern reagieren können, müssen sie auf der CLP-Oberfläche zugänglich sein. Dies ist im split-Core-System wesentlich erleichtert gegenüber dem konventionellen kontinuierlichen System.

Eine Reihe von Modell-Fremdsequenzen wurde im split-Core-System exprimiert, alle getesteten Insertionen bildeten CLPs. Für einige wurde die Interaktionsfähigkeit direkt nachgewiesen.

Das split-Arrangement macht auf der CLP-Oberfläche neue Termini für weitere Derivatisierung zugänglich. Die Figur 5 zeigt ein split-Core Dimer mit an coreN fusioniertem ACID-Peptid, welches mit dem komplementären BASE-Peptid interagieren kann. Wurde das BASE-Peptid an ein Molekül X fusioniert, bindet X an die ACID-CLPs. Durch die split-Anordnung ist ACID flexibel; ACID inseriert im kontinuierlichen Core-System interagiert mit BASE-Peptid, da sich aber eine starre coiled-coil Doppelhelix zwischen ACID- und BASE-Peptid ausbildet [O'Shea et al., 1993], wird die Struktur des Core-Trägers instabil, die CLPs zerfallen. In der split-Anordnung sollten die CLPs dagegen stabil bleiben. Diese Koppelung

ist generalisierbar: Statt des ACID-Peptides kann ein anderes interaktionsfähiges Molekül A an coreN, oder coreC fusioniert sein. Entsprechende CLPs können dann mit B, einem spezifischen Interaktionspartner von A, interagieren. Solche Interaktionspartner-Paare sind His6 - Ni-NTA, Biotin-Akzeptor-Peptid - Streptavidin; Z33 - Immunglobulin.

Andere Koppelungsmöglichkeiten sind nachfolgend näher erläutert:

a) His6-tag:

An coreN via Gly2-Linker fusioniert; bildet Partikel, die an Ni²⁺NTA Agarose binden. His-tag im Gegensatz zum kontinuierlichen System frei zugänglich. Vergleichbares Insert (His7) in c/e1 des kontinuierlichen Core-Systems ergab keine nennenswerten Mengen Protein, weil dort der His6-tag Rest sterisch nicht zugänglich ist.

b) Biotin-Akzeptor (BA)-Peptid:

Biotin-Akzeptor (BA23) ist ein artifizielles 13 AS langes Peptid (GLNDIFEAQKIEWH)), welches durch die Biotin-Ligase BirA aus E. coli biotinyliert wird. Effiziente Biotinylierung erfordert freie Zugänglichkeit; diese ist im split-core-System gegeben, im kontinuierlichen core-System nicht oder nur eingeschränkt. Das split-Core-BA Fusionprotein bildet CLPs und wird in E. coli biotinyliert. An das CLP-präsentierte Biotin kann Avidin/Streptavidin bzw. deren Konjugate gebunden werden.

c) Z 33 Domäne aus Protein A

S. aureus Protein A bindet Immunglobuline mit hoher Affinität ("Protein-A-Sepharose" für Immunpräzipitation). Protein A besteht aus 5 strukturell ähnlichen Ig-Bindedomänen. Z33 ist eine einzige solche Domäne mit modifizierter AS-Sequenz; Gesamtlänge 33 AS). Z33 hat immer noch hohe Affinität zu Ig's (Kd 40 nM). Die Bindung an unterschiedliche Ig's setzt strukturelle Flexibilität voraus, zudem Zugänglichkeit. Die Z33 Sequenz wurde über eine lange Linkersequenz an coreN fusioniert:

GGGGSGGGVEDGGGGSGGGGT-FNMQQRRFYALHDPNLNEEQRNAKIKSIREDP.

Im split-core-System bildeten sich CLPs. Nach Zugabe eines FITC-markierten Immunglobulins und erneuter Sedimentation konnte ein Anteil der Fluoreszenz in den Partikel-typischen Gradientenfraktionen nachgewiesen werden. Dies war nicht der Fall, wenn

statt der Z33-Fusion wt-Core-Protein CLPs eingesetzt wurden.

d) ACID Peptid Insert

Das ACID-Peptid inseriert in das kontinuierliche core-System interagiert mit dem komplementären BASE Peptid ("Peptid-Velcro"= Peptid-Klettverschluss) via coiled-coil Formation der Peptide; [O'Shea et al., 1993] unter massiven Veränderungen der Corestruktur bis hin zum Zerfall; Ursache ist, dass das ACID Peptid alleine eine flexible Struktur annimmt, die keinen "Stress" auf die Corestruktur ausübt. Zugabe des BASE-Peptides führt zur Interaktion mit der ACID-Sequenz, wodurch beide Peptidsequenzen eine starre helicale coiled-coil Konformation annehmen. Wegen der beidseitigen Verknüpfung entsteht Spannung. Ein nur einseitig fixiertes ACID-Peptid sollte spannungsfrei mit zugesetztem BASE-Peptid, oder BASE-Peptid fusioniert an weitere Partner interagieren können. Auf jeden Fall bleibt die Partikelstruktur des ACID-Fusionsproteins im herkömmlichen kontinuierlichen core-System nach Spaltung der C-terminalen Verbindung zum Core-Träger mittels TEV-Protease erhalten.

Split-Cores mit exponierter ACID-Sequenz können mit Fremdproteinen beladen werden, an die das komplementäre BASE-Peptid gekoppelt ist. Dies ist eine von mehreren Möglichkeiten, split-Core CLPs nachträglich mit Fremdmolekülen der Wahl zu beladen.

Durch die oben näher erläuterten Kupplungsmechanismen ist es möglich, CLPs mit einem Teil der Kupplung bereitzustellen. Das an den anderen Teil der Kupplung fusionierte Protein kann leicht an die CLPs gekoppelt werden. Auf diese Art können beispielsweise nicht peptidartige Strukturen dem Immunsystem präsentiert werden.

e) Simultane Fusion unterschiedlicher Fremdmoleküle an coreN und coreC

In den bisherigen Beispielen wurde jeweils *eine* Fremdsequenz an entweder coreN oder coreC fusioniert. Grundsätzlich können auch *zwei unterschiedliche* Fremdmoleküle simultan an coreN und coreC fusioniert werden; dies könnten bspw. die beiden Untereinheiten eines heterodimeren Fremdproteins sein. Als Modell wurde ein zweigeteiltes GFP benutzt, wovon das eine Segment, die ersten 10 der 11 β -Stränge von GFP enthaltend, an coreN und das zweite Segment, den 11. β -Strang von GFP enthaltend, an coreC fusioniert wurde. Ko-Expression von einem Typ1-Vektor führte zur Entstehung grün fluoreszierender CLPs. Somit

hatten sich sowohl die coreN- und coreC-Domänen zu einer assemblierungsfähigen CLP-Struktur zusammengelagert wie auch die beiden GFP-Teile zu einer korrekten 3D-Struktur unter Ausbildung des GFP-Chromophors ergänzt.

Das Beispiel zeigt, dass die simultane Fusion unterschiedlicher Fremdsequenzen an coreN und coreC unter Ausbildung funktioneller Strukturen sowohl des split-Core-Trägers wie auch der beiden unterschiedlichen an coreN und coreC fusionierten Fremdsequenzen möglich ist.

Beispiel 12

Split-Core-CLPs, welche die GB1-Domäne von Protein G auf der Oberfläche präsentieren

Als weiteres konkretes Beispiel wurde die sog. GB1 Domäne aus dem mit Protein A funktionell verwandten *Streptococcus spp.* Immunglobulin-Bindeprotein "Protein G" in das HBV split-Core System eingeführt. Protein G besteht ebenfalls aus mehreren ähnlichen Domänen. Protein G bindet wie Protein A an Immunglobuline, hat jedoch andere Spezifitäten bez. Speziesherkunft und Subtypen der IgGs. Wegen dieser Komplementarität zwischen Protein G und Protein A werden beide als kommerzielle Reagenzien für immunologische Nachweise angeboten, die auf der Bindung an unterschiedlichen Immunglobuline beruhen. Wie Protein A bindet auch Protein G und die von ihm abgeleitete GB1-Domäne den konstanten (Fc) Teil des Antikörpers, der variable, Antigen-erkennende Teil des Antikörpers bleibt für die Bindung an das Antigen zugänglich. Somit kann eine Vielzahl unterschiedlicher Antikörper gebunden werden.

In diesem Beispiel wurde die GB1-Domäne in das split-core-System eingeführt und gezeigt, dass (i) CLPs gebildet werden und (ii), dass diese CLPs - aber nicht Kontroll-CLPs ohne GB1 - in der Lage sind, unterschiedliche Antikörper zu binden.

Analog den zuvor genannten Beispielen wurde die GB1-Domäne genetisch mit coreN bzw. mit coreC fusioniert und mit den jeweils fehlenden coreC bzw. coreN-Fragmenten ko-exprimiert.

Die spezifische AS-Sequenz entspricht den AS 229 bis 284 des Protein G Precursor-Proteins (Swiss Prot Accession: P06654); die 3D-Struktur ist bekannt (PDB Accession:

1PGA; Ref: Gallagher T, Alexander P, Bryan P, Gilliland GL (1994) Two crystal structures of the B1 immunoglobulin-binding domain of streptococcal protein G and comparison with NMR Biochemistry 33: 4721-4729)

coreD78 - GGGGSGGGGTQ -

YKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTDATKTFTVTEDP

Die Verknüpfung an coreN erfolgte über einen G4SG4TQ-Linker; die vorletzte AS K wurde durch D (Kleinschreibung in der Sequenz) ersetzt; der vorhergehende E-Rest ist die letzte in der Röntgenstruktur sichtbare AS; durch den K>D-Austausch lässt sich auf Nukleotid-Ebene eine BamHI-Schnittstelle einführen (GAg gat cct TAG); diese befindet sich damit an homologer Position zu den vorbeschriebenen Konstrukten.

Wie in den anderen Beispielen kam es zur effizienten Bildung von Partikeln, nachgewiesen durch Sucrose-Gradienten-Sedimentation, Laufverhalten im nativen Agarosegel, und durch Elektronen-Mikroskopie.

Zum Nachweis der Interaktionsfähigkeit der CLP-getragenen GB1-Domänen wurden die Partikel mit Immunglobulinen inkubiert, anschließend erneut im Sucrose-Gradienten sedimentiert; dabei kam es zur eindeutigen Ko-Sedimentation der Antikörper mit den Partikeln. Zusätzlich ließ sich die IgG-Bindung dadurch nachweisen, dass das coreN-GB1 Fragment auf Western Blots nach SDS-Gelelektrophorese direkt mit Sekundär-Antikörper-Konjugaten reagierte. Auch im Kontext der Fusion an coreN war daher das modifizierte GB1 in der Lage, effizient zu renaturieren und an seinen Liganden, IgG, zu binden.

Zum Nachweis, dass tatsächlich das Partikel-gebundene GB1 Immunglobulin bindet, wurde ein Aliquot der entsprechenden Sucrose-Gradientenfraktion mit einem mit 5 nm Gold-markierten Antikörper inkubiert, ungebundener Antikörper durch erneute Sedimentation im Sucrosegradienten abgetrennt, und die Partikelfraktion elektronenmikroskopisch analysiert. Die elektronendichten Gold-Partikel zeigten die Bindung des Antikörpers an intakte CLPs.

Beispiel 13

Selbst-fluoreszierende GFP- und GFP-Varianten-haltige split-Core-CLPs, welche interaktionsfähige Fremdmoleküle präsentieren

Beispiel 11 e) erläutert Fusionen mit Bedeutung jenseits von Vakzine-Anwendungen, wobei ein in zwei Teilen vorliegendes GFP-Molekül (GFP β 1-10 und GFP β 11) unter Ausbildung der nativen GFP-Struktur (grüne Fluoreszenz) und unter Bildung von CLPs assoziiert, wenn GFP β 1-10 an coreN, und GFP β 11 an coreC fusioniert ist.

Im vorliegenden Beispiel wurden an GFP β 11 zusätzliche Fremdsequenzen fusioniert, nämlich die GB1-Domäne aus Protein G bzw. die PräS-Domänen der Oberflächenproteine des humanen HBV, des Enten-HBV, und des Reiher-HBV. Dies ist nur möglich, weil durch die split-Anordnung ein neuer N-Terminus an GFP β 11 entsteht, der zudem durch die spezifische Orientierung auf den split-Core-CLPs von der CLP-Oberfläche nach außen zeigt. Die Konstruktion der jeweiligen Expressionsplasmide erfolgte analog den zuvor beschriebenen Verfahren in split-Core-Vektoren, die für separate coreN und coreC-Fragmente kodieren, in diesem Fall bereits verknüpft mit GFP β 1-10 an coreN, und GFP β 11 an coreC.

Anhand der bisherigen Daten war erwartet worden, dass der N-Terminus des GFP β 11-Segments weg von der CLP-Oberfläche nach außen zeigen würde, und somit die Anwesenheit einer weiteren Fremd-domäne nicht grundsätzlich mit der Fähigkeit der coreN und coreC-Anteile zur Bildung einer Partikel-Struktur, und nicht mit der Fähigkeit der GFP β 1-10 und GFP β 11-Anteile zur Bildung der nativen GFP-Struktur interferieren würde. Das Ergebnis dieses Beispiels ist in Fig. 7 schematisch dargestellt. In dieser Figur zeigen die Teilabbildungen A-D folgendes:

A. Grundstruktur der beiden Teile des Fusionsproteins. GFP β 1-10 ist an coreN fusioniert, GFP β 11 an coreC. Da GFP β 11 einen neuen N-Terminus bereitstellt, kann an diesen eine weitere Domäne, hier GB1 fusioniert werden. B. Selbst-fluoreszierende CLPs entstehen durch Interaktion von coreN mit coreC (CLP-Bildung), und von GFP β 1-10 mit GFP β 11 (Bildung des GFP-Chromophors), wie bereits vorher gezeigt. Zusätzlich präsentieren diese jedoch GB1 in zugänglicher Form auf ihrer Oberfläche. C. An die GB1-Domänen können unterschiedliche Antikörper über ihren Fc-Teil gebunden werden. D. Über ihre unterschiedlichen variablen Teile können die Antikörper ihrerseits mit ihrem spezifischen Antigen interagieren. Split-Core-GFP-GB1-CLPs können somit den üblichen Fluoreszenz-markierten Sekundärantikörper in der indirekten Immunfluoreszenz ersetzen. Eine

Anwendung zur Fluoreszenz-Markierung von Tubulin in zwei unterschiedlichen Zellen ist in Abb. 7 gezeigt. Analog sind andere Anwendungen möglich, die auf der Bindung von Antikörper an Antigen beruhen.

Tatsächlich ließen sich die weiter modifizierten Fragmente effizient in *E. coli* ko-exprimieren und bildeten laut Sedimentation im Sucrose-Gradienten sowie Laufverhalten im nativen Agarosegel CLPs. Zusätzlich zeigten diese CLPs die typische GFP-Fluoreszenz, somit hatten sich auch die GFP-Anteile in korrekter Weise zusammengefunden. Das Beispiel zeigt, dass auch die GB1-Anteile korrekt gefaltet und auf der CLP-Oberfläche zugänglich vorlagen.

Zum Nachweis der Bindungsfähigkeit der selbstfluoreszierenden GB1-präsentierenden CLPs an Antigen-gebundene Antikörper wurden HeLa (aus Mensch) bzw. LMH-Zellen (aus Huhn) permeabilisiert, dann mit einem Maus-Erst-Antikörper gegen Tubulin inkubiert. In einem Fall wurden die Zellen konventionell mit einem chemisch Fluoreszenz-markierten (Cy2) Zweit-Antikörper (Ziege-anti-Maus), oder alternativ mit den selbst-fluoreszierenden, GB1-präsentierenden split-Core CLPs inkubiert, dann gewaschen; schließlich wurden die Zellen fluoreszenz-mikroskopisch observiert.

In beiden Fällen wurden vergleichbare Fluoreszenz-Bilder (wie erwartet, cytoplasmatische Anfärbung unter Aussparung des Zellkerns) erhalten. Somit müssen die GB1-Domänen zur Bindung an den an Tubulin gebundenen anti-Tubulin-Erst-Antikörper in der Lage gewesen sein.

Selbstfluoreszierende split-Core CLPs, welche GB1 präsentieren, können somit alternativ zu konventionellen Fluoreszenz-markierten Sekundär-Antikörper-Konjugaten zum Nachweis eines Antigen-gebundenen Erst-Antikörpers eingesetzt werden.

Dies belegt, dass die GB1-Domänen nach wie vor in der Lage sind, mit IgG zu interagieren, sie müssen daher korrekt gefaltet vorliegen. Zudem belegt es, dass die selbstfluoreszierenden, GB1-präsentierenden CLPs als generelles Detektionsreagenz (vergleichbar einem fluoreszenz-markierten Sekundärantikörper) eingesetzt werden können.

Analoge Konstrukte wurden mit Farbvarianten von GFP erhalten, in welchen bekannte Mutationen, die das Absorptions- und Emissionsspektrum verändern, durch gerichtete Mutagenese in das vorhandene GFP-Konstrukt eingeführt wurden. Spezifisch wurden gelb-fluoreszierende (Yellow fluorescent protein; YFP) und cyan-fluoreszierende (Cyan fluorescent protein; CFP) Varianten hergestellt. Beide bildeten CLPs, die die jeweils

typischen Absorptionsspektren zeigten. Somit können GB1-präsentierende split-Core-GFP-CLPs mit unterschiedlichen Chromophor-Eigenschaften erzeugt werden, die für Mehrfach-Immunfluoreszenz-Anwendungen geeignet sind.

Das split-core System erlaubt die Herstellung selbst-fluoreszierender CLPs, welche auf ihrer Oberfläche ein interaktionsfähiges weiteres Fremdmolekül präsentieren. Ist dieses Fremdmolekül GB1, so ist es weiterhin in der Lage, mit Immunglobulinen spezifisch zu interagieren, auch wenn diese an ihr Ziel-Antigen gebunden sind. Hieraus ergibt sich eine neue, über Vakzine-Anwendungen hinausgehende Anwendungsmöglichkeit, nämlich der direkte Nachweis, via Fluoreszenz, von unterschiedlichen Antigenen mittels der selbstfluoreszierenden CLPs. Besonders geeignet sind solche selbstfluoreszierenden, interaktionsfähigen Split-Core-CLPs für diagnostische und analytische Anwendungen.

Selbst-fluoreszierende split-Core-CLPs, welche andere Fremddomänen als GB1-aus Protein G präsentieren

Die o.g. Ergebnisse zeigen, dass auf split-Core-CLPs, welche zusätzlich ein zweigeteiltes GFP enthalten, eine weitere Fremddomäne, GB1, in funktioneller, d.h. mit dem spezifischen Liganden IgG interaktionsfähiger, Form präsentiert werden können. Allerdings ist GB1 mit weniger als 60 AS eine relativ kleine Domäne. Um die Generalisierbarkeit nachzuweisen, wurden homologe Konstrukte hergestellt, in denen GB1 durch die PräS-Domänen der großen Hüllproteine der HBVs von Mensch (HBV), Ente (DHBV) und Reiher (HHBV). Diese Domänen vermitteln die spezifische Bindung der jeweiligen Viren an ihre natürlichen Zielzellen, d.h. Hepatozyten der entsprechenden Wirtsspezies. Mit 108 AS für HBV, und ca. 160 AS für DHBV und HHBV PräS sind sie deutlich größer als GB1 und zudem in ihrer Sequenz von GB1, aber auch untereinander unterschiedlich.

Alle drei Domänen wurden analog GB1 in split-Core-GFP Fusionsproteine inseriert (vgl. Fig. 7; statt GB1 die entsprechenden PräS-Domänen). Alle 3 bildeten effizient CLPs, wie durch Sucrose-Gradienten Sedimentation, Laufverhalten in der nativen Agarose-Gelelektrophorese, sowie durch Elektronenmikroskopie nachgewiesen wurde; die CLPs zeigten die typische GFP-Fluoreszenz (d.h. die GFP-Anteile waren zur korrekten 3D-Struktur von GFP assoziiert); zudem reagierten die HBV-PräS- und DHBV-PräS-CLPs mit monoklonalen Antikörpern gegen HBV-PräS und DHBV-PräS; ein entsprechender Antikörper gegen das Reiher-Virus-PräS ist derzeit nicht verfügbar.

Eine weitere Anwendung ist das Aufspüren der zellulären Rezeptoren für die jeweiligen Viren. Es lässt sich annehmen, dass durch die große Anzahl (240) PräS-Domänen pro Partikel die CLPs eine enorm gesteigerte Avidität für die Rezeptoren besitzen, so dass diese stabil gebunden und so auch identifiziert werden können. Rezeptor-tragende Zellen würden via GFP-Fluoreszenz der CLPs mittels Fluoreszenz-Mikroskopie oder FACS identifiziert; solubilisierte Rezeptormoleküle aus solchen Zellen würden mit den CLPs inkubiert und sollten sich bei nachfolgender Sedimentation in Sucrose-Gradienten in den Partikel-typischen Gradienten-Fraktionen anreichern.

Beispiel 14

Split-CoreOspA CLPs mit OspA an coreN erzeugen höhere, mit OspA an coreC niedrigere Titer an *protektiven* anti-OspA Antikörpern als LipOspA

Wie in der Figur 5 gezeigt, lässt sich durch Verknüpfung an entweder coreN oder coreC die Orientierung eines präsentierten Fremdproteins gezielt beeinflussen. Im Fall von OspA wird dadurch entweder das C terminal gelegene Epitop des bekannt neutralisierenden monoklonalen Antikörpers LA-2 besonders gut exponiert (Fusion an coreN) oder stattdessen der N terminale OspA-Bereich, in dem keine bekannten neutralisierenden Epitope liegen (Fusion an coreC). Wie in der Figur 4 gezeigt, ergeben beide split-Core-OspA CLPs (dort Gruppe 2 für coreN-, und Gruppe 6 für coreC-Fusion) hohe Gesamt-anti-OspA Antikörpertiter, der Anteil LA-2-äquivalenter Antikörper ist jedoch - wie aus der Struktur erwartet - bei Fusion an coreN wesentlich höher.

Allerdings handelte es sich hierbei um in vitro Daten. Um direkt das protektive Potential der induzierten Antikörper in vivo zu bestimmen, wurde die Fähigkeit der jeweiligen Immunseren zur Neutralisation einer artifiziell gesetzten *B. burgdorferi* Infektion in einem etablierten Mausmodell erfasst (vgl. Nassal et al., European J. Immunol. 2005; S. 655-665). SCID-Mäuse wurden mit den entsprechenden Immunseren i.p. inokuliert, 2 Stunden später erfolgte s.c. challenge mit je 10^3 /Maus Spirochaeten. In ungeschützten Mäusen führt dies regelmäßig zur Infektion, die sich in Arthritis-artiger Symptomatik äußert, welche im linken und rechten Tibiotarsal-Gelenk semiquantitativ erfasst werden kann. Als Negativkontrolle diente eine Gruppe von 3 Mäusen, die lediglich Normal-Maus-Serum erhielten. Als Positivkontrolle dienten Gruppen von je 6 Mäusen, die entweder den gereinigten, bekannt neutralisierenden monoklonalen Antikörper LA2, oder mit LipOspA induzierte Antikörper erhielten. Die Testgruppen (ebenfalls je 6 Tiere) erhielten Immunseren, die durch split-CoreOspA mit OspA

an coreN bzw. dito an coreC erhalten worden waren. Um quantitative Unterschiede zu erfassen, erfolgte die passive Immunisierung einmal mit 5 µg und einmal mit 1 µg LA2 bzw. der diesen LA2-Mengen äquivalenten Volumina an Immunseren von LipOspA bzw. split-Core-OspA mit OspA an coreN vakzinierten Tieren. Die Ergebnisse sind in Fig. 8 dargestellt.

Wegen des geringen Anteils an LA2-äquivalenten Antikörpern in den mittels split-Core-OspA mit OspA an coreC erhaltenen Immunseren wurden hiervon äquivalente Volumina wie von split-Core-OspA mit OspA an coreN eingesetzt, ohne Rücksicht auf den LA2 äquivalenten Titer. Arthritis scores wurden an Tag 13, 17, 21, 28, 35, 45 und 52 bestimmt. Die Ergebnisse für Tag 52 in Figur 8 zusammengefasst.

Alle mit Normal-Maus-Serum inokulierten Tiere entwickelten, wie erwartet, Arthritis. Die Protektion durch den monoklonalen Antikörper LA-2 verringerte sich bei Dosis-Reduzierung von 5 auf 1 µg von 4/6 auf 1/6 Tiere, durch die LipOspA Immunseren wurden 5/6 Tieren geschützt. Passive Immunisierung mit via split-CoreOspA mit OspA an coreN erhaltenen Immunseren schützte alle (6/6) Tiere, auch bei der geringen Dosierung, vor Infektion. OspA an coreC hatte eine deutlich geringere protektive Aktivität (4/6 Tieren bei der hohen, 1/6 Tieren bei der niedrigen Dosierung).

Split-Core-OspA CLPs mit OspA an coreN führen zu hohen Titern an LA2-äquivalenten Antikörpern und diese sind in vivo protektiv. Das protektive Potential übersteigt dasjenige von LipOspA-induzierten Antikörpern. Split-Core-OspA-CLPs mit OspA an coreC induzieren hohe Gesamttiter an anti-OspA-Antikörpern, diese sind jedoch nur zu einem geringen Anteil LA-2-äquivalent und besitzen nur ein geringeres neutralisierendes Potential. Fusion eines Fremdantigens an coreN bewirkt somit eine qualitativ distinkte Immunantwort von der Fusion an coreC.

Beispiel 15

Cirumsporozoiten-Protein (CSP) des Malaria-Erregers *Plasmodium falciparum*

Voll-Längen CSP im split-Core-System induziert eine stärkere und breitere Immunantwort als die immundominante CSP-repeat-Sequenz im konventionellen, kontinuierlichen Core-System

Bisher war gezeigt worden, dass sich das komplette CSP mit einer Länge von 319 AS ausschließlich im split-Core-System unter CLP-Bildung exprimieren ließ; im konventionellen kontinuierlichen System war dies nicht möglich. Eine verkürzte Version ohne die Cysteine-reiche C terminal Domäne (nachfolgend CSPshort) ließ sich zwar im konventionellen kontinuierlichen System auch darstellen, das Protein zeigte jedoch nach wenigen Tagen eine ausgeprägte Präzipitationstendenz - im split-Core-System trat diese nicht auf, was überlegene protein-chemische Eigenschaften belegt.

Eine Besonderheit des CSP ist die Anwesenheit einer mehrfachen Wiederholung der Tetrapeptid-Motive NANP und NVDP (in der hier benutzten Vollängen-Version 24 NANP und 3 NVDP-Motive); diese sind bekannt immunogen und eine experimentelle Vakzine basierend auf dem konventionellen kontinuierlichen Core-System ist beschrieben. Für eine vergleichende Immunogenitätsstudie wurde daher ein entsprechendes konventionelles Konstrukt mit der repeat-Sequenz NANPNVDP(NANP)₃NVDP zwischen Core-AS D78 und S81 hergestellt, das - wie erwartet, da die Insertion klein ist - CLPs bildete. Dieses diente in einer Immunogenitätsstudie als Vergleich für Vollängen-CSP und das C terminal verkürzte CSPshort. Mäuse wurden mit jeweils gleichen Mengen der jeweiligen CLPs immunisiert (20 µg/Maus); nach 2, 4, 6 und 8 Wochen wurden die gebildeten Antikörper-Mengen per ELISA bestimmt; dann erfolgte eine boost-Immunisierung (analog der ersten), und die Titer wurden 2, 4, und 6 Wochen nach boost erneut bestimmt. Als Testantigene dienten rekombinantes CSP (als Fusionprotein aus E. coli), NANP und NVDP-Peptide, sowie rekombinantes HBcAg, um die B-Zellantwort gegen den Träger zu bestimmen. Die Antigene wurden auf ELISA-Platten immobilisiert, dann mit 2-fachen Verdünnungsreihen der Immunseren getestet. Die größtmögliche Verdünnung, die ein über dem Hintergrund liegendes Signal ergab, ist in der Figur 9 angegeben.

Hieraus ergeben sich die folgenden Schlussfolgerungen:

a) B-Zell-Antwort gegen rekombinantes CSP

Alle drei Immunogene ergeben eine äußerst starke Antwort gegen CSP; für Voll-Längen-CSP liegt diese jedoch noch 4-fach höher (Titer: 1 : 12 x 10⁶ vs. 1 : 3 x 10⁶ für repeat bzw. verkürztes CSP); dies bedeutet, dass eine 12-Millionen-fache Verdünnung (!) des Immunserums noch in der Lage ist, CSP Protein zu erkennen.

W3

b) B-Zell-Antworten gegen repeat-Peptide NANP und NVDP

Die Antworten gegen das NANP-Peptid sind erwartungsgemäß für alle Konstrukte ähnlich; Die Antworten gegen das weniger oft im repeat vorhandene NVDP-Peptid sind ähnlich, beim Voll-Längen-CSP-Konstrukt jedoch schon nach der ersten Immunisierung (ohne boost) deutlich höher (Titer $1 : 2.5 \times 10^4$ vs. $1 : 5 \times 10^3$).

c) B-Zell-Antworten gegen nicht im repeat enthaltene CSP-Sequenzen

Ausschließlich CSPshort und Voll-Längen-CSP induzieren eine Antwort gegen die Peptide CSP12 (EPPSDKHIEQYLKKIQNSLS; Pos. 246-264 im Voll-Längen split-Core-Konstrukt) und CSP8 (GNGIQVRIKPGSANKPKDELD; Pos. 274-294 im Voll-Längen split-Core-Konstrukt). Das Fehlen entsprechender Antworten nach Immunisierung mit dem repeat-Peptid im kontinuierlichem Core-System ist erwartet, da dort die CSP8 und CSP12 Sequenzen nicht enthalten sind. Die Bedeutung des Ergebnisses liegt darin, dass insbesondere das Voll-Längen-CSP ausschließlich im split-Core-System hergestellt werden kann; Mit dem split-Core-System können also zusätzliche Epitope angesprochen werden, die zur Schutzwirkung einer Impfung beitragen können.

d) B-Zell-Antworten gegen den Core-Träger

Sowohl CSPshort wie auch Voll-Längen-CSP im split-core-System führen zu einer ca. 25-fach geringeren Antwort gegen den Core-Träger als das repeat-Peptid im kontinuierlichen System (Titer $1 : 1,25 \times 10^5$ vs. 3×10^6). Da die erwünschte Antwort gegen das Fremdprotein mindestens genauso hoch (CSPshort) oder noch höher (Voll-Längen-CSP) ist wie bei Verwendung des repeat-Peptides im kontinuierlichen Core-System, ist die spezifische Immunogenität bez. der inserierten Fremdsequenz bei Verwendung des verkürzten, und besonders des Voll-Längen-CSP im split-Core-System wesentlich verbessert. Hinzu kommt, dass eine starke Antwort gegen den Core-Träger die Differenzial-Diagnostik von anti-HBsAg-positiven Personen (Infektion vs. anti-HBsAg durch Vakzinierung) erschwert; eine geringe anti-Core-Träger-Antwort ist daher erwünscht.

e) Voll-Längen-CSP im split-Core-System aktiviert T-Zellen

Zum Nachweis der Induktion einer T-Zell-Antwort wurden Milzzellen der jeweilig immunisierten Mäuse mit rec. HBcAg bzw. einem bekannten T-Zell-Peptid (HBc 120-140) oder mit rec. CSP bzw. den CSP-Peptiden NANP und CSP8 stimuliert, dann die IL-2-Produktion gemessen. Alle Konstrukte führten zum Auftreten von T-Zellen, die durch HBcAg, HBc120-140, rec. CSP und NANP aktivierbar waren. Ausschließlich Milzzellen von mit Voll-Längen-CSP im split-core-System immunisierten Mäusen waren jedoch durch das CSP8 Peptid zur IL-2-Produktion anzuregen.

Auch auf T-Zell-Ebene führt daher das Voll-Längen-CSP Konstrukt zu einer breiteren Antwort als das kontinuierliche Nur-repeat-Peptid-Konstrukt.

Universitätsklinikum Freiburg**Patentansprüche**

1. Split-Core-Trägersystem, das als getrennte Polypeptide die Core N- und die Core C-Domäne des Core-Proteins eines Hepatitis B-Virus und wenigstens ein Fremdmolekül, gegen das eine Immunantwort hervorgerufen werden sollen, aufweist, wobei das Fremdmolekül an den C-Terminus der Core N-Domäne oder an den N-Terminus der Core C-Domäne fusioniert ist und das Core-Protein capsid-like-particles binden kann und wobei das Fremdmolekül an eine Aminosäure des Core-Proteins fusioniert ist, die zwischen der Position 73 und 94 des Core-Proteins liegt.
2. Split-Core-Trägersystem nach Anspruch 1, dadurch gekennzeichnet, dass das Hepatitis B-Virus für Säugetiere spezifisch ist.
3. Split-Core-Trägersystem nach Anspruch 2, dadurch gekennzeichnet, dass das Core-Protein die Sequenz eines humanen Hepatitis B-Virus aufweist.
4. Split-Core-Trägersystem nach Anspruch 2, dadurch gekennzeichnet, dass das Core-Protein die Aminosäuresequenz des WHV aufweist.
5. Split-Core-Trägersystem nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, dass das Fremdmolekül eine Aminosäuresequenz eines Proteins eines pathogenen Bakteriums ist.
6. Split-Core-Trägersystem nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, dass das Fremdmolekül eine Aminosäuresequenz eines Proteins eines pathogenen Eukaryonten, insbesondere des Malaria-Erregers Plasmodium falciparum ist.
7. Split-Core-Trägersystem nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, dass das Fremdmolekül eine Aminosäuresequenz eines Proteins eines Virus ist.
8. Split-Core-Trägersystem nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, dass das Fremdmolekül eine Aminosäuresequenz eines Proteins mit eigenem Pathogenitäts-bezogenen Potential, insbesondere ein Tumormarkerprotein, ist.

Cf

9. Split-Core-Trägersystem nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, dass das Fremdmolekül ausgewählt ist aus der Gruppe bestehend aus

- a) der Sequenz des Biotin-Akzeptor-Peptides
- b) der Sequenz des ACID-Peptides
- c) der Sequenz der Z33-Domäne abgeleitet von Protein A
- d) der Sequenz der GB1-Domäne von Protein G.

10. Verfahren zur Herstellung eines Trägers für Fremdmoleküle, insbesondere Fremdproteine, gegen die eine Immunantwort induziert werden soll, der einerseits eine Proteinsequenz des Core-Antigens eines Hepatitis B-Virus aufweist und andererseits ein Fremdmolekül, insbesondere eine heterologe Proteinsequenz,

wobei das Fremdmolekül, insbesondere die heterologe Proteinsequenz nach der Position 73 oder vor der Position 94 der Hepatitis B-Coreregion an die Core N oder die Core C Domäne anfusioniert wird und,

wobei die Polypeptidkette des Trägers entweder nach der Domäne Core N oder vor der Domäne Core C unterbrochen wird, dadurch gekennzeichnet, dass

entweder die Domäne Core N und das Fremdmolekül, insbesondere die heterologe Proteinsequenz oder die Domäne Core C und das Fremdmolekül, insbesondere die heterologe Proteinsequenz separat als Fusionsproteine exprimiert werden

oder, dass zwischen Core N und Fremdmolekül, insbesondere heterologer Proteinsequenz oder zwischen Fremdmolekül, insbesondere heterologer Proteinsequenz und Core C eine Erkennungssequenz für eine Protease eingefügt wird und der Split-Core-Impfstoff nach der Expression, aber vor der Verwendung mit der Protease in zwei Polypeptide gespalten wird, und,

dass capsid-like particles gebildet werden.

11. Verfahren nach Anspruch 10, dadurch gekennzeichnet, dass die heterologe Proteinsequenz nach der Aminosäure 74 oder vor die Aminosäure 85 des Core-Antigens von Hepatitis B anfusioniert wird.

12. Verfahren nach einem der Ansprüche 10-11, dadurch gekennzeichnet, dass die

4A

heterologe Fremdproteinsequenz wenigstens 40 Aminosäuren lang ist.

13. Verfahren nach Anspruch 10, dadurch gekennzeichnet, dass die heterologe Fremdproteinsequenz wenigstens 120 Aminosäuren lang ist.

14. Verfahren nach einem der Ansprüche 9-12, dadurch gekennzeichnet, dass zwei separate Polypeptide mit Hilfe eines bicistronischen Vektors exprimiert werden, wobei das eine Polypeptid die Core N-Domäne und das andere Polypeptid die Core C-Domäne beinhaltet und die heterologe Proteinsequenz entweder mit dem C-Terminus der Core N-Domäne oder mit dem N-Terminus der Core C-Domäne fusioniert wird.

15. Arzneimittel, das Capsid-ähnliche Partikel aufweist, dadurch gekennzeichnet, dass diese Partikel ein Split-Core-Trägersystem nach einem der Ansprüche 1 bis 8 aufweisen.

16. Arzneimittel nach Anspruch 14, dadurch gekennzeichnet, dass es sich um einen Impfstoff handelt.

17. Diagnostisches Mittel, das Capsid-ähnliche Partikel aufweist, dadurch gekennzeichnet, dass diese Partikel ein Split-Core-Trägersystem nach einem der Ansprüche 1 bis 8 aufweisen.



Fig. 1: Schematische Darstellung der Coreprotein-Struktur

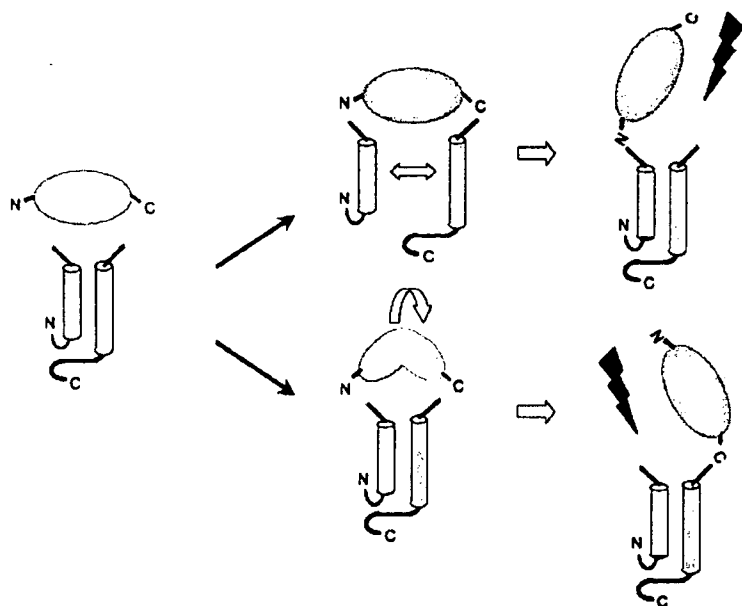


Fig. 2: Prinzip des split-Core-Systems

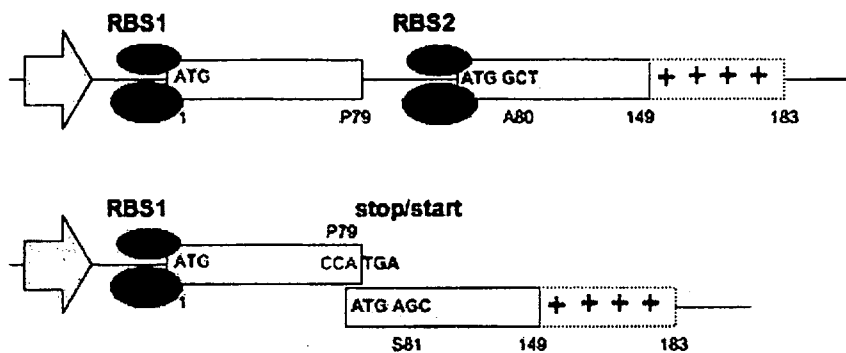
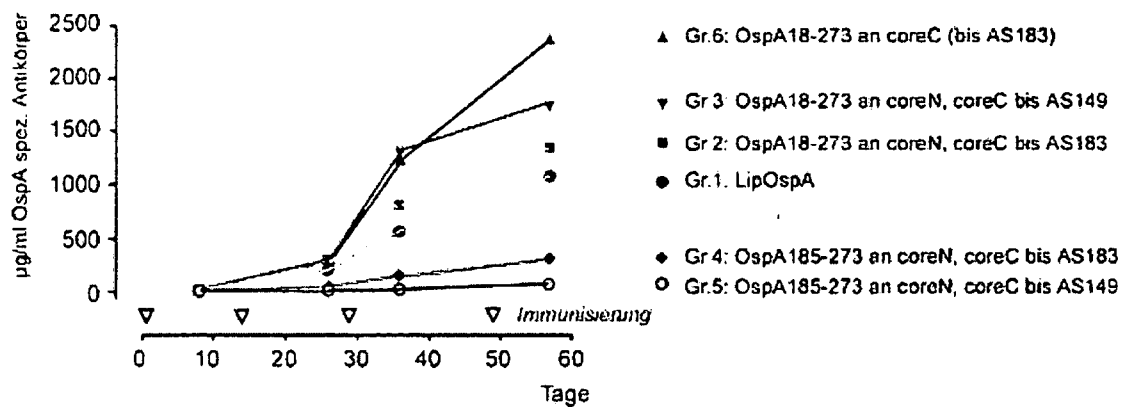


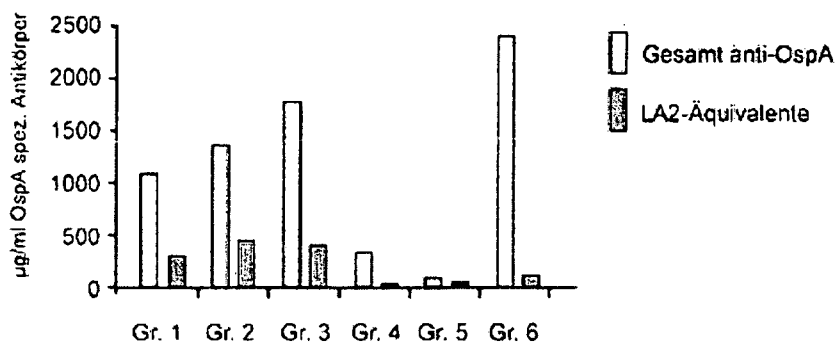
Fig. 3 Schematische Darstellung zweier Realisationen von bakteriellen Expressionsvektoren für annähernd äquimolare Produktion der beiden coreN- und coreC-Fragmente

Kinetik der Induktion OspA-spezifischer Antikörper



Gesamt anti-OspA vs. LA2-äquivalente Antikörper

(Serumproben von Blutung 4)



Figur 4

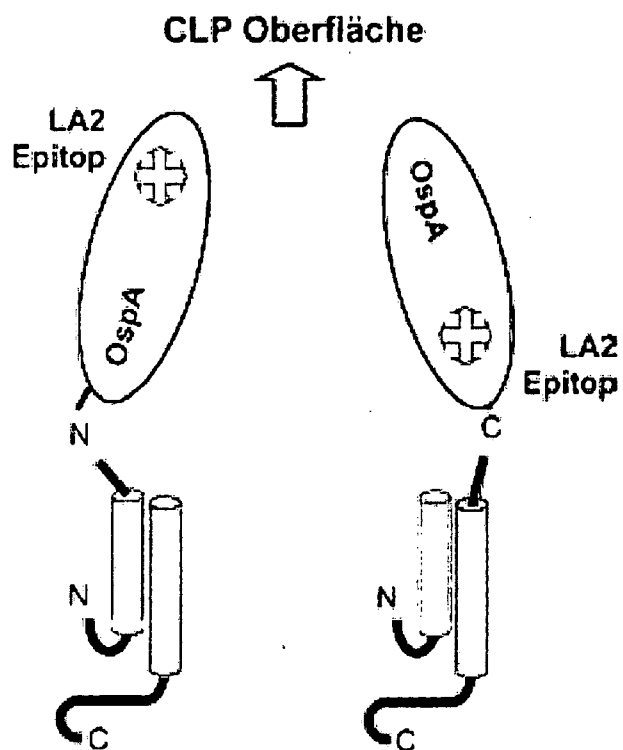


Fig. 5: Prinzip der regionsspezifisch gerichteten Induktion von Antikörpern gegen ein Fremdprotein im split-Core-System.

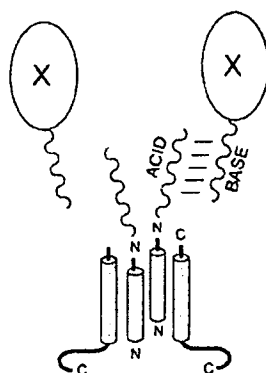


Fig. 6: Split-Core Fusion mit Interaktionsfähigem, flexibel exponiertem Fremdmolekül

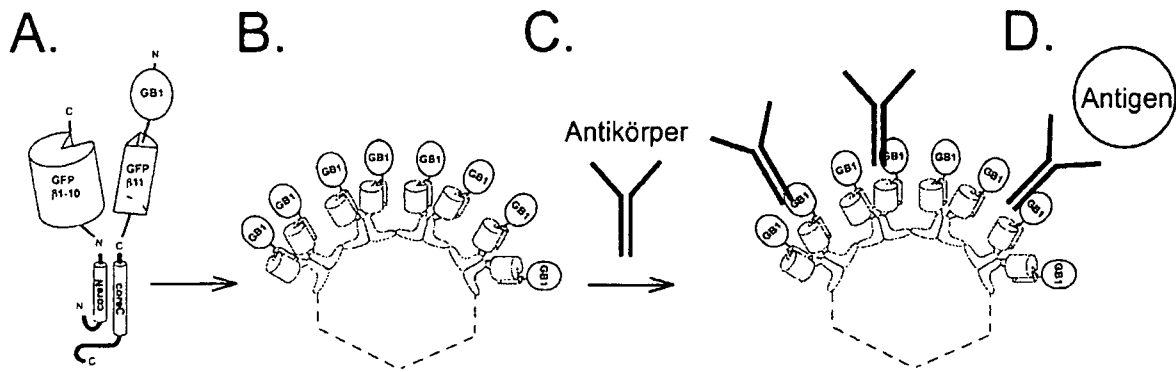


Fig. 7: Prinzip der selbst-fluoreszierenden split-Core-CLPs, welche ein interaktionsfähiges Fremdmolekül, hier GB1, auf ihrer Oberfläche präsentieren.

Mausgruppe passiv immunisiert mit	Arthritis, Tag 52		geschützt vor Infektion
	ausgeprägt	Schwach	
LA-2, 5 µg	1/6	1/6	4/6
LA-2, 1 µg	2/6	3/6	1/6
LipOspA, 5 µg	0/6	1/6	5/6
LipOspA, 1 µg	1/6	0/6	5/6
Split-coreOspA an coreN, 5 µg LA-2 äq. Antikörper	0/6	0/6	6/6
Split-coreOspA an coreN, 1 µg LA-2 äq. Antikörper	0/6	0/6	6/6
Split-coreOspA an coreC, Vol.. Äquiv. zu 5 µg LA-2 äq. mit OspA an coreN	2/6	0/6	4/6
Split-coreOspA an coreC, Vol.. Äquiv. zu 1 µg LA-2 äq. mit OspA an coreN	3/6	2/6	1/6
Maus-Normalserum	3/3	0/3	0/3

Figur 8: Protektives Potential unterschiedlicher anti-OspA-Immunisierungen gegen challenge von SCID-Mäusen mit B. burgdorferi

Immuno- gen	Woche (pb = post- boost)	Rec. CSP	NANP	NVDP	Rec. HBc	CSP12 Peptid	CSP8 Peptid
HBc149 CSP- repeat kont.	wk 8	Nicht best.	$1,25 \times 10^5$	5×10^3	$6,25 \times 10^5$	Nicht best.	Nicht best.
	wk 6 pb	3×10^5	3×10^5	$6,25 \times 10^5$	3×10^5	0	0
Split- Core149- CSP short	wk 8	Nicht best.	$1,25 \times 10^5$	$5,0 \times 10^3$	$1,25 \times 10^5$	Nicht best.	Nicht best.
	wk 6 pb	3×10^5	3×10^5	$6,25 \times 10^5$	$1,25 \times 10^5$	$2,5 \times 10^4$	0
Split- Core149- CSP Full- length	wk 8	Nicht best.	$1,25 \times 10^5$	$2,5 \times 10^4$	$2,5 \times 10^4$	Nicht best.	Nicht best.
	wk 6 pb	12×10^5	3×10^5	$6,25 \times 10^5$	$1,25 \times 10^5$	2×10^2	$2,5 \times 10^4$

Figur 9: B-Zell-Anworten gegen CSP und Core-Träger nach einmaligen bzw. zweimaliger Immunisierung von B10 Mäusen mit den angegebenen Immunogenen

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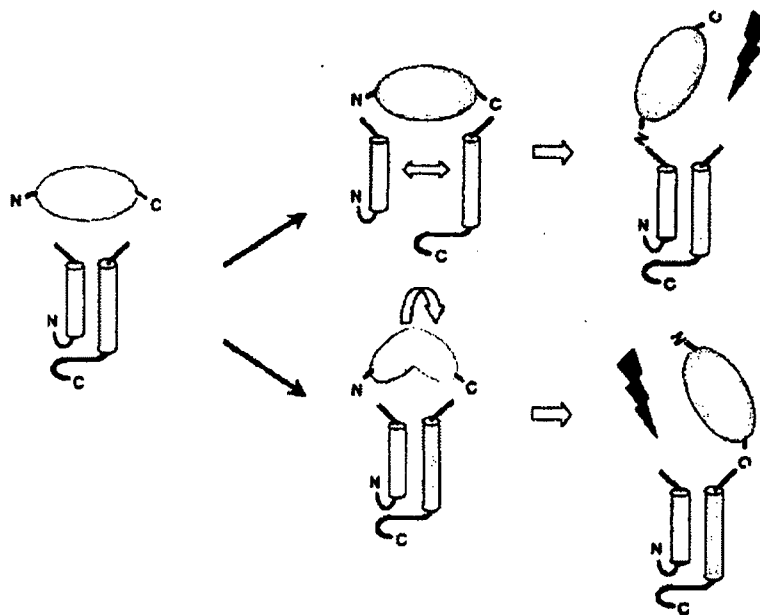
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(54) Título: PARTÍCULAS DE NÚCLEO DIVIDIDO PARA LA PRESENTACIÓN DE MOLÉCULAS EXTRAÑAS, ESPECIALMENTE PARA APLICACIONES EN VACUNAS, Y MÉTODO PARA PRODUCIRLAS

(57) Resumen:



Principio del sistema de núcleo dividido

Se describen sustancias portadoras de núcleo dividido ("split-core") que, como polipéptidos separados, tienen el dominio nuclear N y el dominio nuclear C de la proteína nuclear de un virus de la hepatitis B, y al menos una molécula extraña contra la cual ha de provocarse una respuesta inmune. De acuerdo con la invención, la molécula extraña, especialmente la secuencia de aminoácidos extraña heteróloga, está fusionada al extremo C del dominio nuclear N o el extremo N del dominio nuclear C, y la proteína nuclear puede formar partículas similares a cápsides. La invención también se relaciona con un método de producción asociado.

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PCT/EP2007/006190

PARTÍCULAS DE NÚCLEO DIVIDIDO PARA LA PRESENTACIÓN DE
MOLÉCULAS EXTRAÑAS, ESPECIALMENTE PARA APLICACIONES EN
VACUNAS, Y MÉTODO PARA PRODUCIRLAS

La presente invención se relaciona con nuevos portadores para vacunas, pero también para otras moléculas, basados en el antígeno nuclear del virus de la hepatitis B. En el antígeno nuclear del virus de la hepatitis se incorporan secuencias de aminoácidos extrañas que pueden provenir de patógenos. La vacuna ha de permitir la producción de anticuerpos contra estas secuencias proteicas, preferiblemente anticuerpos protectores o neutralizadores. Las partículas de la invención también pueden estimular la respuesta inmune celular (células T). La proteína nuclear de la hepatitis B tiene la propiedad de formar partículas similares a cápsides al agruparse varias copias. Estas partículas similares a cápsides (CLP) son particularmente apropiadas para la producción de vacunas, debido a que estimulan el sistema inmune, y en consecuencia, incrementan la producción de anticuerpos. En esta solicitud, la expresión "vacuna" preferiblemente hace referencia a un sistema portador que puede provocar respuestas inmunes humorales y celulares.

Las cápsides de los virus de la hepatitis B son

nanopartículas de simetría icosaédrica (diámetro: aproximadamente 30 nm) que consisten en 180 ó 240 copias de la proteína nuclear viral. La proteína nuclear se denomina HBcAg. Sirven como portadores particulados para moléculas extrañas, y preferiblemente se aplican como portadores antigénicos para vacunas que potencian la respuesta inmune. La modificación apropiada de la proteína nuclear permite presentar las moléculas extrañas en la superficie de la partícula (también dentro de ella, de ser necesario). La exposición en la superficie se optimiza al unir la molécula extraña a residuos de aa en la secuencia de aa de la proteína nuclear (una región de aproximadamente entre los aa 73 y 94), que incluye el epítopo inmunodominante para células B "c/e1" y tiene la mayor exposición en la estructura tridimensional de la superficie de la partícula (aa denota aminoácidos).

En WO 01/77158 se describen proteínas de fusión del antígeno nuclear de la hepatitis B donde se han insertado epítopos heterólogos, preferiblemente en la región entre las posiciones 61 y 90. En US 2003/0198649 se describen partículas del antígeno nuclear del virus de la hepatitis B donde los inmunógenos están conectados con la proteína nuclear del virus de la hepatitis B (HBV) a través de estructuras de unión. Kratz et al. [PNAS (1999), 1915-1920] describen la presentación de GFP (proteína fluorescente

verde) en la superficie de la proteína nuclear del HBV. En este caso, los aminoácidos 79 y 80 de la proteína nuclear fueron reemplazados por la secuencia de 238 aminoácidos de la GFP.

Nassal et al. [Eur. J. Immunol. (2005), pp. 655-665] describen un producto de fusión de la proteína OspA completa de *Borrelia burgdorferi* y la proteína de la cápside del virus de la hepatitis B. También en este caso, se reemplazaron los aminoácidos 79 y 80 por los aminoácidos 18 a 273 de OspA, pero con la incorporación de secuencias conectoras entre la proteína nuclear y OspA. Skamel et al. (Journal of Biological Chemistry, 2006, páginas 17474-17481) describen la presentación de la proteína OspC completa de *Borrelia burgdorferi* con la ayuda de partículas similares a cápsides del virus de la hepatitis B. Sin embargo, se ha descubierto que las proteínas nucleares modificadas de las soluciones descritas en los antecedentes técnicos ya no tienden a formar partículas similares a cápsides (CLP) de la manera deseada, por lo que ya no estimulan la respuesta inmune de modo satisfactorio.

Esto significa que, cuando se insertan moléculas extrañas basadas en péptidos o proteínas en la proteína nuclear por manipulación genética, la secuencia extraña se conecta a través de su extremo N y su extremo C a la proteína nuclear. Recientemente, se ha establecido que la

unión a ambos lados limita drásticamente el tipo y la selección de secuencias extrañas apropiadas.

El sistema de núcleo dividido ("split-core-system") que se describe en la presente permite superar estas limitaciones, ya que permite generar la proteína nuclear en dos partes separadas, que sorprendentemente se unen de manera espontánea y forman partículas de cápside de un modo similar a la cadena proteica continua. Las moléculas extrañas pueden fusionarse con el fragmento N-terminal ("coreN") o el fragmento C-terminal ("coreC"), por lo que quedan unidas a la proteína portadora solamente a través de un extremo. Como resultado, esencialmente se eliminan las limitaciones estructurales provocadas por la unión a ambos lados, relacionadas con la inserción de secuencias extrañas en la cadena peptídica continua de la proteína nuclear.

Por consiguiente, el sistema de núcleo dividido de la invención permite

- (i) presentar moléculas extrañas que, debido a su tamaño y/o su estructura, solamente pueden presentarse en el contexto continuo convencional de la proteína nuclear con un rendimiento muy bajo, o no pueden presentarse en lo absoluto;
- (ii) presentar proteínas extrañas heterodiméricas;
- (iii) presentar moléculas extrañas interactivas en una forma flexible y fácilmente accesible, que facilita

sustancialmente la interacción con las moléculas asociadas deseadas;

- (iv) presentar moléculas extrañas adicionales a través de los extremos N y C adicionales expuestos en la superficie, que están presentes en el sistema de núcleo dividido, pero no en el sistema de núcleo continuo convencional.

Los virus de la hepatitis B (HBV) son virus con cápside. La nucleocápside interna también se conoce como partícula nuclear ("núcleo"), y en términos serológicos, se define como el antígeno nuclear de la hepatitis B (HBcAg). El núcleo consiste en 180 ó 240 copias de la proteína nuclear de 183-185 aa (dependiendo del subtipo de HBV). La proteína nuclear puede expresarse de manera heteróloga (en bacterias, levaduras, células eucariotas), con la formación espontánea de "partículas similares a cápsides" (CLP). Estas últimas no son infecciosas, ya que no contienen el genoma viral o la cápside externa. Estas CLP también pueden obtenerse a partir de HBV de mamíferos (por ejemplo, de marmota, ardilla, y otros animales), relacionados más estrechamente, y HBV de aves (por ejemplo, pato, garza, y otras), relacionados de manera más distante. De acuerdo con la invención, en principio, es posible emplear cualquier secuencia de HBV, pero es preferible emplear secuencias de HBV capaces de infectar seres humanos.

Se ha descrito la secuencia de aminoácidos y la secuencia de nucleótidos de la proteína nuclear del HBV [Galibert et al., Nature (1979), pp. 646-650; Nassal, Gene (1988), páginas 279-294 o WO 01/77158], y puede hacerse referencia a estas publicaciones. Resulta preferible emplear la secuencia de la proteína nuclear del subtipo ayw, pero también es posible usar variantes similares, modificaciones de secuencias de HBV, tales como secuencias de otros HBV de mamíferos o HBV de aves. Las secuencias se almacenan en bibliotecas de genes de acceso público.

Aparte del virus de la hepatitis B que es patogénico para el ser humano (HBV en sentido estricto), hay una cantidad de virus de la hepatitis B relacionados, específicos para animales, por ejemplo, el HBV de la marmota norteamericana (nombre zoológico: *Marmota monax*; virus de la hepatitis B de la marmota = WHV), el HBV de la ardilla de California (nombre zoológico: *Spermophilus beecheyi*; virus de la hepatitis B de la ardilla = GSHV), y otros.

Estos virus poseen una estructura genética similar a la del HBV humano, pero presentan diferencias características en su secuencia de nucleótidos, y por lo tanto, en la secuencia de aminoácidos de sus proteínas.

El uso del núcleo del HBV humano como portador de vacunas tiene dos posibles limitaciones:

1. Los sujetos que sufren una infección crónica de HBV pueden presentar tolerancia potencial a antígenos del HBV (esta carencia de respuesta inmune es responsable en parte de la persistencia de la infección). El núcleo también actúa como antígeno dependiente de las células T. Por consiguiente, los epitopes de las células T en la secuencia de la proteína nuclear participan en la fuerte inmunogenicidad de las vacunas basadas en las CLP nucleares del HBV. Entonces, es posible que dichas vacunas no tengan efecto alguno sobre los individuos con infecciones crónicas de HBV, debido a la tolerancia de sus células T al núcleo del HBV. Sin embargo, no hay una tolerancia de esta índole al núcleo del WHV y los HBV de otros animales (véase Billaud JN. et al. *Advantages to the use of rodent hepadnavirus core proteins as vaccine platforms*. Vaccine. 19 de Febrero de 2007;25(9): 1593-606). Por ende, el uso de una proteína nuclear de HBV de origen animal como portador puede ser ventajoso para este grupo particular de sujetos que pueden ser potencialmente vacunados.

2. Debido a su inmunogenicidad extraordinariamente fuerte, el núcleo provoca una fuerte respuesta contra el núcleo en las células B (anti-HBcAg) en todos los sujetos con infecciones agudas o crónicas de HBV. Además, las infecciones curadas se caracterizan por la aparición de anticuerpos contra las proteínas nucleares del HBV (anti-

HBsAg). Las vacunas profilácticas actuales contra la infección de HBV se basan exclusivamente en la proteína de la cápside del HBV (HBsAg). Por lo tanto, una vacunación exitosa provoca la aparición de anti-HBsAg, al igual que una infección que ha sido neutralizada. La determinación del origen de la adquisición de anti-HBsAg por un sujeto, es decir, por infección o vacunación con HBsAg, resulta de interés para el diagnóstico en algunos casos, y puede realizarse detectando anti-HBcAg, que solamente aparece después de la infección.

Una vacuna basada en el núcleo del HBV humano también provocaría una determinada respuesta contra el núcleo (anti-HBcAg), además de la respuesta deseada contra la proteína extraña insertada. Al haber anti-HBsAg presente al mismo tiempo, se complicaría aún más la distinción entre la infección de HBV y la vacunación con HBsAg.

La producción de anti-HBcAg ha sido minimizada en el sistema de núcleo dividido, ya que el epítopo de mayor importancia, es decir, c/e1, ha sido separado físicamente, por lo que ya no es reconocido por la mayoría de los anticuerpos anti-HBcAg. Sin embargo, hay una respuesta anti-HBcAg residual contra otras regiones de la secuencia de la proteína nuclear. Esta respuesta puede prevenirse o reducirse aún más usando la proteína nuclear de un HBV no

humano, preferiblemente la proteína nuclear del WHV, como base portadora.

Los componentes esenciales del sistema portador de núcleo dividido de la invención son, en primer lugar, la proteína nuclear de un virus de la hepatitis B, y en segundo lugar, la molécula extraña contra la cual se produce una respuesta inmune.

En su realización más amplia, la proteína nuclear deriva de cualquier virus de la hepatitis B. Se conocen diversos virus de la hepatitis B. De acuerdo con la invención, es preferible emplear aquellas secuencias que derivan de virus de la hepatitis B que han sido aislados de mamíferos y son específicas de éstos. En particular, es preferible emplear HBV humano. Sin embargo, además de lo anterior, también es posible que los virus de la hepatitis B que provean la secuencia de la proteína nuclear se originen en otros animales, por ejemplo, aves, tales como patos o garzas.

El otro componente, es decir, la molécula extraña contra la cual ha de generarse una respuesta inmune, puede ser en principio cualquier molécula. En este caso, se prefiere el uso de secuencias de proteínas, y en particular, se prefieren aquellas secuencias de proteínas que se hallan en la superficie de los patógenos, y que entran en contacto con el sistema inmune. Si el sistema inmune es capaz de

producir anticuerpos contra las estructuras superficiales de estos patógenos, estas últimas son inactivadas después del contacto con los componentes individuales del sistema inmune del paciente vacunado, donde el invasor patogénico normalmente es destruido.

Al realizar una comparación entre las secuencias del virus de la hepatitis B de la marmota (WHV) disponibles en bases de datos y los aminoácidos de la proteína nuclear del HBV (subtipo ayw), es posible establecer una identidad de aproximadamente 60%. Las diferencias entre las secuencias son particularmente prominentes en la región entre las posiciones de aminoácidos 66 y 94, y esta secuencia incluye el epítipo c/e1, y en el sistema de núcleo dividido del HBV de la invención, el sitio de división entre la región N-terminal y la región C-terminal de la proteína nuclear. En una realización particularmente preferida, este sitio de división se halla entre los aminoácidos 79 y 81.

Los WHV de los sistemas portadores de núcleo dividido de la invención se prepararon separando la secuencia de la proteína nuclear del WHV a nivel de los aminoácidos, entre la posición 79 (codifica el aminoácido Glu) y la posición 80 (codifica el aminoácido glutamina). A nivel de los nucleótidos, se agregó una secuencia que codificaba el sitio de corte para BamH1 en el extremo carboxilo del segmento N-terminal de la proteína nuclear del WHV (abreviado WcoreN).

Le siguió un segundo sitio de unión a ribosomas y un sitio de corte para la endonucleasa de restricción Nde1, que a su vez, estaba superpuesto con el codón de inicio del segmento coreC del WHV (WcoreC). Esta disposición es idéntica a la disposición preferida en las construcciones de núcleo dividido preferidas del HBV. Esto permite transferir inserciones directamente de un sistema al otro, con la ayuda de una digestión con BamH1-Nde1. En el caso que se describió con anterioridad, WcoreN contiene una sustitución conservativa, es decir, E79D, y además, una P como aminoácido en el extremo C, debido al sitio de corte introducido para BamH1. Esto no tiene efectos adversos sobre la capacidad de formar partículas, y la prolina en el extremo C, debido a su resistencia a la proteasa, incrementa la estabilidad de la proteína dividida. Al igual que el HBV de núcleo dividido, WcoreC contiene una metionina de inicio adicional, en este caso, antes de Q80 (S81 en HBV). Esta construcción de núcleo dividido del WHV formó partículas de manera idéntica a la construcción de núcleo dividido del HBV correspondiente.

En otra realización de la presente invención, la región C-terminal y la región N-terminal de la proteína nuclear pueden originarse en distintos virus de la hepatitis B. En este caso, el sistema portador híbrido de núcleo dividido de la invención tiene esencialmente tres componentes: la región

N-terminal de la proteína nuclear de un virus de la hepatitis B, por ejemplo, un virus de la hepatitis B humano, la molécula extraña contra la cual puede provocarse una respuesta inmune, y la región C-terminal de la proteína nuclear originada en otro virus de la hepatitis B, por ejemplo, el WHV. Esta construcción híbrida de núcleo dividido también formó partículas sin problemas. Como las secuencias de aminoácidos de las proteínas nucleares de distintos virus de la hepatitis B son diferentes, los epitopes para las células B y T también difieren. Por lo tanto, el sistema híbrido de núcleo dividido puede influir específicamente en la respuesta inmune contra el portador particulado.

Los sistemas portadores híbridos de núcleo dividido preferidos de la invención se prepararon separando la secuencia de la proteína nuclear del WHV a nivel de los ácidos nucleicos, entre la posición 79 (codifica el aminoácido Glu) y la posición 80 (codifica el aminoácido glutamina). A nivel de los nucleótidos, se agregó una secuencia que codificaba un sitio de corte para BamH1 en el extremo carboxilo del segmento N-terminal de la proteína nuclear del WHV (abreviado WcoreN). Le siguió un segundo sitio de unión a ribosomas y un sitio de corte para la endonucleasa de restricción Nde1, que a su vez, estaba superpuesto con el codón de inicio del segmento coreC del

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El HBcAg natural y las CLP recombinantes del HBV son altamente inmunogénicos (se trata de antígenos independientes de las células T y dependientes de las células T). La estructura particulada simétrica y multimérica de la partícula nuclear es esencial en este caso. Dicha estructura puede observarse por microscopía electrónica de coloración negativa convencional, y también por microscopía electrónica con congelamiento. La naturaleza particulada puede detectarse por medios bioquímicos, por sedimentación en gradientes de sacarosa (coeficiente de

sedimentación: 60-80 S, dependiendo de la variante; compárese con los coeficientes de las proteínas monoméricas solubles: aproximadamente 1-5 S o las subunidades de los ribosomas eucariotas: 40S + 60S). Además, las partículas tienen un comportamiento migratorio distintivo en la electroforesis en gel nativo, en geles de agarosa, que es diferente del de las formas no particuladas. Esto posibilita determinar si pueden formarse las CLP o si hay proteínas presentes sin la estructura de las CLP.

La proteína nuclear del HBV tiene 183 ó 185 aminoácidos (la proteína del WHV tiene 187 aminoácidos) y consiste en dos dominios: los primeros 140 aa, aproximadamente, son necesarios y suficientes para el ensamblaje de las CLP (dominio de ensamblaje); la región del extremo C es rica en aa básicos y se une a los ácidos nucleicos. Una variante de la proteína nuclear con un corte en el extremo C (aa 1-149) se expresó en los sistemas de expresión originales en *E. coli* de una manera sustancialmente mejor que la proteína completa. Por lo tanto, las CLP de la proteína nuclear 1-149, es decir, sin el dominio de unión a aminoácidos, fueron utilizadas para realizar estudios de EM estructural de resolución media alta. De esta manera, fue posible determinar la estructura de las CLP y la proteína. Como se ha predicho en términos bioquímicos, la proteína nuclear

forma dímeros simétricos muy estables, los cuales se ensamblan para producir CLP que comprenden 90 ó 120 dímeros.

Las partículas nucleares tienen prolongaciones ("spikes") conspicuas; cada dímero forma una prolongación cuya estructura consiste en un haz de 4 hélices, donde cada monómero provee dos hélices α en el centro de la secuencia de aa. Los extremos de la prolongación comprenden los aminoácidos (aa) en la región de los aa 74-85. Esta región también contiene el epítipo inmunodominante para células B de HBcAg (epítipo c/el), y su inmunogenicidad particularmente elevada puede explicarse debido a la exposición de estos residuos en la superficie.

En la figura 1 se ilustra un diagrama de la estructura de la proteína nuclear. La secuencia primaria de la proteína nuclear del HBV se ilustra en el lado izquierdo de la figura 1. La proteína completa consiste en 183 aa (185 aa en algunos subtipos del HBV); solamente los 140 aa del extremo N, aproximadamente, son necesarios para la formación de las CLP (dominio de ensamblaje); una posición de aa preferida en el extremo C es 149; puede fusionarse una marca His en esta posición, y con cualquier aa subsiguiente, hasta el aa.183, sin alterar las CLP. El epítipo c/el está localizado en la región los aa 78-83, y forma parte del rizo entre las hélices α centrales (parte inferior izquierda). Las hélices mismas pueden estar cortadas, y es probable que los residuos

de aa hasta la posición Gly73 en el núcleo N y los residuos desde el aa Gly94 hacia delante en coreC sean los mínimos necesarios. En el lado derecho de la figura 1 se ilustra una representación diagramática de una proteína nuclear dimérica. Solamente se ilustran las dos hélices centrales de cada monómero. El epítopo c/e1 está localizado en la superficie de la partícula.

Para que sea posible formar partículas con éxito, la secuencia de aa del núcleo debe incluir al menos la posición 140, preferiblemente al menos la posición 149, pero también debe extenderse más allá, hasta los aa 183 ó 185 en el extremo C, dependiendo del subtipo de HBV. La secuencia de aa después de la posición 140, o mejor, 149, puede reemplazarse por secuencias extrañas, que luego quedan localizadas usualmente dentro de las CLP. En principio, no se producen anticuerpos contra estos aminoácidos extraños, pero éstos pueden tener otras funciones.

Debido a la inmunogenicidad extraordinariamente elevada de HBcAg, las CLP recombinantes del HBV son atractivas para usar como portadores potenciadores de la inmunogenicidad para antígenos extraños.

Una solución alternativa es la unión química de moléculas extrañas a CLP formadas con antelación. En la presente invención se provee una fusión genética de secuencias de aa extrañas con la proteína nuclear. Sobre la

base de evaluaciones empíricas, la región del epítopo c/el que se provee en la presente era la más apropiada para inducir respuestas potentes en las células B, lo que se estableció incluso antes de que se resolviera la estructura por cristalografía de rayos X. Ahora, sobre la base de dicha estructura, está claro que estas fusiones representan inserciones en el rizo entre las hélices centrales. Como este rizo no tiene una función estructural per se, al menos en principio, la estructura tridimensional de la proteína nuclear puede retenerse insertando secuencias extrañas. Dependiendo de la secuencia extraña insertada, algunas de estas proteínas de fusión puede continuar formando CLP regulares, aunque esto no está siempre garantizado, ya que la secuencia de aminoácidos extraña puede tener un efecto adverso sobre la formación de la estructura de la proteína de fusión. Por su parte, la estructura particulada es esencial para obtener una inmunogenicidad elevada.

Con anterioridad, se han insertado múltiples secuencias extrañas en la proteína nuclear, y en algunos casos, también se ha detectado una inmunogenicidad incrementada por medios experimentales [Ulrich et al., Adv. Virus Res. (1998), pp. 141-182]. Sin embargo, como muchos intentos de insertar secuencias extrañas relativamente largas, con más de aproximadamente 40 aa, han fallado (ya no se formaban partículas), se asumió que había una capacidad de recibir

secuencias extrañas de hasta 40 aa, o en un caso especial, hasta 120 aa, limitada de manera natural.

Se estableció que las secuencias de aminoácidos extrañas fusionadas en el extremo C y el extremo N de la proteína nuclear solamente formaban partículas si se cumplían al menos dos criterios esenciales:

- (i) la estructura 3D de la proteína extraña debe ser tal que su extremo N y su extremo C se ajusten con la posición espacial de los puntos de unión en la proteína nuclear (extremo C de la porción N-terminal del núcleo, es decir, los aa nucleares entre aproximadamente 1 y aproximadamente 78 [coreN]; extremo N de la porción C-terminal del núcleo, es decir, los aa nucleares entre aproximadamente 80 y 149 ó 183 [coreC]);
- (ii) si la proteína extraña propiamente dicha produce interacciones homoméricas (dímeros, trímeros, etcétera), la estructura de estos homo-oligómeros debe ajustarse igualmente con la estructura de las dos porciones de la proteína nuclear.

En consecuencia, las proteínas extrañas cuyos extremos N y C se hallan muy próximos en la estructura tridimensional nativa son apropiadas para la presentación en las proteínas de fusión descritas con anterioridad. GFP cumple ambos requerimientos, pero muchas otras proteínas no lo hacen; un ejemplo de estas últimas es la proteína de la superficie

externa A (OspA) del patógeno causante de la enfermedad de Lyme, *Borrelia burgdorferi*, cuyos extremos N y C se hallan en extremos opuestos de la estructura tridimensional larga.

La inserción de OspA u otras proteínas con una estructura desfavorable similar resulta en tensiones en la proteína de fusión. Puede suceder que la proteína extraña permanezca plegada correctamente e impida que se aproximen las dos porciones de la proteína nuclear, lo que evitaría su plegamiento y la dimerización subsiguiente, y por lo tanto, la formación de partículas; o puede suceder que el plegamiento de la porción de la proteína nuclear altere el plegamiento de la proteína extraña, que ya no sea nativa (> antigenicidad alterada), o de haber un plegamiento incorrecto masivo, se produzca una agregación.

En el caso de OspA, este problema fue solucionado solamente de manera parcial al utilizar secuencias conectoras muy largas. Estas secuencias conectoras representan un problema potencial, especialmente para las aplicaciones en vacunas, ya que podrían presentar una antigenicidad propia indeseable, que posiblemente resultaría en reacciones cruzadas dañinas, por ejemplo, con antígenos endógenos. Más aún, las proteínas de proteínas correspondientes solamente presentaron una capacidad muy limitada de formar CLP regulares.

Además, el impedimento estérico general podría ser un problema con las proteínas extrañas muy grandes o las proteínas extrañas que tuvieran una estructura que se desviara en gran medida de la globular, debido a la estructura dimérica de la proteína nuclear portadora y la superficie geoméricamente limitada de las partículas portadoras (el espacio disponible en la "superficie esférica" de las CLP es limitado). El tamaño máximo de la proteína extraña depende de diversos factores. Con el método de la invención, ha sido posible presentar una proteína extraña de aproximadamente 320 aa (CSP).

En consecuencia, la presente invención se relaciona con una vacuna de núcleo dividido que tiene polipéptidos separados, el dominio nuclear N y el dominio nuclear C de la proteína nuclear de un virus de la hepatitis B, y al menos una secuencia de aminoácidos extraña contra la cual puede provocarse una respuesta humoral, y cuando sea apropiado, una respuesta inmune celular, donde dicha secuencia de aminoácidos extraña está fusionada al extremo C del dominio nuclear N o el extremo N del dominio nuclear C, y donde la proteína nuclear es capaz de unirse a partículas similares a cápsides. Se prefiere un sistema portador que provoca la producción de anticuerpos neutralizadores.

Es esencial que la proteína nuclear en la vacuna de núcleo dividido de la invención esté interrumpida, es decir,

separada, en el epitope c/e1, vale decir, aproximadamente entre los aminoácidos 73 y 94. Luego, la secuencia de aminoácidos extraña puede fusionarse con el extremo C de la región nuclear N. En este caso, el extremo N de la parte nuclear C comienza en el aminoácido donde se separó la proteína nuclear N. También es posible suprimir parte del epitope c/e1, es decir, eliminar uno o más aminoácidos de la región entre los aminoácidos 73 y 94. Como alternativa, es posible producir la secuencia de aminoácidos extraña contra la cual han de generarse anticuerpos fusionada al extremo N de la región nuclear C. En este caso, la región C-terminal del núcleo N termina en el aminoácido donde se ha interrumpido la proteína nuclear. Es necesario que la vacuna de núcleo dividido retenga un efecto sustancial, es decir, que las partículas de la vacuna de núcleo dividido que contengan las secuencias de aminoácidos extrañas fusionadas aún puedan formar las partículas nucleares similares a cápsides. Esto puede evaluarse en un gradiente de sacarosa o una electroforesis en un gel de agarosa nativa. También es posible realizar una prueba con la ayuda de un microscopio electrónico.

La secuencia de aminoácidos extraña contra la cual han de producirse anticuerpos preferiblemente es una secuencia que se origina en estructuras superficiales de microorganismos. Estos microorganismos usualmente provocan

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enfermedades en los seres humanos. Si es posible producir anticuerpos neutralizadores contra estas estructuras, es posible que la defensa inmune pueda eliminar los microorganismos invasores muy rápidamente. Una bacteria para la cual se ha avanzado en el desarrollo de una vacuna es *Borrelia burgdorferi*, el patógeno causante de la enfermedad de Lyme. Las secuencias de aminoácidos extrañas que preferiblemente se emplean dentro del alcance de la invención son las proteínas superficiales de *Borrelia burgdorferi* OspA y OspC. Sin embargo, las secuencias de aminoácidos extrañas también pueden ser otras secuencias que se originen en organismos patogénicos. Un ejemplo de dichas secuencias es el patógeno de la malaria, *Plasmodium falciparum*.

Otras secuencias de aminoácidos extrañas contra las que pueden producirse anticuerpos son secuencias que se originan en virus. Se prefiere el uso de aquellas secuencias de aminoácidos que se originan en proteínas de virus que entran en contacto con el sistema inmune del organismo huésped. Estas proteínas son primariamente proteínas superficiales, ya que éstas usualmente son las primeras que entran en contacto en el sistema inmune del organismo huésped. Sin embargo, como alternativa, también pueden ser proteínas que sean liberadas durante el ciclo vital del virus. Dependiendo

del tipo de virus, pueden ser, por ejemplo, proteínas nucleares virales o proteínas de la nucleocápside.

Sin embargo, la presente vacuna de núcleo dividido puede usarse para generar no solamente anticuerpos contra secuencias de aminoácidos exógenas, sino también anticuerpos contra estructuras endógenas indeseables, tales como, por ejemplo, marcadores tumorales. En las células tumorales frecuentemente se expresan marcadores superficiales diferentes de los presentes en las células saludables. Por consiguiente, si estas secuencias de aminoácidos endógenas se presentan en la vacuna de núcleo dividido, se desencadenará una respuesta inmune que provocará que el cuerpo produzca cada vez más anticuerpos contra dichas células tumorales. Estos anticuerpos permiten que la defensa inmune reconozca rápidamente y finalmente elimine las células tumorales. Sin embargo, el sistema portador de la invención también puede inducir una respuesta inmune celular, que puede mejorar la eficiencia.

Esta solicitud también se relaciona con un método para preparar una vacuna contra una secuencia proteica heteróloga. La vacuna de núcleo dividido consiste en dos partes del antígeno nuclear de un virus de la hepatitis B, donde la secuencia proteica heteróloga está fusionada a la región nuclear N, o donde la proteína nuclear C está fusionada al extremo C de la secuencia proteica heteróloga.

La posición en la cadena de aminoácidos donde se separan los dos dominios está localizada entre la posición 73 y la posición 94. Los aminoácidos individuales de esta región de aminoácidos que representan el epítopo c/el pueden ser eliminados.

En principio, el método de la invención puede llevarse a cabo de dos maneras diferentes. Si las dos partes del antígeno de núcleo dividido solamente se expresan como una única cadena polipeptídica, se inserta una secuencia de reconocimiento para una proteasa de acuerdo con la invención en la posición deseada. Esto permite que el polipéptido expresado sea cortado por la proteasa en dos partes definidas. En esta realización, también es posible coexpresar la proteasa que ha de realizar el corte en el sitio de corte deseado. La secuencia genética que la codifica puede estar localizada en el mismo vector o en un vector separado.

Sin embargo, en otra realización preferida, los dos polipéptidos mencionados con anterioridad se expresan usando un vector especial. Se prefiere el uso de un vector "bicistrónico". Esto significa que una parte de la vacuna de núcleo dividido se expresa como una cadena polipeptídica y que hay un codón de detención en el extremo C. Sin embargo, poco después, los ribosomas pueden unirse nuevamente para que se exprese el segundo polipéptido. Esto permite asegurar

que ambas partes de la vacuna de núcleo dividido sean producidas en cantidades aproximadamente iguales, y no es necesario procurar que el organismo huésped no pierda alguno de los vectores, lo que podría resultar en la producción de solamente una parte de la vacuna de núcleo dividido.

La expresión "proteína de fusión" o "fusionados" denota que dos proteínas o polipéptidos diferentes están conectados uno con otro a través de un enlace peptídico. Estas proteínas de fusión se producen expresando las secuencias de ácidos nucleicos que las codifican, que están conectadas en serie.

En la presente invención, se descubrió que la unión de la proteína extraña insertada en ambos extremos, a través de sus extremos N y C, era el problema proteico-químico principal que limitaba la utilidad del sistema portador de HBV-CLP (específicamente como portador para vacunas, generalmente para la presentación de moléculas extrañas). La cantidad y el tipo de proteínas extrañas que pueden insertarse aumenta drásticamente si es posible disolver uno de los dos enlaces covalentes (en los extremos N o C del inserto) y aún se forman partículas, debido a que las porciones separadas de la proteína nuclear (de núcleo dividido) se encuentran y se pliegan correctamente. Este es un aspecto esencial de la presente invención.

En el diagrama de la figura 2 se ilustra que la inserción de una proteína extraña con una estructura tridimensional desfavorable (por ejemplo, OspA) puede prevenir la formación de una estructura de la proteína nuclear portadora que sea competente para la formación de partículas (vía superior). Como alternativa, la formación de la estructura tridimensional correcta de la proteína nuclear portadora puede prevenir la formación de la estructura tridimensional nativa de la proteína extraña (vía inferior). Esto tendría un efecto adverso sobre la unión de los anticuerpos deseados. Este problema estérico se elimina si se disuelve cualquiera de los dos enlaces covalentes (flecha) entre la proteína extraña insertada y el portador (en el extremo C o el extremo N del inserto). Una manera para lograr esto comprende el corte subsiguiente de una proteína de fusión continua, para lo cual es necesario introducir un sitio de fusión adicional para una proteasa específica. Entonces, es preferible expresar los dos fragmentos como entidades separadas desde el principio.

La solución de acuerdo con la invención tiene otras consecuencias:

- (i) como el epítopo c/el está localizado en la superficie de la partícula, se forman nuevos extremos N y C en esta superficie, los cuales pueden someterse a una derivatización adicional. Por ejemplo, una molécula

extraña X puede fusionarse al fragmento coreN, y una molécula extraña Y puede fusionarse al fragmento coreC. Una aplicación posible es la presentación de moléculas extrañas heterodiméricas. Sin embargo, en este caso es necesario tener en cuenta que las dos partes unidas en la fusión no produzcan un impedimento estérico.

- (ii) las subregiones específicas de una secuencia extraña insertada, por ejemplo, un epítopo particularmente importante, podrán orientarse hacia la superficie de la partícula o hacia el exterior, dependiendo de la unión a través del extremo N o el extremo C. Un ejemplo es el "epítopo LA2" de OspA de *B. burgdorferi*, del que se ha descubierto que está localizado en la región C-terminal. Los anticuerpos que reconocen este epítopo tienen una acción neutralizadora.

Esto también es importante para insertar secuencias extrañas que por su parte presentan superficies de interacción adicionales (la unión de terceras moléculas puede consultarse en los ejemplos). Como la secuencia extraña capaz de interactuar está unida solamente en un lado, no se fuerza una estructura tridimensional artificial en ella; sino que, dependiendo del tipo, es flexible o puede formar la estructura tridimensional correcta sin interferencias. Esto facilita sustancialmente las interacciones con los otros miembros de la interacción.

La presente invención también se relaciona con medicamentos, en particular vacunas, que comprenden partículas similares a cápsides basadas en el sistema de núcleo dividido.

Los medicamentos de la invención incluyen las partículas similares a cápsides de la invención, y preferiblemente tienen una influencia positiva sobre el sistema inmune. Usualmente, el sistema portador de núcleo dividido de la invención le presenta al sistema inmune una secuencia de aminoácidos extraña, contra la cual el sistema inmune produce anticuerpos, preferiblemente anticuerpos neutralizadores. Posteriormente, estos anticuerpos tienen una participación decisiva en el control del microorganismo o el virus que ha invadido el cuerpo, o contribuyen a la destrucción específica de las células tumorales indeseables.

En otra realización, el sistema portador de núcleo dividido de la invención puede emplearse como un medio de diagnóstico y/o análisis. En los ejemplos 12 y 13 se ilustran estas aplicaciones con mayor detalle. Con estos medios, es posible, por ejemplo, preparar partículas capaces de unirse a anticuerpos accesibles, con fluorescencia propia, que por su parte, pueden unirse a los antígenos y tornarlos visibles merced a su fluorescencia. En otra realización, los sistemas portadores de núcleo dividido de la invención pueden comprender secuencias de péptidos que se

unen específicamente a iones metálicos/lantánidos determinados. Si estos péptidos se incorporan en el sistema de núcleo dividido, se proveerán partículas que comprendan múltiples átomos/iones señalizadores de este tipo por partícula individual, por lo que serán más fáciles de detectar.

Ejemplo 1

Proteína nuclear tipo salvaje con una secuencia insertada para el reconocimiento por la proteasa del TEV

Se introdujo una secuencia de reconocimiento para la proteasa del virus del grabado del tabaco (TEV) en el epítipo c/el de la proteína nuclear tipo salvaje 1-149 (sustitución de los aa P79+A80 por GGGGT-ENLYFQGT-GGGG; los residuos de G son conectores para asegurar el acceso de la secuencia de reconocimiento a la proteasa). La proteína expresada por medios recombinantes formó partículas. Éstas fueron incubadas con la proteasa del TEV, y el éxito de la reacción de corte se verificó por PAGE en SDS. Después, la mezcla resultante de la digestión por la proteasa se hizo sedimentar en un gradiente de sacarosa. Hubo un corte virtualmente completo en la posición esperada, ya que se produjeron dos fragmentos con un tamaño prácticamente igual. A pesar del corte, la proteína sedimentó en el gradiente, aproximadamente en la misma posición que la proteína nuclear tipo salvaje 1-149 sin cortar. Se obtuvieron resultados

comparables usando una proteína de fusión basada en la proteína nuclear completa 1-183.

Ejemplo 2

Variantes de proteínas nucleares con secuencias extrañas insertadas relativamente grandes

A modo de ejemplo de una proteína de fusión con una inserción moderadamente grande, se insertó la secuencia de un péptido artificial, "ACID" (rodeada por conectores ricos en Gly, con 65 aa en total), que puede interactuar con un péptido complementario, "BASE", en el epítipo c/el [O'Shea et al., Curr. Biol. (1993), 656-667], seguida por la secuencia de reconocimiento para la proteasa del TEV GGGGSGGGVEDGGGGSGGGT-AQLEKELQALEKENAQLEWELQALEKELAQTG-ENLYFQGTGGGG.

La proteína de fusión formó CLP. Estas fueron aisladas e incubadas con la proteasa del TEV como se describió con anterioridad. También en este caso, hubo un corte específico, pero las partículas permanecieron intactas. En este caso, los fragmentos resultantes del corte con la proteasa del TEV tienen distintos tamaños, por lo que pueden ser distinguidos directamente en el gel de SDS. A pesar del corte, los fragmentos co-sedimentan en las fracciones del gradiente específicas para CLP.

Con este ejemplo se demuestra que las CLP compuestas por una proteína nuclear, con la inserción de un péptido de

65 aa (conector + ACID + sitio de corte para la proteasa del TEV), también pueden ser cortadas de manera específica, pero retienen la estructura particulada.

Ejemplo 3

Otro ejemplo detallado es un experimento correspondiente donde se usa una proteína nuclear que contiene la secuencia de aa 18-273 de la proteína OspA de *Borrelia burgdorferi*. La proteína correspondiente sin el sitio de corte para la proteasa del TEV se describe en Nassal et al., 2005; solamente una fracción de ella formó partículas regulares, y estos resultados fueron evidentes en una amplia distribución en el gradiente de sacarosa. Por lo tanto, la vacuna sin cortar solamente puede usarse en una extensión limitada. Más aún, esta vacuna sin cortar no podría aplicarse en seres humanos/con fines médicos, ya que serían necesarias secuencias conectoras adicionales, muy largas, con un potencial antigénico intrínseco indeseable, para obtener al menos una pequeña proporción de la preparación en forma de CLP.

Después de introducir un sitio de corte para la proteasa del TEV, la proteína de fusión se trató con la proteasa del TEV, al igual que en el ejemplo 2, y luego se hizo sedimentar en el gradiente de sacarosa. También en este caso, hubo un corte específico, pero éste no fue completo (presumiblemente, debido a un impedimento estérico provocado

por OspA, y a que el sitio de corte para la proteasa del TEV se hallaba menos accesible); y se clasificaron los fragmentos por medio de anticuerpos monoclonales que reconocían específicamente la porción del extremo N o el extremo C del núcleo, lo que resultó en la reacción del anticuerpo a-coreN con la proteína de fusión sin cortar y el fragmento obtenido con el corte en el extremo N. Aún así, el material parcialmente cortado presentó un comportamiento migratorio típico de una partícula. Estos datos permiten concluir que la formación de partículas se vio marcadamente mejorada por la apertura de cualquiera de las dos conexiones entre la proteína extraña insertada y el portador nuclear.

Con este ejemplo, se comprueba que una proteína nuclear de fusión con la inserción de una proteína extraña grande (OspA de 255 aa + conector + sitio de corte para la proteasa del TEV) puede ser cortada de manera específica (aunque con menor eficiencia). Si bien la proteína de fusión nuclear continua OspA descrita con anterioridad está distribuida ampliamente a lo largo del gradiente (Nassal et al., 2005), la proteína de fusión (parcialmente) cortada se halla concentrada de manera distinguishable en las fracciones del gradiente típicas de las partículas. Esta es una indicación de que la eficiencia de la formación de las partículas está siendo mejorada por el corte.

En otro experimento, se demostró que la proteasa del TEV coexpresada (a partir de un plásmido compatible) corta las proteínas nucleares de fusión que contienen sitios de corte para la proteasa del TEV, algunas veces con mayor eficiencia que la que podría observarse en el corte in vitro subsiguiente. También se detectó la formación de partículas por medio de un gradiente de sedimentación de sacarosa para las proteínas que ya habían sido cortadas en las bacterias.

En los ejemplos 1 a 3, se demostró que las CLP formadas con antelación permanecen intactas después del corte en la región del epítopo c/el. Esto puede ser explicado en términos estructurales, por el hecho de que el rizo conector entre las hélices formadoras de prolongaciones no tiene una función estructural per se, pero se trata de algo que no podría haberse previsto de este modo. Esto resultó en la realización más preferida, donde, en lugar del corte subsiguiente de la cadena proteica continua, las partes coreN y coreC se expresan directamente como fragmentos proteicos separados, proceso durante el cual obviamente entran en contacto de manera espontánea y forman partículas. Este abordaje tiene al menos tres ventajas sustanciales:

- (i) simplificación: el paso de corte adicional con la proteasa del TEV (o una proteasa diferente) ya no es necesario;

- (ii) no se necesita una secuencia peptídica adicional en la proteína de fusión para que haya un reconocimiento específico por parte de la proteasa;
- (iii) para que se formen partículas, aún si esto sucede solamente de manera parcial, con una proteína extraña desfavorable desde el punto de vista estérico, tal como OspA, se necesitan secuencias conectoras muy largas; este problema podría solucionarse si se evitaran los impedimentos estéricos, ya que no se necesitaría la unión a ambos lados con la proteína nuclear portadora.

Los puntos (ii) y (iii) son particularmente importantes para las aplicaciones en vacunas, ya que cualquier secuencia adicional puede resultar en consecuencias inmunológicas impredecibles (nuevos epitopes debido a secuencias adicionales, posiblemente reacciones cruzadas con epitopes endógenos).

Ejemplo 4

Construcciones de expresión para obtener una expresión aproximadamente equimolar de los fragmentos coreN y coreC

La cadena peptídica continua inevitablemente contiene cantidades equimolares de los productos de corte finales. Con una expresión separada, las dos partes también deberían ser producidas en cantidades aproximadamente equimolares para obtener un ensamblaje eficiente. En el primer

experimento, se emplearon dos plásmidos compatibles separados para expresar las dos partes de las proteínas portadoras, pero esto solamente tuvo un éxito moderado.

En una realización preferida, se emplean vectores bicistrónicos, que tienen la ventaja adicional de que permiten evitar la selección con otro antibiótico (para retener el segundo plásmido, que sería necesario en la primera alternativa).

Las **construcciones tipo 1** incluyen, detrás de un promotor compartido (en este caso, el promotor de la ARN polimerasa del fago T7), dos cassettes de expresión, cada uno con un sitio de unión a ribosomas (RBS; secuencia Shine-Dalgarno). En los vectores que se proveen en este caso, la traducción del fragmento coreN es detenida por un codón de detención artificial después del aa prolina 79 (P79), y un codón de inicio artificial antes del aa serina 81 (S81) permite que comience la traducción del segundo fragmento. Una estructura de operón de este tipo puede hallarse con frecuencia en bacterias ("ARNm policistrónicos"; los ribosomas pueden unirse a cada RBS en el ARNm de manera relativamente independiente para iniciar la traducción del gen localizado hacia el extremo 3'). En las construcciones que se proveen, el segundo RBS es una copia exacta del primer RBS, que deriva de los vectores pET originales; también son posibles otras secuencias RBS. El codón de

inicio ATG del segundo cistron es parte de un sitio de corte para Nde (CATATG), que facilita la clonación de secuencias extrañas.

Las **construcciones tipo 2** ("detención/inicio"; "stop/start") no contienen un segundo RBS separado, sino que hay un segundo codón de inicio para el gen hacia el extremo 3' (NNA TGA) superpuesto con el codón de detención del gen hacia el extremo 5' (NNN TGA), en un marco de lectura con una desviación de 1. En este caso, los ribosomas pueden leer el primer gen, y luego pueden recomenzar directamente en el codón ATG del segundo gen; esta disposición permite la co-expresión de dos genes; es posible hallar disposiciones de detención-inicio similares en algunos fagos que afectan bacterias. Dicha disposición también está implementada en algunos retrotransposones eucariotas distintos de LTR (unión ORF1/ORF2). Sobre la base de las secuencias conocidas, por ejemplo, en el operón Trp de *E. coli*, podrían ser igualmente apropiadas otras combinaciones de codones de detención/inicio adyacentes o superpuestos (por ejemplo, TAA TG, TGA TG, etcétera).

Los vectores de expresión preferidos se ilustran en un diagrama en la fig. 3.

Parte superior: Construcciones tipo 1. Un promotor, por ejemplo, para la ARN polimerasa T7, resulta en la transcripción de un ARNm bicistrónico (puede insertarse una

secuencia terminadora hacia el extremo 3', para obtener una terminación eficiente). El primer cistron codifica la secuencia de los aa 1-79 de la proteína nuclear del HBV. Un sitio de unión a ribosomas hacia el extremo 5' (RBS1) permite que comience la traducción (las subunidades ribosomales pequeña y grande están representadas como óvalos). Hay un codón de detención localizado más allá del extremo 3' del codón para P79. Hay otro RBS (RBS2), para iniciar la traducción del fragmento coreC, localizado en el extremo 5' del segundo cistron. Además, este fragmento posee su propio codón de inicio (ATG). En el ejemplo ilustrado, la secuencia coreC comienza con Ala80 y puede extenderse hasta la posición de aa 140, o mejor 149, o hasta el extremo auténtico, en la posición 183. La secuencia de aa desde la posición 140, o mejor 149, en adelante también puede ser reemplazada por secuencias extrañas.

Parte inferior: Construcciones tipo 2. Éstas solamente contienen un RBS antes del primer cistron. La traducción del fragmento coreC se realiza por reinicio, y el codón de inicio artificial de coreC (ATG) está superpuesto con el codón de detención de coreN (TGA), como se ilustra. En el ejemplo, la secuencia coreC comienza con Ser81.

Los aa 77, 78 y 79 de la proteína nuclear son glutamato (E; codones: GAA, GAG), aspartato (D; codones: GAC o GAT) y 79 prolina (P; codones: CCN). En las construcciones tipo 1

provistas, se seleccionó la secuencia de nucleótidos GAG GAT CCN para obtener la secuencia de aa EDP, que genera un sitio de corte para BamHI (GGATCC); este sitio de corte único para BamHI posibilita la clonación simple de distintos fragmentos de ADN que codifican coreN y coreC. El nucleótido N en el codón de prolina CCN no está definido; el codón de detención puede ser TAA, TAG o TGA.

La secuencia hasta el final del primer gen en las construcciones tipo 2 es G GAT CCa TGA. Esta secuencia también contiene un sitio de corte para BamHI (subrayado), para clonar distintos fragmentos de ADN que codifican coreN o coreC. P79 en las construcciones tipo 2 provistas debe tener el codón CCa, y el codón de detención debe ser TGA (> CCa TGa), para permitir que haya un codón de inicio ATG superpuesto para generar el segundo gen. El codón para el primer aa del segundo gen después del codón de inicio ATG debe comenzar con A. Otras secuencias de nucleótidos de detención/inicio serían TGA TG o TAA TG, como es el caso del operón Trp de *E. coli*, entre TrpE y TrpD, o bien TrpB y TrpA [Das y Yanofsky (1989), Nucl. Acids Res., pp. 9333-9340; Oppenheim y Yanofsky (1960), Genetics, pp. 785-795].

El esqueleto de los vectores provistos (pET28a2-xxx) se basa en el vector comercial pET-28, donde, sin embargo, el gen de resistencia a kanamicina ha sido reemplazado por el gen de resistencia a ampicilina. Sin embargo, ciertamente

también es posible aplicar otros esqueletos de vectores (distintos orígenes de replicación, distintos genes de resistencia, distintos promotores, distintos RBS). La disposición bicistrónica de los genes para coreN y coreC, por medio de dos RBS separados o mediante una disposición de detención/inicio, es esencial. En los experimentos realizados, fue posible expresar varias proteínas de núcleo dividido a partir de construcciones tipo 1 y tipo 2, aunque la cantidad de construcciones tipo 2 (detención/inicio) donde este fue el caso fue menor. Además, hay una menor variabilidad de secuencia posible en las construcciones tipo 2 (véase la descripción anterior). Por consiguiente, las construcciones tipo 1 tienen una aplicación más amplia.

El aa seleccionado para el extremo C de la porción coreN fue P79, ya que la prolina es particularmente resistente a las proteasas, por lo que permite minimizar la degradación del fragmento. Debido a la estructura tridimensional conocida y las variantes de supresión descritas en esta región central, puede asumirse que

- (i) el extremo del fragmento coreN podría estar localizado en la región que abarca los aa aproximadamente entre las posiciones 72 y 74, particularmente Gly73,
- (ii) el comienzo del fragmento coreC podría estar localizado en la región que abarca los aa aproximadamente entre las posiciones 78 y 86, posiblemente hasta Gly94, sin

que se obtuvieran resultados sustancialmente diferentes de los que se obtendrían usando los límites seleccionados en este caso (extremo de coreN en P79, comienzo de coreC en S81, con un codón de inicio ATG hacia el extremo 5').

Debido a los distintos mecanismos de transcripción y traducción, las construcciones descriptas no pueden aplicarse de esta manera para expresarlas en células eucariotas: es necesario usar un promotor eucariota en lugar de un promotor procariota (por ejemplo, CMV-ES DECIR, y muchos otros), y debe introducirse una señal de poliadenilación (por ejemplo, del virus SV40, o muchos otros) más allá del extremo 3' de los marcos abiertos de lectura.

En eucariotas, es posible obtener una expresión aproximadamente igual de coreN y coreC de las siguientes maneras:

- (i) mediante la co-transfección de dos plásmidos (uno para coreN, uno para coreC);
- (ii) mediante la transfección de un plásmido con dos cassettes de expresión independientes;
- (iii) mediante la transfección de un plásmido con ARNm funcionalmente bicistrónico; por ejemplo, el segundo gen podría ser expresado a partir de un sitio de ingreso a ribosomas interno hacia el extremo 5' (IRES);

(iv) una disposición de detención/inicio similar al vector tipo 2 también podría ser funcional en eucariotas.

Ejemplo 5

La expresión separada de fragmentos coreN y coreC tipo salvaje produce CLP intactas

Se expresó la proteína nuclear tipo salvaje por medio de vectores de expresión basados en el tipo 1 (figura 3) en células de *E. coli* BL21, en un caso, sobre la base de los aa 1-149, y en el otro, sobre la base de los aa 1-183. Ambos fragmentos esperados fueron producidos y ensamblados para obtener CLP intactas. Éstas fueron detectadas usando gradientes de sacarosa y electroforesis en gel de agarosa nativa. La formación de las CLP se detectó directamente, realizando una comparación por microscopía electrónica (coloración negativa) de partículas compuestas por una cadena continua de los aa 1-183, con una marca de His6 en el extremo C, con partículas compuestas por el sistema de núcleo dividido correspondiente. No se detectaron diferencias con esta resolución.

Las CLP del HBV tipo salvaje son muy robustas y resistentes a concentraciones molares de urea; las CLP compuestas por la proteína nuclear 1-149, con un corte en el extremo C, pueden disociarse en dímeros con urea 3-5 M, y pueden reasociarse nuevamente para formar CLP in vitro, mediante el cambio de las condiciones de amortiguación

100

(remoción de la urea, pH neutro, concentración de sal incrementada). En un estudio comparativo de la estabilidad de las CLP compuestas por núcleos divididos, se demostró una estabilidad similarmente elevada de las CLP de núcleo dividido. Esto indica que la afinidad mutua entre las porciones coreN y coreC es elevada e importante para el uso de dichas porciones como un sistema portador de moléculas extrañas.

Ejemplo 6

La expresión separada de fragmentos coreN fusionados a proteínas extrañas y fragmentos coreC tipo salvaje, y viceversa, permite producir CLP intactas

Se usó GFP fusionada a coreN o coreC como modelo para demostrar que el sistema de núcleo dividido era apropiado en principio para la formación de CLP y la presentación de proteínas extrañas. Se empleó un vector tipo 1 (segundo RBS) o tipo 2 (detención/inicio) para la construcción coreN. Todas las construcciones resultaron en la expresión de proteínas fluorescentes verdes que formaron partículas, de acuerdo con la sedimentación en gradientes de sacarosa.

Con el fin de comprobar también la asociación física entre los fragmentos coreN y coreC particulares, se sometieron alícuotas de las fracciones del gradiente a electroforesis en geles de agarosa nativa. En este caso, las partículas permanecieron intactas, al igual que el cromóforo

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de la GFP. Las fracciones de GFP en coreN y GFP en coreC producen una banda fluorescente verde distinguible; la proteína de fusión de GFP no ensamblada permaneció en las fracciones superiores del gradiente, y presentó un comportamiento migratorio diferente y una distribución más difusa (difusión más rápida en el gel debido a su estructura sustancialmente más pequeña, en comparación con las partículas compuestas por 180 ó 240 subunidades). CoreC también fue detectado con el anticuerpo α -coreC en la banda fluorescente verde (es decir, que contenía GFP), lo cual fue válido para GFP en coreN y GFP en coreC.

Con este ejemplo, se demuestran dos ítems:

- (i) En el sistema de núcleo dividido, puede fusionarse una proteína extraña de aproximadamente 240 aa, en este caso, GFP, tanto con coreN como con coreC, sin que esto interfiera con la formación de las CLP.
- (ii) Los vectores de núcleo dividido tipo 1 y tipo 2 son apropiados para la co-expresión de coreN y coreC con secuencias extrañas unidas por fusión.

Ejemplo 7

Presentación de proteínas extrañas de relevancia médica con CLP en el sistema de núcleo dividido, lo que por medios convencionales, resulta imposible o es posible en una extensión muy limitada

OspA de *Borrelia burgdorferi*

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Como se explicó inicialmente, la unión de la proteína extraña insertada a ambos lados con la proteína portadora, que era necesaria en el sistema precedente, resulta en restricciones topológicas de importancia. En contraste con la GFP, OspA no tiene un "ajuste" natural. Por consiguiente, se usó OspA como precedente para una proteína extraña con una estructura desfavorable en el sistema de núcleo dividido, y se la fusionó con coreN o coreC. En contraste con la construcción continua anterior [descrita por Nassal et al., 2005], que presentaba una distribución amplia en el gradiente, ambas proteínas de fusión se acumularon de manera distintiva en las fracciones típicas de las partículas. Con un análisis de microscopía electrónica, se demostró una eficiencia drásticamente mejorada para la formación de CLP, tanto para la fusión de OspA con coreN como para la fusión de OspA con coreC, en comparación con la construcción continua anterior.

Con este ejemplo, se demuestra que

- (i) el sistema de núcleo dividido permite presentar como CLP proteínas extrañas cuya estructura interfiere con la formación de CLP en el sistema de núcleo continuo convencional;
- (ii) el sistema de núcleo dividido permite formar CLP de manera eficiente, mediante la fusión de la proteína extraña a coreN y coreC.

De manera similar, también fue posible presentar OspC de *Borrelia burgdorferi* de manera exitosa en el sistema de núcleo dividido de la invención.

Ejemplo 8

Proteína del circumsporozoito del patógeno causante de la malaria, *Plasmodium falciparum*

Otra proteína patogénica de gran relevancia para las vacunas es la proteína del circumsporozoito (CSP) del patógeno causante de la malaria, *Plasmodium falciparum*. La CSP (la forma usada en este caso tenía una longitud total de 319 aa) contiene una repetición de aproximadamente 110 aa del motivo tetrapeptídico NANP/NVDP. La estructura de la CSP es desconocida; el dominio del extremo C, que tiene aproximadamente 50 aa y 4 residuos de Cys, probablemente (sobre la base de la homología de secuencia) se pliega de manera similar a las repeticiones de la trombospondina tipo 1. Aunque la repetición es inmunogénica, las otras regiones de la CSP posiblemente también contengan epitopes importantes. No ha sido posible preparar una CSP completa en forma de CLP usando el sistema de núcleo continuo convencional, pero esto fue fácilmente posible usando el sistema de núcleo dividido. Actualmente, hay datos no ambiguos disponibles para la fusión de CSP con coreN; la co-expresión con coreC (como las construcciones 1-149 y 1-183)

resulta en la producción eficiente de estructuras particuladas.

Se preparó una serie de construcciones (basadas en los núcleos 1-149 y 1-183) que solamente contenían la secuencia repetida o la CSP truncada sin el dominio rico en Cys, en cada caso, en el sistema de núcleo continuo o el sistema de núcleo; y adicionalmente, se preparó una CSP completa fusionada a coreN. Las preparaciones de CLP se usaron en un estudio comparativo de inmunogenicidad en ratones, que aún no ha sido completado. Los datos hasta el momento permiten concluir que la vacuna preparada en el sistema de núcleo dividido tiene propiedades superiores. Esto puede atribuirse al hecho de que la CSP completa solamente puede formar CLP cuando se aplica el sistema portador de la invención. En contraste, con otros sistemas, no se producen CLP. Como resultado, las vacunas de CSP preparadas con la ayuda del sistema portador de la invención deberían presentar una inmunogenicidad superior e inducir particularmente neutralizadores anticuerpos.

Ejemplo 9

Las CLP de núcleo dividido como portadores para proteínas extrañas mejoran la respuesta de las células B a las proteínas extrañas

En un estudio de inmunogenicidad en ratones, se comparan 5 construcciones que comprenden el núcleo-OspA en

el sistema de núcleo dividido con OspA modificada con lípidos (LipOspA) que no ha sido fusionada a un núcleo; las vacunas Lymerix comerciales están basadas en ella, y son las vacunas más avanzadas de uso actual en la enfermedad de Lyme. La porción de lípido en LipOspA (tris-palmitoil-cisteína (Pam3-Cys)) es esencial para esta inmunogenicidad relativamente alta, ya que OspA no modificada con lípidos solamente presenta una inmunogenicidad muy débil (véase Nassal et al., 2005). Tres de las construcciones que comprenden el núcleo-OspA contienen OspA completa (aa 18-273), y otras dos contienen OspA truncada (que comienza en el aa 185 y se extiende hasta el aa 273).

Para este propósito, se inmunizaron 6 grupos, cada uno con 5 ratones BALB/c, en 4 ocasiones, en cada caso con 10 μ g de antígeno (días 0, 14, 29, 49):

Grupo 1: LipOspA

Grupo 2: OspA completa (aa 18-273) en coreN, coreC
comprende los aa del núcleo 81 a 183

Grupo 3: OspA completa (aa 18-273) en coreN, coreC
comprende los aa del núcleo 81 a 149

Grupo 4: OspA truncada (aa 185-273) en coreN, coreC
comprende los aa del núcleo 81 a 183

Grupo 5: OspA truncada (aa 185-273) en coreN, coreC
comprende los aa del núcleo 81 to 149

Grupo 6: OspA completa (aa 18-273) en coreC hasta el aa del núcleo 183, coreN entre los aa 1 y 79.

Los anticuerpos inducidos fueron determinados tomando muestras de sangre el día -1 (= 1 día antes de la primera inmunización, es decir, suero preinmune) y los días 8, 26, 36 y 57. Los siguientes parámetros fueron determinados por medio de un ELISA: la cinética de la producción de anticuerpos específicos contra OspA; la proporción particular de anticuerpos equivalentes a LA2, donde LA2 es un anticuerpo monoclonal conocido como neutralizador, que reconoce un epítipo de conformación complejo de secuencias de aa discontinuas hacia el extremo C del aa 185 de OspA.

Los resultados de este experimento se ilustran en detalle en la fig. 4.

En la fig. 4A se ilustra la cinética de la inducción de anticuerpos anti-OspA totales (en μg de anticuerpo específico por ml de suero). Los sueros de los ratones inmunizados con las construcciones de núcleo dividido con OspA completa contienen titulaciones detectables de anti-OspA, aún después de la segunda inmunización, que son comparables a las que provoca LipOspA. Después de las 3ª y 4ª inmunizaciones, las titulaciones de anti-OspA en los ratones inmunizados con OspA completa de núcleo dividido excedieron marcadamente las de los ratones inmunizados con LipOspA; las titulaciones son particularmente elevadas

después de la inmunización con OspA18-273 en coreC (grupo 6). OspA truncada 185-273 en el contexto de core149 (grupo 5) y core183 (grupo 4) solamente provoca una respuesta específica contra OspA sustancialmente menor.

En la fig. 4B se comparan directamente las titulaciones de anticuerpos anti-OspA totales con las de anticuerpos dirigidos contra el epítipo neutralizador conocido LA2 en la región C-terminal de OspA. OspA completa en coreN, en el contexto de core149 y core183 (grupos 2 y 3, respectivamente), induce titulaciones de anticuerpos equivalentes a anti-LA2 más elevadas que LipOspA, y OspA completa en coreC produce titulaciones marcadamente menores. En la actualidad, se continúa con el estudio del contenido de anticuerpos neutralizadores particulares (protección contra el ataque del patógeno *B. burgdorferi*).

Con este ejemplo, se demuestra que las CLP de núcleo dividido inducen anticuerpos específicos contra la proteína extraña OspA completa presentada, y las titulaciones son mayores que con la vacuna establecida LipOspA, donde la porción lipídica es esencial para la inmunogenicidad. La proporción diferente de anticuerpos equivalentes a LA2 después de la inmunización con OspA en coreN (aproximadamente 30%) versus OspA en coreC (< 5%) permite demostrar que el tipo de unión afecta el tipo de anticuerpos producidos; véase el ejemplo 10.

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Ejemplo 10

Inducción específica de anticuerpos con especificidad de región contra una proteína extraña por medio de una fusión alternativa con coreN o coreC

Con proteínas cuyos extremos N y C no se hallan inmediatamente adyacentes, tal como en GFP, la fusión con coreN resulta en una orientación diferente con relación a la superficie de la partícula que la fusión con coreC (coreN: extremo C de la proteína extraña "out"; coreC: extremo N de la proteína extraña "out"). Cuando se las use como vacunas para células B, es de esperar que las unidades proteicas que se extiendan en mayor medida hacia el solvente provoquen una respuesta de anticuerpos más fuerte. Entonces, es posible inducir distintos anticuerpos con especificidad de región fusionando la proteína extraña con coreN (extremo C de la proteína extraña "out") o coreC (extremo N de la proteína extraña "out").

En el diagrama de la figura 5 se ilustra una manera posible para determinar qué parte de la molécula se extiende más allá de la superficie de una CLP formada con una proteína extraña que tiene una estructura elongada, tal como OspA en la presente, mediante la fusión con coreN o coreC. Un epítopo bien mapeado en OspA es LA2, que se halla en la región C-terminal y es reconocido por el anticuerpo monoclonal neutralizador LA2. Como era de esperarse, las CLP

de núcleo dividido con OspA en coreN (preparadas de manera que el extremo C de OspA quedara expuesto) indujeron una fuerte respuesta equivalente a LA2, y las CLP con OspA en coreC solamente indujeron una débil respuesta equivalente a LA2. Por el contrario, la respuesta a otras regiones de OspA se vio mejorada.

Se esperaba que una fusión de OspA como proteína extraña con coreN proveyera un buen acceso al epítopo LA2 en la región C-terminal de OspA, y por otro lado, se esperaba que una fusión con coreC proveyera un buen acceso a la región N-terminal de OspA, que no había sido bien caracterizada desde el punto de vista inmunológico hasta el momento. Con los datos mencionados con anterioridad, fue posible confirmar este concepto de manera destacable (se obtuvieron titulaciones anti-OspA totales muy altas, pero las titulaciones de anticuerpos equivalentes a LA2 para OspA en coreC fueron bajas).

Por ende, el sistema de núcleo dividido tiene una actividad potenciadora de la respuesta inmune elevada con la fusión de la proteína extraña con coreN y la fusión de la proteína extraña con coreC.

La especificidad de tipo/región de los anticuerpos inducidos puede controlarse seleccionando el sitio de unión (coreN versus coreC). Para obtener resultados óptimos en la vacunación, pueden emplearse mezclas de vacunas de núcleo

dividido, donde una parte de la secuencia de aminoácidos extraña heteróloga se fusione con la porción y la otra parte se fusione con la porción coreC, de manera de obtener una presentación óptima de diversos epitopes.

Ejemplo 11

Fusiones con significación más allá de las aplicaciones en vacunas

Aparte de la significación directa como portadores particulados potenciadores de la respuesta inmune para péptidos, y en particular, como vacunas antigénicas proteicas, pueden concebirse diversas aplicaciones para esta plataforma portadora. En cada caso, se colocan varias moléculas presentadas (180 ó 240) muy próximas, de manera simétrica. Si dichas moléculas son moléculas extrañas capaces de interactuar (las vacunas son un caso especial: interactúan con anticuerpos), la partícula que tiene múltiples copias de la molécula extraña presenta una actividad drásticamente incrementada en comparación con la molécula extraña monomérica (véanse los parámetros de las IgM naturales, y también los tetrámeros del MHC en el inmunodiagnóstico). Estas moléculas extrañas capaces de interactuar deben hallarse accesibles en la superficie de la CLP para que puedan reaccionar con los otros miembros de la interacción. El sistema de núcleo dividido facilita esto de

manera sustancial, en comparación con el sistema continuo convencional.

Se expresó una cantidad de secuencias extrañas modelo en el sistema de núcleo dividido, y todas las inserciones evaluadas formaron CLP. Las capacidades de interacción de algunas de ellas fueron detectadas directamente.

La disposición dividida permite que haya nuevos extremos accesibles en la superficie de la CLP, los cuales podrán ser sometidos a una derivatización adicional. En la figura 5 se ilustra un dímero de núcleo dividido con un péptido ACID fusionado a coreN, péptido que puede interactuar con el péptido complementario BASE. Una molécula X, con la cual se ha fusionado dicho péptido BASE, se fusiona a las CLP con ACID. La disposición dividida le confiere flexibilidad a ACID; ACID insertado en el sistema de núcleo continuo interactúa con el péptido BASE, pero como los péptidos ACID y BASE forman una hélice doble en forma de espiral enrollada [O'Shea et al., 1993], la estructura del núcleo portador se torna inestable y las CLP se desintegran. Sin embargo, en la disposición dividida, las CLP deberían permanecer estables. Esta unión puede generalizarse: una molécula capaz de interactuar, A, distinta del péptido ACID, puede fusionarse con coreN o coreC. Luego, las CLP correspondientes pueden interactuar con B, un miembro de interacción específico para A. Estos pares de miembros de

interacción son His6-Ni-NTa, el péptido aceptor de biotina-estreptavidina; Z33-inmunoglobulina.

A continuación, se explican con mayor detalle otras uniones posibles.

a) Marca de His6

Fusionada con coreN a través de un conector Gly2, forma partículas que se unen a Ni²⁺NTA agarosa. La marca de His se halla libremente accesible, en contraste con el sistema continuo. Un inserto comparable (His7) en c/el en el sistema de núcleo continuo no produce cantidades significativas de proteína, ya que el residuo de la marca de His6 no se halla accesible desde en este sitio debido a impedimentos estéricos.

b) Péptido aceptor de biotina (BA)

El aceptor de biotina (BA23) es un péptido artificial de 13 aa (GLNDIFEAQKIEWH) que es biotinilado por la biotina ligasa de *E. coli*, BirA. Para que haya una biotinilación eficiente, es necesario que haya un acceso libre, lo que se da el sistema de núcleo dividido, pero no en el sistema de núcleo continuo - y si se sucede, es solamente en una medida limitada. La proteína de fusión de núcleo dividido-BA forma CLP y es biotinilada en *E. coli*. La avidina/estreptavidina o sus conjugados pueden unirse a la biotina presentada por las CLP.

c) Dominio Z33 de la proteína A

La proteína A de *S. aureus* se une a las inmunoglobulinas con gran afinidad ("proteína A sefarosa" para la inmunoprecipitación). La proteína A consiste en 5 dominios de unión a Ig con estructuras similares. Z33 constituye uno solo de estos dominios, y tiene una secuencia de aa modificada con una longitud total de 33 aa. Z33 aún presenta una afinidad elevada por las Ig (con una Kd de 40 nm). Para que se dé la unión a las distintas Ig, es necesario que haya flexibilidad estructural, y más aún, acceso. La secuencia de Z33 se fusionó con coreN a través de una secuencia conectora larga:

GGGSGGGVEDGGGSGGGGT-FNMQQRRFYEALHDPNLNEEQRNAKIKSIREDP.

Se formaron CLP en el sistema de núcleo dividido. Después de agregar una inmunoglobulina marcada con FITC y realizar otra precipitación, se detectó una proporción de la fluorescencia en las fracciones típicas para partículas en el gradiente. Este no fue el caso cuando se usaron CLP basadas en proteínas nucleares tipo salvaje en lugar de la fusión Z33.

d) Inserto del péptido ACID

El péptido ACID insertado en el sistema de núcleo continuo interactúa con el péptido complementario BASE ("Velcro peptídico") mediante la formación de espirales enrolladas entre los péptidos [O'Shea et al., 1993], lo que

resulta en modificaciones masivas en la estructura nuclear, hasta su desintegración. Esto es provocado por el péptido ACID propiamente dicho, que adopta una estructura flexible que no afecta la estructura del núcleo. La adición del péptido BASE resulta en la interacción con la secuencia ACID, como resultado de lo cual, ambas secuencias peptídicas adoptan una conformación de hélice en espiral enrollada rígida. Surge tensión debido a la unión a ambos lados. Un péptido ACID fijado solamente a un lado debería poder interactuar sin tensión con el péptido BASE agregado o el péptido BASE fusionado a otros miembros. En cualquier caso, la estructura de partícula de la proteína de fusión ACID en el sistema de núcleo continuo convencional permanecería intacta después del corte de la conexión C-terminal con el portador nuclear por medio de la proteasa del TEV.

Los sistemas de núcleo dividido con una secuencia ACID expuesta pueden cargarse con proteínas extrañas, con las cuales se une el péptido complementario BASE. Esta es una de las alternativas posibles para cargar posteriormente las CLP de núcleo dividido con las moléculas extrañas seleccionadas.

Con los mecanismos de unión detallados con anterioridad, es posible proveer CLP que actúen como parte de la asociación. La proteína fusionada a la otra parte de la asociación puede unirse con facilidad a dichas CLP. De esta manera, es posible, por ejemplo, presentarle al sistema

immune estructuras no peptídicas.

e) Fusión simultánea de distintas proteínas extrañas con coreN y coreC

En cada uno de los ejemplos anteriores, se fusionó una secuencia extraña con coreN o coreC. En principio, también es posible fusionar dos moléculas extrañas diferentes con coreN y coreC de manera simultánea. Un ejemplo posible de esta situación es la fusión de dos subunidades de una proteína extraña heterodimérica. El modelo utilizado fue una GFP de dos partes, cuyo primer segmento, que contiene las primeras 10 de las 11 cadenas β de GFP, se fusionó con coreN, y el segundo segmento, que contiene la 11ª cadena β de GFP, se fusionó con coreC. La co-expresión a partir de un vector tipo 1 resultó en CLP con fluorescencia verde. Por consiguiente, los dominios coreN y coreC se combinaron para obtener una estructura de CLP capaz de ensamblarse, y las dos partes de GFP se complementaron para producir una estructura tridimensional correcta, lo que resultó en la formación del cromóforo de GFP.

Con este ejemplo, se demuestra que es posible realizar la fusión simultánea de distintas secuencias extrañas con coreN y coreC, para formar estructuras funcionales con el portador de núcleo dividido y las dos secuencias extrañas diferentes fusionadas con coreN y coreC.

Ejemplo 12

CLP de núcleo dividido que presentan el dominio GB1 de la proteína G en la superficie

Otro ejemplo específico comprendió la introducción del dominio "GB1" de la proteína capaz de unirse a la inmunoglobulina de *Streptococcus spp.*, la "proteína G", que está relacionada desde el punto de vista funcional con la proteína A, en el sistema de núcleo dividido del HBV. De la misma manera, la proteína G consiste en múltiples dominios similares. Como la proteína A, la proteína G se une a las inmunoglobulinas, pero presenta especificidades diferentes con relación al origen y los subtipos de las especies de IgG. Debido a esta complementariedad entre la proteína G y la proteína A, ambas se hallan disponibles comercialmente como reactivos para ensayos de detección inmunológica basados en la unión a distintas inmunoglobulinas. Como la proteína A, la proteína G y el dominio GB1 derivado de ella también se unen a la parte constante (Fc) del anticuerpo, de modo que la parte variable del anticuerpo, capaz de reconocer el antígeno, permanece accesible para unirse al antígeno. Por lo tanto, es posible realizar la unión con una variedad de anticuerpos diferentes.

En este ejemplo, se introdujo el dominio GB1 en el sistema de núcleo dividido, y se demostró que (i) se formaban CLP, y (ii) que estas CLP, pero no las CLP control sin GB1, eran capaces de unirse a distintos anticuerpos.

De una manera similar a los ejemplos mencionados con anterioridad, el dominio GB1 se fusionó por manipulación genética a coreN o coreC, y fue co-expresado con los fragmentos faltantes de coreC y coreN respectivos.

La secuencia de aa específica corresponde a los aa 229 a 284 de la proteína precursora de la proteína G (acceso Swiss Prot P06654); la estructura tridimensional es conocida (acceso PDB: 1PGA; Ref: Gallagher T, Alexander P, Bryan P, Gililand GL (1994) Two crystal structures of the B1 immunoglobulin-binding domain of streptococcal protein G and comparison with NMR Biochemistry 33: 4721-4729):

coreD78-GGGGSGGGGTQ-

YKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTEdP.

La unión con coreN se realizó a través de un conector G4SG4TQ; el penúltimo aa, K, fue reemplazado por D (en minúscula en la secuencia); el residuo E hacia el extremo C es el último aa visible en la estructura de rayos X; la sustitución K por D permite introducir un sitio de corte para BamHI a nivel de los nucleótidos (Gag gat cct TAG); por lo que esta última posición es homóloga a las construcciones descriptas con anterioridad.

Al igual que en los otros ejemplos, se formaron partículas de manera eficiente, lo cual fue detectado por sedimentación en un gradiente de sacarosa, analizando el

comportamiento migratorio en un gel de agarosa nativa y por microscopía electrónica.

La capacidad de interacción de los dominios GB1 en las CLP se detectó incubando las partículas con inmunoglobulinas, y luego haciéndolas sedimentar nuevamente en un gradiente de sacarosa. En el proceso, los anticuerpos co-sedimentaron claramente con las partículas. También se detectó la unión con IgG por el hecho de que el fragmento coreN-GB1 reaccionó directamente con los conjugados de anticuerpos secundarios en Western Blot, después de realizar una electroforesis en gel con SDS. Por ende, aún en el contexto de la fusión con coreN, el domino GB1 modificado fue capaz de realizar una renaturalización eficiente y unirse a su ligando, IgG.

Para demostrar que GB1 unido a las partículas es capaz de unirse efectivamente a la inmunoglobulina, se incubó una alícuota de la fracción correspondiente del gradiente de sacarosa con un anticuerpo de 5 nm marcado con oro. El anticuerpo no unido se eliminó con una nueva sedimentación en un gradiente de sacarosa, y la fracción de partículas se analizó por microscopía electrónica. Las partículas de oro con alta densidad electrónica sirvieron como indicación de la unión del anticuerpo a las CLP intactas.

Ejemplo 13

GFP con fluorescencia propia y variantes de GFP que contienen CLP de núcleo dividido que presentan moléculas extrañas capaces de interactuar

En el ejemplo 11 e) se ilustran fusiones que tienen una significación más allá de las aplicaciones en vacunas, que comprenden una molécula de GFP en dos partes (GFP β 1-10 y GFP β 11) que se asocian cuando GFP β 1-10 se fusiona con coreN y GFP β 11 se fusiona con coreC, para producir la estructura nativa de GFP (con fluorescencia verde) y formar CLP.

En este ejemplo, se fusionaron secuencias extrañas adicionales a GFP β 11, a saber, el dominio GB1 de la proteína G y los dominios preS de las proteínas superficiales del HBV humano, el HBV de pato y el HBV garza. Esto solamente es posible porque la disposición dividida produce un nuevo extremo N en GFP β 11, que además, sobresale de la superficie de la CLP, debido a la orientación específica de las CLP de núcleo dividido. Los plásmidos particulares empleados para la construcción se construyeron de manera similar a los métodos que se describieron con anterioridad, en vectores de núcleo dividido que codificaban fragmentos coreN y coreC separados, en este caso, ya unidos por GFP β 1-10 a coreN y por GFP β 11 a coreC.

Sobre la base de los datos previos, se esperaba que el extremo N del segmento GFP β 11 sobresaliera y quedara separado de la superficie de la CLP, por lo que la presencia

de otro dominio extraño no interferiría en principio con la capacidad de las porciones coreN y coreC de formar la estructura de una partícula, ni con la capacidad de las porciones GFP β 1-10 y GFP β 11 de formar la estructura nativa de GFP. El resultado de este ejemplo se ilustra en el diagrama de la fig. 7. En las partes A-D de esta figura se ilustran los siguientes detalles:

A. La estructura básica de las dos partes de la proteína de fusión. GFP β 1-10 está fusionado con coreN, GFP β 11 está fusionado con coreC. Como GFP β 11 provee un nuevo extremo N, con él puede fusionarse otro dominio, GB1 en este caso.

B. Se producen CLP con fluorescencia propia mediante la interacción de coreN con coreC (formación de CLP) y la interacción de GFP β 1-10 con GFP β 11 (formación del cromóforo de GFP), como se demostró con anterioridad. Sin embargo, también presentan GB1 en una forma accesible en su superficie.

C. Es posible unir distintos anticuerpos con los dominios GB1 a través de su parte Fc.

D. Por su parte, los anticuerpos pueden interactuar con sus antígenos específicos a través de sus partes variables diferentes.

Por consiguiente, las CLP de núcleo dividido GFP-GB1 pueden usarse en reemplazo del anticuerpo secundario usual, marcado con fluorescencia, en los estudios de

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inmunofluorescencia indirecta. En la fig. 7 se ilustra una aplicación en la marca fluorescente de tubulina en dos células diferentes. De manera similar, son factibles otras aplicaciones basadas en anticuerpos que se unen a antígenos.

En efecto, fue posible co-expresar los fragmentos sometidos a modificaciones adicionales de manera eficiente en *E. coli*, donde formaron CLP, de acuerdo con la sedimentación en un gradiente de sacarosa y el comportamiento migratorio en un gel de agarosa nativa. Además, estas CLP presentaron la fluorescencia típica de la GFP, lo que significa que las porciones de la GFP también se ensamblaron de manera correcta. Con este ejemplo, se demuestra que las porciones GB1 también se unieron correctamente y estuvieron accesibles en la superficie de la CLP.

La capacidad de las CLP con fluorescencia propia que presentan GB1 de unirse a anticuerpos unidos a antígenos se detectó permeabilizando células HeLa (humanas) o LMH (de pollo), que luego fueron incubadas con un anticuerpo primario de ratón contra tubulina. En un caso, las células se incubaron de manera convencional con un anticuerpo secundario (de cabra anti-ratón) marcado con fluorescencia por medios químicos (Cy2), o como alternativa, con las CLP de núcleo dividido con fluorescencia propia presentadoras de

GB1, y luego se lavaron. Finalmente, las células se observaron por microscopía de fluorescencia.

En ambos casos, se obtuvieron imágenes de fluorescencia comparables (como era de esperarse, la coloración se localizó en el citoplasma, excluyendo el núcleo). Por consiguiente, los dominios GB1 deben haber sido capaces de unirse a los anticuerpos primarios anti-tubulina unidos a la tubulina.

Por lo tanto, las CLP de núcleo dividido con fluorescencia propia que presentan GB1 pueden emplearse como una alternativa a los conjugados de anticuerpos secundarios marcados con fluorescencia usados comúnmente para detectar un anticuerpo primario unido a un antígeno.

Esto permite comprobar que los dominios GB1 aún son capaces de interactuar con la IgG, y que en consecuencia, deben estar plegados correctamente. Más aún, permite comprobar que las CLP con fluorescencia propia presentadoras de GB1 pueden emplearse como un reactivo de detección general (comparable a un anticuerpo secundario marcado con fluorescencia).

Se obtuvieron construcciones similares con variantes de color de GFP introduciendo, en la construcción de GFP anterior, mutaciones conocidas, que permiten alterar los espectros de absorbancia y emisión, por mutagénesis dirigida. Específicamente, se prepararon variantes con la

proteína fluorescente amarilla (YFP) y la proteína fluorescente cian (CFP). Ambas variantes formaron CLP, que en cada caso, presentaron los espectros de absorción típicos. Por lo tanto, es posible generar CLP de núcleo dividido que presentan GB1 con GFP con distintas propiedades cromofóricas, que son apropiadas para aplicaciones de multi-inmunofluorescencia.

El sistema de núcleo dividido permite preparar CLP con fluorescencia propia que presentan una molécula extraña adicional, capaz de interactuar en su superficie. Si es GB1, esta molécula extraña aún es capaz de interactuar específicamente con las inmunoglobulinas, incluso cuando éstas están unidas a su antígeno blanco. Como resultado, hay una nueva aplicación posible que va más allá de las aplicaciones en vacunas: la detección directa de distintos antígenos por fluorescencia mediante el uso de CLP con fluorescencia propia. Estas CLP de núcleo dividido que presentan fluorescencia propia y son capaces de interactuar son particularmente apropiadas para aplicaciones de diagnóstico y análisis.

CLP de núcleo dividido con fluorescencia propia que presentan dominios de proteína G extraños, distintos de GB1

A partir de los resultados anteriores, puede concluirse que es posible presentar otro dominio extraño, GB1, en una forma funcional, es decir, capaz de interactuar con su

ligando específico, IgG, en CLP de núcleo dividido que adicionalmente contienen una GFP de dos partes. Sin embargo, GB1, con menos de 60 aa, es un dominio relativamente pequeño. Con el fin de proveer una mayor capacidad de generalización, se prepararon construcciones homólogas donde se reemplazó GB1 por los dominios preS de las proteínas de la cápsula mayores de los HBV humano (HBV), de pato (DHBV) y garza (HHBV). Estos dominios median en la unión específica del virus respectivo a sus células blanco naturales, es decir, los hepatocitos de la especie huésped correspondiente. Con 108 aa en el HBV y aproximadamente 160 aa en el DHBV y el HHBV, los dominios preS son marcadamente más grandes que GB1, y más aún, sus secuencias son diferentes de la de GB1, pero también difieren entre sí.

Los tres dominios fueron insertados en las proteínas de fusión de núcleo dividido con GFP, de manera similar a GB1 (véase la fig. 7, pero reemplácese GB1 por los dominios preS correspondientes). Los tres formaron CLP de manera eficiente, lo cual fue detectado por sedimentación en gradiente de sacarosa, a partir de su comportamiento migratorio, por electroforesis gel de agarosa nativa y microscopía electrónica. Las CLP presentaron la fluorescencia típica de la GFP (es decir, las porciones de la GFP se habían asociado para proveer la estructura tridimensional correcta de la GFP). Más aún, las CLP con

HBV-preS y DHBV-preS reaccionaron con anticuerpos monoclonales contra HBV-preS y DHBV-preS; en la actualidad, no hay disponible un anticuerpo correspondiente contra el dominio preS del virus de garza.

Otra aplicación comprende identificar los receptores celulares para el virus particular. Debido a la gran cantidad (240) de dominios preS por partícula, puede asumirse que las CLP tienen una avidez mucho mayor por sus receptores, lo que permite unir estos últimos de manera estable, y también identificarlos de este modo. Las células que presentaran receptores serían identificadas a partir de la fluorescencia de GFP emitida por las CLP por microscopia de fluorescencia o FACS. Se incubarían moléculas receptoras solubilizadas de estas células con las CLP, las cuales posteriormente deberían acumularse en las fracciones típicas de las partículas en sedimentaciones en gradientes de sacarosa subsiguientes.

Ejemplo 14

Las CLP de núcleo dividido con OspA en coreN generan mayores titulaciones de anticuerpos protectores anti-OspA que LipOspA, las CLP de núcleo dividido con OspA en coreC generan titulaciones menores

Como se ilustra en la figura 5, la orientación de una proteína extraña presentada puede modificarse de manera deliberada al unirla a coreN o coreC. En el caso de OspA,

esto resulta en una exposición particularmente buena del epitope C-terminal del anticuerpo monoclonal LA-2, del que se sabe que es neutralizador (fusión con coreN), o la región N-terminal de OspA, que no contiene epitopes neutralizadores conocidos (fusión con coreC). Como se ilustra en la figura 4, las CLP de núcleo dividido con OspA (grupo 2 para la fusión con coreN y grupo 6 para la fusión con coreC) produjeron titulaciones de anticuerpos totales, pero la proporción de anticuerpos equivalentes a LA-2 es sustancialmente mayor con la fusión con coreN, lo que era de esperar teniendo en cuenta la estructura.

Sin embargo, en este caso, los datos son datos in vitro. Con el fin de determinar directamente el potencial protector de los anticuerpos inducidos in vivo, se registró la capacidad del suero inmune particular de neutralizar una infección de *B. burgdorferi* inducida por medios artificiales en un modelo establecido en ratón (véase Nassal et al., European. J. Immunol. 2005; pp. 655-665). Se sometieron ratones SCID a una inoculación i.p. con el suero inmune apropiado, a lo que siguió un ataque s.c. con 10^3 espiroquetas por ratón 2 horas más tarde. Usualmente, esto resulta en una infección en los ratones no protegidos, infección que se manifiesta con síntomas similares a los de la artritis, que pueden registrarse de manera semicuantitativa en las articulaciones tibiotalares

izquierda y derecha. Como control negativo, se usó un grupo de 3 ratones que solamente habían recibido suero normal de ratón. Como control positivo, se formaron grupos de 6 ratones, cada uno de los cuales recibió el anticuerpo monoclonal LA2 purificado, del que se sabe que es neutralizador, o anticuerpos inducidos por LipOspA. Los grupos de prueba (también de 6 animales cada uno) recibieron un suero inmune que había sido producido con un sistema de núcleo dividido de OspA en coreN o un sistema de núcleo dividido de OspA en coreC. Con el objeto de registrar las diferencias cuantitativas, se realizó una inmunización pasiva, primero con 5 μ g y luego con 1 μ g de LA2, con volúmenes de LA2 de suero inmune de animales vacunados con LipOspA equivalentes a las cantidades anteriores, o con el sistema de núcleo dividido de OspA en coreN. Los resultados se ilustran en la fig. 8.

Debido a la proporción de anticuerpos equivalentes a LA2 en el suero inmune producido con el sistema de núcleo dividido con OspA en coreC, se usaron volúmenes equivalentes del sistema de núcleo dividido con OspA en coreN, independientemente de la titulación equivalente de LA2. Se establecieron las calificaciones de la artritis los días 13, 17, 21, 28, 35, 45 y 52. Los resultados del día 52 se resumen en la figura 8.

Como era de esperarse, todos los animales inoculados con suero de ratón normal desarrollaron artritis. La protección debida al anticuerpo monoclonal LA-2 se redujo desde 4/6 hasta 1/6 animales, la reducción de la dosis fue desde 5 hasta 1 μ g, y 5/6 animales fueron protegidos por el suero inmune LipOspA. La inmunización pasiva con suero inmune obtenido con el sistema de núcleo dividido con OspA en coreN protegió a todos los animales (6/6) de la infección, aún con una dosificación baja. OspA en coreC tuvo una actividad de protección marcadamente inferior (4/6 animales como máximo, 1/6 animales con la dosificación más baja).

Las CLP de núcleo dividido con OspA en coreN producen titulaciones elevadas de anticuerpos equivalentes a LA2 que confieren protección in vivo. El potencial de protección excede el de los anticuerpos inducidos por LipOspA. Las CLP de núcleo dividido con OspA en coreC inducen titulaciones totales elevadas de anticuerpos anti-OspA; solamente una pequeña proporción de éstas son equivalentes a las de LA-2, y presentan un potencial de neutralización inferior. Por ende, la fusión de un antígeno extraño con coreN provoca una respuesta inmune que es cuantitativamente diferente de la que produce la fusión con coreC.

Ejemplo 15**Proteína del circumsporozoito (CSP) del patógeno causante de la malaria, *Plasmodium falciparum***

La CSP completa en el sistema de núcleo dividido induce una respuesta inmune que es más fuerte y más amplia que la que induce la secuencia repetida de la CSP inmunodominante en el sistema de núcleo continuo convencional.

Con anterioridad, se había demostrado que la CSP completa, con una longitud de 319 aa, solamente podía ser expresada en el sistema de núcleo dividido para formar CLP. Esto no era posible en el sistema continuo convencional. Aunque fue posible preparar una versión truncada sin el dominio C-terminal rico en cisteína (de aquí en adelante: CSPshort), también en el sistema continuo convencional, la proteína presentó una tendencia pronunciada a precipitar después de unos pocos días, lo que no ocurrió en el sistema de núcleo dividido, lo cual es evidencia de propiedades proteico-químicas superiores.

Una variante especial de CSP comprende una repetición múltiple de los motivos tetrapeptídicos NANP y NVDP (24 motivos NANP y 3 motivos NVDP en la versión completa utilizada en la presente), donde dichos motivos son inmunógenos conocidos, y se ha descrito una vacuna experimental basada en el sistema de núcleo continuo convencional. Entonces, un estudio comparativo de

(30)

inmunogenicidad comprendió la preparación de una construcción convencional correspondiente, donde la secuencia repetida NANPNVDP(NANP)₃NVDP se localizó entre los aa del núcleo D78 y S81, y que formó CLP, como era de esperarse, ya que el inserto es pequeño. Dicha construcción se usó en un estudio de inmunogenicidad, a modo de comparación con la CSP completa y CSPshort, con el corte C-terminal. En cada caso, se inmunizaron ratones con las mismas cantidades de las CLP particulares (20 µg/ratón), se determinaron las cantidades de anticuerpos producidos por ELISA después de 2, 4, 6 y 8 semanas, luego se realizó una inmunización de refuerzo (similar a la primera), y se determinaron las titulaciones nuevamente, 2, 4 y 6 semanas después del refuerzo. Como antígenos, se usó CSP recombinante (como proteína de fusión de *E. coli*), los péptidos NANP y NVDP, y HBcAg recombinante, con el fin de determinar la respuesta de las células B al portador. Los antígenos se inmovilizaron en placas de ELISA, y luego se los evaluó usando diluciones al medio en serie del suero inmune. La mayor dilución posible que resultó en una señal superior al fondo se indica en la figura 9.

Se arribó a las conclusiones que se indican a continuación.

a) Respuesta de las células B a CSP recombinante

Los tres inmunógenos resultaron en una respuesta extremadamente fuerte a la CSP. Sin embargo, la respuesta a la CSP completa aún es 4 veces mayor (titulación: $1:12 \times 10^6$ versus $1:3 \times 10^5$ para la repetición y la CSP truncada, respectivamente), lo que significa que el suero inmune sometido a una dilución al 12 millonésimo (!) aún puede reconocer la proteína CSP.

b) Respuesta de las células B a los péptidos repetidos NANP y NVDP

Las respuestas al péptido NANP son similares para todas las construcciones, como era de esperarse. Las respuestas al péptido NVDP, que es menos común en la repetición, son similares, pero son distintivamente mayores para la construcción de CSP completa, aún después de la primera inmunización (sin refuerzo) (titulación: $1:2,5 \times 10^4$ versus $1:5 \times 10^3$).

c) Respuesta de las células B a secuencias de CSP que no están presentes en la repetición

Solamente CSPshort y la CSP completa inducen una respuesta a los péptidos CSP12 (EPPSDKHIEQYLKKIQNSLS; pos. 246-264 en la construcción de núcleo dividido completa) y CSP8 (GNGIQVRIKPGSANKPKDELD; pos. 274-294 en la construcción de núcleo dividido completa). La ausencia de respuestas correspondientes después de la inmunización con el péptido repetitivo en el sistema de núcleo continuo es esperable, ya

que las secuencias CSP8 y CSP12 no están presentes. El resultado es importante ya que indica que solamente es posible producir específicamente la CSP completa en el sistema de núcleo dividido. Por consiguiente, al usar el sistema de núcleo dividido, es posible afectar epitopes adicionales, que pueden contribuir a la acción protectora de la vacunación.

d) Respuesta de las células B al portador nuclear

Tanto CSPshort como la CSP completa en el sistema de núcleo dividido resultan en una respuesta aproximadamente 25 más débil al portador nuclear que el péptido repetido en el sistema continuo (titulación: $1:1,25 \times 10^5$ versus 3×10^6). Como la respuesta deseada a la proteína extraña es al menos tan alta (CSPshort) o aún más alta (CSP completa) que con el uso del péptido repetido en el sistema de núcleo continuo, la inmunogenicidad específica con relación a la secuencia extraña insertada se ve sustancialmente mejorada cuando se usa la CSP truncada, y en particular, la CSP completa en el sistema de núcleo dividido. Además, una respuesta fuerte al portador nuclear complica el diagnóstico diferencial de las personas positivas para anti-HBsAg (infección versus anti-HBsAg por vacunación), por lo que resulta deseable una respuesta baja contra el portador nuclear.

e) La CSP completa en el sistema de núcleo dividido activa las células T

Se detectó la inducción de la respuesta en las células T estimulando células de bazo de ratones inmunizados específicamente con HBcAg recombinante, con un péptido conocido de las células T (HBc 120-140), o con CSP recombinante o los péptidos de CSP NANP y CSP8, y luego midiendo la producción de IL-2. Todas las construcciones provocaron la aparición de células T que pudieron ser activadas por HBcAg, HBc120-140, CSP recombinante y NANP. Sin embargo, la producción de IL-2 solamente pudo ser estimulada con el péptido CSP8 en las células de bazo de los ratones inmunizados con la CSP completa en el sistema de núcleo dividido.

Entonces, la construcción con la CSP completa resulta en una respuesta más amplia que la construcción continua que solamente contiene la repetición, también a nivel de las células T.

REIVINDICACIONES

1. Un sistema portador de núcleo dividido que, como polipéptidos separados, tiene el dominio nuclear N y el dominio nuclear C de la proteína nuclear de un virus de la hepatitis B, y al menos una molécula extraña contra la cual ha de provocarse una respuesta inmune, donde dicha molécula extraña está fusionada al extremo C del dominio nuclear N o el extremo N del dominio nuclear C, donde la proteína nuclear es capaz de unirse para formar partículas similares a cápsides, y donde la molécula extraña está fusionada a un aminoácido de la proteína nuclear que está localizado entre las posiciones 73 y 94 de la proteína nuclear.
2. El sistema portador de núcleo dividido reivindicado en la reivindicación 1, caracterizado porque el virus de la hepatitis B es específico para mamíferos.
3. El sistema portador de núcleo dividido reivindicado en la reivindicación 2, caracterizado porque la proteína nuclear tiene la secuencia de un virus de la hepatitis B humano.
4. El sistema portador de núcleo dividido reivindicado en la reivindicación 2, caracterizado porque la proteína nuclear tiene la secuencia de aminoácidos del WHV.
5. El sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 4, caracterizado porque la molécula extraña es la secuencia de aminoácidos de

una proteína de una bacteria patogénica.

6. El sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 4, caracterizado porque la molécula extraña es la secuencia de aminoácidos de una proteína de un eucariota patogénico, en particular, el patógeno causante de la malaria, *Plasmodium falciparum*.

7. El sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 4, caracterizado porque la molécula extraña es la secuencia de aminoácidos de una proteína de un virus.

8. El sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 4, caracterizado porque la molécula extraña es la secuencia de aminoácidos de una proteína que tiene su propio potencial basado en patogenicidad, en particular, una proteína marcadora tumoral.

9. El sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 4, caracterizado porque la molécula extraña se selecciona del grupo que consiste en

- a) la secuencia del péptido aceptor de biotina
- b) la secuencia del péptido ACID
- c) la secuencia del dominio Z33 derivado de la proteína A
- d) la secuencia del dominio GB1 de la proteína G.

10. Un método para preparar un portador para moléculas extrañas, en particular, proteínas extrañas, contra las cuales ha de inducirse una respuesta inmune, donde el portador, primero, tiene la secuencia proteica del antígeno nuclear de un virus de la hepatitis B, y segundo, tiene una molécula extraña, en particular, una secuencia proteica heteróloga,

donde la molécula extraña, en particular, la secuencia proteica heteróloga, está fusionada con el dominio nuclear N o el dominio nuclear C, después de la posición 73 o antes de la posición 94 de la región nuclear de la hepatitis B, y donde la cadena polipeptídica del portador está interrumpida después del dominio nuclear N o antes del dominio nuclear C, caracterizado porque comprende

expresar el dominio nuclear N y la molécula extraña, en particular, la secuencia proteica heteróloga, o el dominio nuclear C y la molécula extraña, en particular, la secuencia proteica heteróloga, como proteínas de fusión por separado, o insertar una secuencia de reconocimiento para una proteasa entre el núcleo N y la molécula extraña, en particular, la secuencia proteica heteróloga, o entre la molécula extraña, en particular, la secuencia proteica heteróloga, y el núcleo C, y cortar la vacuna de núcleo dividido con una proteasa, para obtener dos polipéptidos, después de la expresión pero antes de su uso, y

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formar partículas similares a cápsides.

11. El método reivindicado en la reivindicación 10, caracterizado porque la secuencia proteica heteróloga se une por fusión después del aminoácido 74 o antes del aminoácido 85 del antígeno nuclear de la hepatitis B.

12. El método de acuerdo con cualquiera de las reivindicaciones 10-11, caracterizado porque la secuencia de la proteína extraña heteróloga tiene al menos 40 aminoácidos de longitud.

13. El método reivindicado en la reivindicación 10, caracterizado porque la secuencia de la proteína extraña heteróloga tiene al menos 120 aminoácidos de longitud.

14. El método de acuerdo con cualquiera de las reivindicaciones 9-12, caracterizado porque se expresan dos polipéptidos separados con la ayuda de un vector bicistrónico, donde el primer polipéptido comprende el dominio nuclear N y el segundo polipéptido comprende el dominio nuclear C, y donde la secuencia proteica heteróloga está fusionada al extremo C del dominio nuclear N o el extremo N del dominio nuclear C.

15. Un medicamento que tiene partículas similares a cápsides, caracterizado porque dichas partículas tienen un sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 8.

16. El medicamento reivindicado en la reivindicación 14, caracterizado porque es una vacuna.

17. Un agente de diagnóstico que tiene partículas similares a cápsides, caracterizado porque dichas partículas tienen un sistema portador de núcleo dividido de acuerdo con cualquiera de las reivindicaciones 1 a 8.

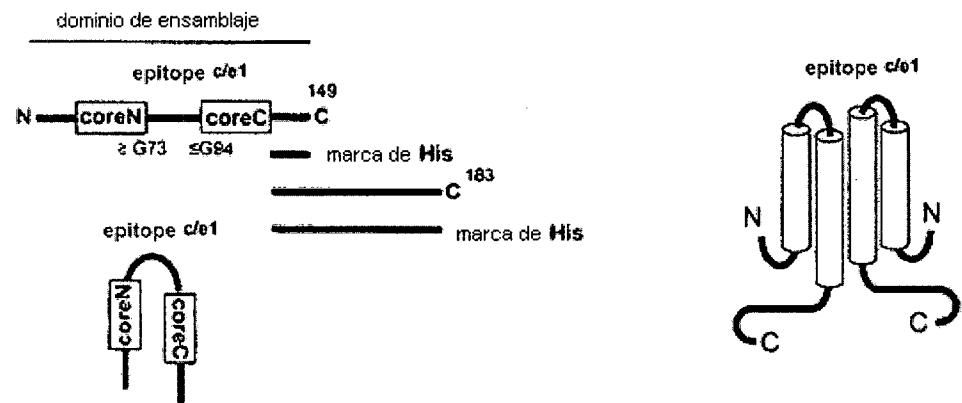


Figura 1. Representación diagramática de la estructura de la proteína nuclear

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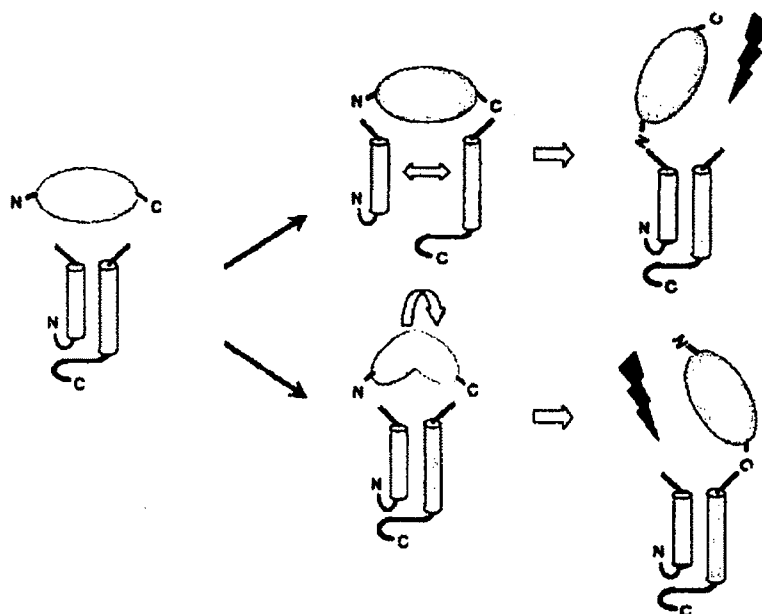


Figura 2. Principio del sistema de núcleo dividido

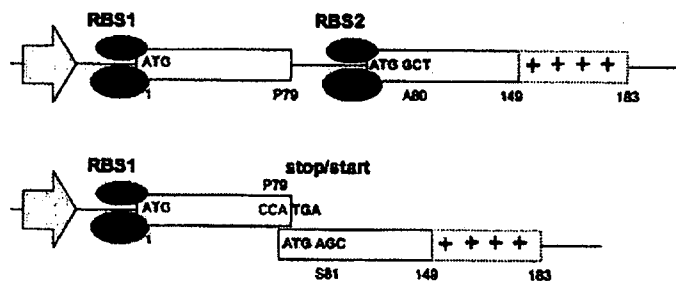
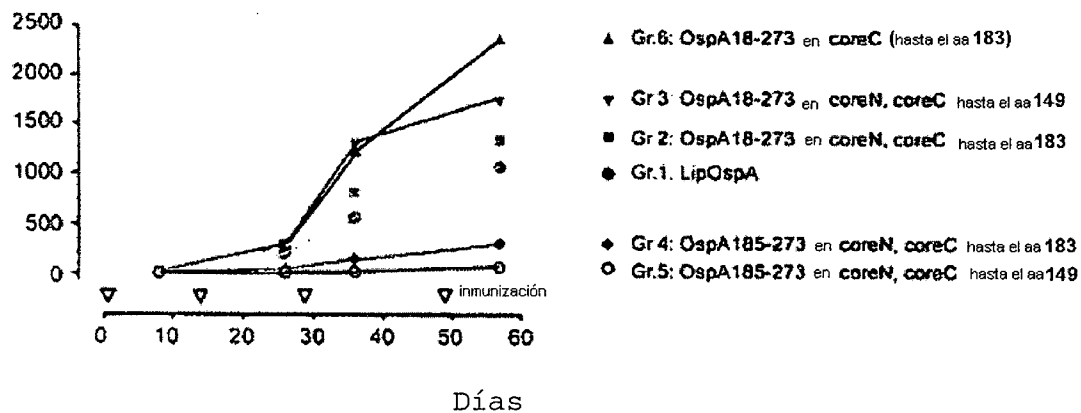


Figura 3. Representación diagramática de dos implementaciones de vectores de expresión para bacterias, para la producción aproximadamente equimolar de los dos fragmentos coreN y coreC

Cinética de la inducción de anticuerpos específicos contra OspA

$\mu\text{g/ml}$ de anticuerpo específico contra OspA



Anti-OspA total versus anticuerpos equivalentes a LA-2

(muestras de suero de la exsanguinación 4)

$\mu\text{g/ml}$ de anticuerpo específico contra OspA

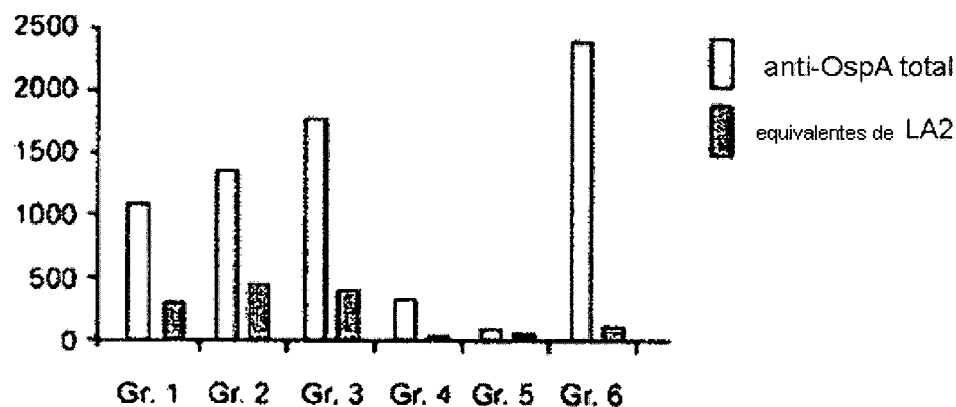


Figura 4

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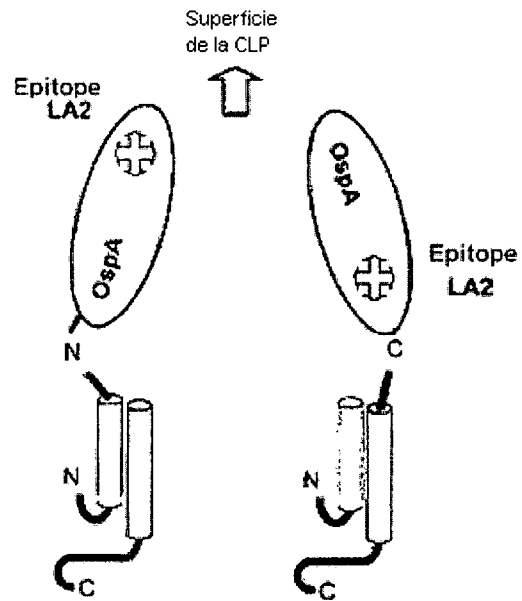


Figura 5. Principio de la inducción dirigida de anticuerpos con especificidad de región contra una proteína extraña en el sistema de núcleo dividido

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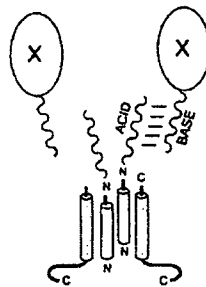


**Figura 6. Fusión de núcleo dividido con una molécula extraña
capaz de interactuar expuesta de manera flexible**

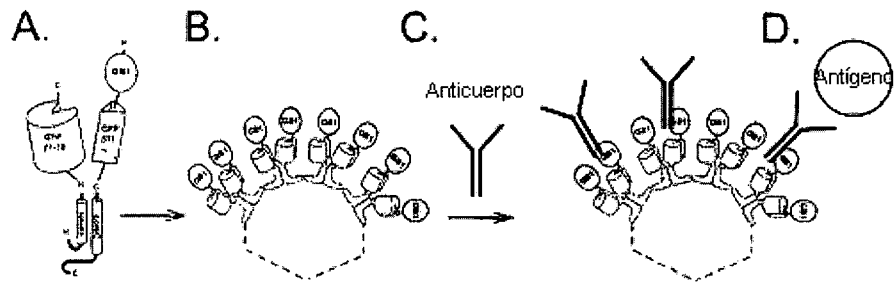


Figura 7. Principio de la CLP de núcleo dividido con fluorescencia propia que presenta una molécula extraña capaz de interactuar, GB1 en este caso, en su superficie

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	Artritis, día 52		Protegidos de la infección
Grupo de ratones sometido a una inmunización pasiva con	Marcad a	Débil	
LA-2, 5 μ g	1/6	1/6	4/6
LA-2, 1 μ g	2/6	3/6	1/6
LipOspA, 5 μ g	0/6	1/6	5/6
LipOspA, 1 μ g	1/6	0/6	5/6
Sistema de núcleo dividido con OspA en coreN, 5 μ g de Anticuerpos equivalentes a LA-2	0/6	0/6	6/6
Sistema de núcleo dividido con OspA en coreN, 1 μ g de Anticuerpos equivalentes a LA-2	0/6	0/6	6/6
Sistema de núcleo dividido con OspA en coreC, volumen equivalente a 5 μ g de	2/6	0/6	4/6

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equivalentes de LA-2 con OspA en coreN			
Sistema de núcleo dividido con OspA en coreC, volumen equivalente a 1 μ g de equivalentes de LA-2 con OspA en coreN	3/6	2/6	1/6
Suero normal de ratón	3/3	0/3	0/3

Figura 8. Potencial de protección de distintos sueros
inmunes anti-OspA contra el ataque de *B. burgdorferi* en
ratones SCID

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Inmunógeno	Semana (pb = después del refuerzo)	CSP recombinante	NANP	NVDP	HBc recombinante	Péptido CSP12	Péptido CSP8
CSP HBc149, repetición continua	Semana 8	No determinada	1,25 × 10 ⁵	5 × 10 ³	6,2 × 10 ⁵	No determinado	No determinado
	Semana 6 pb	3 × 10 ⁶	3 × 10 ⁶	6,25 × 10 ⁵	3 × 10 ⁶	0	0
Sistema de núcleo dividido 149-CSPshort	Semana 8	No determinada	1,25 × 10 ⁵	5,0 × 10 ³	1,2 × 10 ⁵	No determinado	No determinado
	Semana 6	3 × 10 ⁶	3 × 10 ⁶	6,25 × 10 ⁵	1,2 × 10 ⁵	2,5 × 10 ⁴	0

	pb			10^5	10^5		
Sistema de núcleo dividido 149-CSP completa	Seman a 8	No determin ada	1,25 × 10^5	2,5 × 10^4	2,5 × 10^4	No determi nado	No determi nado
	Seman a 6 pb	12×10^6	3 × 10^6	6,25 × 10^5	1,2 5 × 10^5	2×10^2	2,5 × 10^4

Figura 9. Respuesta de las células B a CSP y el portador nuclear después de una sola inmunización o dos inmunizaciones con los inmunógenos indicados en ratones B10

TRATADO DE COOPERACIÓN INTERNACIONAL EN MATERIA DE PATENTES

Del ORGANISMO DE BÚSQUEDA INTERNACIONAL

PCT

A

ver formulario PCT/ISA/220

INFORME ESCRITO
DEL ORGANISMO DE
BÚSQUEDA INTERNACIONAL

(regla PCT N° 43bis.1)

Fecha de envío (día/mes/año)

ver formulario PCT/ISA/210 (hoja 2)

Referencia del Solicitante o Agente

ver formulario PCT/ISA/220

PROCEDER

Ver abajo, punto 2

Referencia Internacional

PCT/EP 2007/006190

Fecha de Presentación Internacional
(día/mes/año)

12.07.2007

Fecha de Prioridad
(día/mes/año)

08.09.2006

Clasificación Internacional de Patentes (IPK) o clasificación nacional e IPK

INV. C07K14/02 C12N15/62

ADD. A61K39/00 C12N7/04

Solicitante

UNIVERSITÄTSKLINIKUM Freiburg

1. Este informe contiene indicaciones acerca de los siguientes puntos:

- ☒ Campo N° I Fundamento del informe
- ☐ Campo N° II Prioridad
- ☐ Campo N° III No realización de dictamen alguno sobre novedad, actividad inventiva y aplicabilidad industrial
- ☐ Campo N° IV Falta de unidad de la invención
- ☒ Campo N° V Declaración fundada según regla PCT 43bis.1(a)(i) con respecto a la novedad, la actividad inventiva y la aplicabilidad industrial; documentos y declaraciones destinados a apoyar esta resolución.
- ☐ Campo N° VI Documentos específicos expuestos
- ☐ Campo N° VII Falencias específicas de la solicitud internacional
- ☐ Campo N° VIII Observaciones específicas a la solicitud internacional

2. **PROCEDER**

Cuando se solicita un Examen Preliminar Internacional, este informe es válido como informe escrito del organismo encargado de la búsqueda internacional ("IPEA"); esto no es así cuando, según la regla 66.1bis(b), el solicitante elige otro organismo distinto a este como IPEA y el IPEA elegido informó a la Oficina Internacional que los informes escritos de este organismo de búsqueda internacional no serán reconocidos.

Cuando este informe es válido como informe escrito de IPEA, tal como está previsto arriba, el solicitante es intimado a presentar ante IPEA una posición por escrito, y cuando corresponde, modificaciones, en el plazo de 3 meses a partir de la fecha en la que fue remitido el formulario PCT/ISA/220 o en el plazo de 22 meses a partir de la fecha de prioridad, según el plazo que venza más tarde.

Para otras opciones, ver formulario PCT/ISA/220.

3. Para otros detalles, ver las instrucciones del formulario PCT/ISA/220.

Nombre y Dirección Postal del ISA
Oficina de Patentes Europea
D-80298 München
Tel.: +49 89 2399-0; Tx: 523656 epmu d
Fax: +49 89 2399-4455

Formulario PCT/ISA/237 (carátula) (abril 2005)

Fecha de confección de
este informe:
ver formulario
PCT/ISA/210

Funcionario autorizado

Schneider, Patrick

Teléfono N° +49 89 2399-7487

**INFORME ESCRITO DEL
ORGANISMO DE BÚSQUEDA INTERNACIONAL**

Referencia Internacional
PCT/EP 2007/006190

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Campo N° I	Fundamento del Informe
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1. Con respecto al **idioma**, el informe se basa sobre
 - ☒ La solicitud internacional en el idioma en que fue presentada
 - ☐ una traducción del idioma original al siguiente idioma , el que se trata del idioma de la traducción presentada a los efectos de la búsqueda internacional (según las reglas 12.3 y 23.1 b)).
2. ☐ Este informe ha sido confeccionado teniendo en cuenta la **rectificación de un error obvio** autorizada por o notificada a este Organismo bajo la regla 91 (regla 43bis.1(a)).
3. Con respecto a la **secuencia de nucleótidos y/o aminoácidos** revelada en la solicitud internacional y requerida para la invención reivindicada, el informe fue confeccionado sobre la siguiente base:
 - a. Tipo de material
 - ☐ listado de secuencias
 - ☐ tablas del listado de secuencias
 - b. Forma del material
 - ☐ en forma escrita
 - ☐ en forma legible por computadora
 - c. Momento de la presentación
 - ☐ comprendido en la presentación internacional tal como fue presentada
 - ☐ presentado, junto con la solicitud internacional, en forma legible por computadora
 - ☐ suministrado posteriormente ante el organismo a los efectos de la búsqueda
3. ☐ Habiendo presentado más de una versión o copia de un protocolo de secuencias y/o la tabla correspondiente se deben presentar adicionalmente las declaraciones requeridas de que la información de las copias presentadas posteriormente o adicionales coincide con la versión original de la solicitud o que no la supera.
4. Observaciones adicionales

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**INFORME ESCRITO DEL
ORGANISMO DE BÚSQUEDA INTERNACIONAL**Referencia Internacional
PCT/EP 2007/006190

Campo N° V	Resolución fundada según regla PCT 43bis.1(a)(i) con respecto a la novedad, la actividad inventiva y la aplicabilidad industrial; documentos y declaraciones destinados a apoyar esta resolución
-------------------	---

1. Resolución

Novedad

Sí Reivindicaciones:
No Reivindicaciones:1-17

Actividad inventiva

Sí Reivindicaciones:
No Reivindicaciones:1-17

Aplicabilidad industrial

Sí Reivindicaciones:
No Reivindicaciones:1-17

2. Documentos y declaraciones:

ver hoja adjunta

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Con respecto al ítem V

Declaración fundada con respecto a la novedad, la actividad inventiva y la aplicabilidad industrial; documentos y declaraciones destinados a apoyar esta resolución.

1. Novedad, actividad inventiva (Art. PCT 33(2) y 33(3))

El objeto de la presente solicitud enseña un sistema portante para polipéptidos antígenos basados en la proteína núcleo de la hepatitis B, en el que la proteína núcleo se divide en dos partes, donde en una o en las dos partes se enlaza una molécula extraña antígena. Las dos partes de la proteína núcleo están también en condiciones de concurrir en partículas capsid-like (CLP). Lo que es esencial para la respuesta inmune frente al antígeno. Un concepto de este tipo de partición física de la proteína núcleo para la adición simplificada de un antígeno no está descrita, ni indicada ni sugerida en el estado de la técnica, de mane que el objeto de la presente solicitud es novedoso e inventivo en el sentido del Art. PCT 33(2) y 33(3).

2. Claridad, apoyo, revelación (Art. PCT 5, 6)

Cada reivindicación que presenta "molécula extraña" no está clara según el Art. PCT 6, dado que en la solicitud sólo se describen polipéptidos como "moléculas extrañas". La expresión "moléculas extrañas" es, no obstante, de un alcance esencialmente más amplio, con lo que el experto no tiene en claro qué es lo que entra en este concepto y como se lleva a cabo técnicamente.

Se hace notar en este contexto que expresiones como "en particular" no poseen ningún efecto limitante y que lo expresado a continuación de ellas se debe considerar completamente opcional.

La misma objeción de claridad es válida para el objeto de la reivindicación con respecto a la expresión "proteína referida a potencial patogénico propio".

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY
(Chapter I of the Patent Cooperation Treaty)

(PCT Rule 44bis)

Applicant's or agent's file reference ZEE20060628	FOR FURTHER ACTION		See item 4 below
International application No. PCT/EP2007/006190	International filing date (day/month/year) 12 July 2007 (12.07.2007)	Priority date (day/month/year) 08 September 2006 (08.09.2006)	
International Patent Classification (8th edition unless older edition indicated) See relevant information in Form PCT/ISA/237			
Applicant UNIVERSITÄTSKLINIKUM FREIBURG			

1. This international preliminary report on patentability (Chapter I) is issued by the International Bureau on behalf of the International Searching Authority under Rule 44 bis.1(a).

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

In the attached sheets, any reference to the written opinion of the International Searching Authority should be read as a reference to the international preliminary report on patentability (Chapter I) instead.

3. This report contains indications relating to the following items:

☒	Box No. I	Basis of the report
☐	Box No. II	Priority
☐	Box No. III	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
☐	Box No. IV	Lack of unity of invention
☒	Box No. V	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
☐	Box No. VI	Certain documents cited
☐	Box No. VII	Certain defects in the international application
☐	Box No. VIII	Certain observations on the international application

4. The International Bureau will communicate this report to designated Offices in accordance with Rules 44bis.3(c) and 93bis.1 but not, except where the applicant makes an express request under Article 23(2), before the expiration of 30 months from the priority date (Rule 44bis .2).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 82 70	Date of issuance of this report 10 March 2009 (10.03.2009)
	Authorized officer Agnes Wittmann-Regis e-mail: pt06.pct@wipo.int

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

Absender: INTERNATIONALE RECHERCHENBEHÖRDE

An:

siehe Formular PCT/ISA/220

PCT

SCHRIFTLICHER BESCHIED DER INTERNATIONALEN RECHERCHENBEHÖRDE (Regel 43bis.1 PCT)

Absendedatum
(Tag/Monat/Jahr)
210 (Blatt 2)

siehe Formular PCT/ISA/

Aktenzeichen des Anmelders oder Anwalts
siehe Formular PCT/ISA/220

WEITERES VORGEHEN
siehe Punkt 2 unten

Internationales Aktenzeichen
PCT/EP2007/006190

Internationales Anmeldedatum (Tag/Monat/Jahr)
12.07.2007

Prioritätsdatum (Tag/Monat/Jahr)
08.09.2006

Internationale Patentklassifikation (IPC) oder nationale Klassifikation und IPC
INV. C07K14/02 C12N15/62
ADD. A61K39/00 C12N7/04

Anmelder
UNIVERSITÄTSKLINIKUM FREIBURG

1. Dieser Bescheid enthält Angaben zu folgenden Punkten:

- ☒ Feld Nr. I Grundlage des Bescheids
- ☐ Feld Nr. II Priorität
- ☐ Feld Nr. III Keine Erstellung eines Gutachtens über Neuheit, erfinderische Tätigkeit und gewerbliche Anwendbarkeit
- ☐ Feld Nr. IV Mangelnde Einheitlichkeit der Erfindung
- ☒ Feld Nr. V Begründete Feststellung nach Regel 43bis.1 a) i) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung
- ☐ Feld Nr. VI Bestimmte angeführte Unterlagen
- ☐ Feld Nr. VII Bestimmte Mängel der internationalen Anmeldung
- ☐ Feld Nr. VIII Bestimmte Bemerkungen zur internationalen Anmeldung

2. WEITERES VORGEHEN

Wird ein Antrag auf internationale vorläufige Prüfung gestellt, so gilt dieser Bescheid als schriftlicher Bescheid der mit der internationalen vorläufigen Prüfung beauftragten Behörde ("IPEA"); dies trifft nicht zu, wenn der Anmelder eine andere Behörde als diese als IPEA wählt und die gewählte IPEA dem Internationale Büro nach Regel 66.1bis b) mitgeteilt hat, dass schriftliche Bescheide dieser Internationalen Recherchenbehörde nicht anerkannt werden.

Wenn dieser Bescheid wie oben vorgesehen als schriftlicher Bescheid der IPEA gilt, so ist der Anmelder aufgefordert, bei der IPEA vor Ablauf von 3 Monaten ab dem Tag, an dem das Formblatt PCT/ISA/220 abgesandt wurde oder vor Ablauf von 22 Monaten ab dem Prioritätsdatum, je nachdem, welche Frist später abläuft, eine schriftliche Stellungnahme und, wo dies angebracht ist, Änderungen einzureichen.

Weitere Optionen siehe Formblatt PCT/ISA/220.

3. Nähere Einzelheiten siehe die Anmerkungen zu Formblatt PCT/ISA/220.

Name und Postanschrift der
Internationalen Recherchenbehörde



Europäisches Patentamt
D-80298 München
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Datum der Fertigstellung
dieses Bescheids

siehe Formular
PCT/ISA/210

Bevollmächtigter Bediensteter

Schneider, Patrick
Tel. +49 89 2399-7487



Feld Nr. I Grundlage des Bescheids

1. Hinsichtlich der **Sprache** beruht der Bescheid auf
 - ☒ der internationalen Anmeldung in der Sprache, in der sie eingereicht wurde
 - ☐ einer Übersetzung der internationalen Anmeldung in die folgende Sprache , bei der es sich um die Sprache der Übersetzung handelt, die für die Zwecke der internationalen Recherche eingereicht worden ist (Regeln 12.3 a) und 23.1 b)).
2. Hinsichtlich der **Nucleotid- und/oder Aminosäuresequenz**, die in der internationalen Anmeldung offenbart wurde und für die beanspruchte Erfindung erforderlich ist, ist der Bescheid auf folgender Grundlage erstellt worden:
 - a. Art des Materials:
 - ☐ Sequenzprotokoll
 - ☐ Tabelle(n) zum Sequenzprotokoll
 - b. Form des Materials:
 - ☐ in Papierform
 - ☐ in elektronischer Form
 - c. Zeitpunkt der Einreichung:
 - ☐ in der eingereichten internationalen Anmeldung enthalten
 - ☐ zusammen mit der internationalen Anmeldung in elektronischer Form eingereicht
 - ☐ bei der Behörde nachträglich für die Zwecke der Recherche eingereicht
3. ☐ Wurden mehr als eine Version oder Kopie eines Sequenzprotokolls und/oder einer dazugehörigen Tabelle eingereicht, so sind zusätzlich die erforderlichen Erklärungen, dass die Information in den nachgereichten oder zusätzlichen Kopien mit der Information in der Anmeldung in der eingereichten Fassung übereinstimmt bzw. nicht über sie hinausgeht, vorgelegt worden.
4. Zusätzliche Bemerkungen:

**SCHRIFTLICHER BESCHEID DER
INTERNATIONALEN RECHERCHEBEHÖRDE**

Internationales Aktenzeichen
PCT/EP2007/006190

**Feld Nr. V Begründete Feststellung nach Regel 43bis.1 a) i) hinsichtlich der Neuheit, der
erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur
Stützung dieser Feststellung**

1. Feststellung

Neuheit	Ja: Ansprüche <u>1-17</u> Nein: Ansprüche
Erfinderische Tätigkeit	Ja: Ansprüche <u>1-17</u> Nein: Ansprüche
Gewerbliche Anwendbarkeit	Ja: Ansprüche: <u>1-17</u> Nein: Ansprüche:

2. Unterlagen und Erklärungen:

siehe Beiblatt

Zu Punkt V

Begründete Feststellung hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung

1 Neuheit, Erfinderischer Schritt (Art. 33(2) und (3) PCT)

Der Gegenstand der vorliegenden Anmeldung ein Trägersystem für Antigene Polypeptide basierend auf dem Hepatitis B Core-Protein, bei dem das Core-Protein in zwei Teile geteilt wird, wobei an eines oder beide Teile das antigene Fremdmolekül angehängt wird. Die beiden Teile des Core-Proteins sind weiterhin in der Lage, sich zu capsid-like-particles (CLP) zusammenzufinden, was für die Immunantwort gegen das Antigen essentiell ist. Ein derartiges Konzept der physischen Teilung des Core-Proteins zur vereinfachten Anlagerung eines Antigens ist im Stand der Technik weder beschrieben noch angedeutet noch nahegelegt worden, so dass der Gegenstand der vorliegenden Anmeldung neu und erfinderisch im Sinne der Art. 33(2) und (3) PCT ist.

2 Klarheit, Stützung, Offenbarung (Art. 5, 6 PCT)

Jeder Anspruch, der "Fremdmoleküle" aufweist, ist unklar nach Art. 6 PCT, da in der Anmeldung lediglich Polypeptide als "Fremdmoleküle" beschrieben. Der Begriff "Fremdmoleküle" ist jedoch wesentlich breiteren Umfangs, wobei dem Fachmann jedoch nicht klar ist, was darunter fällt und wie dies technisch zu bewerkstelligen ist. In diesem Zusammenhang sei darauf hingewiesen, dass Begriffe wie "insbesondere" keinen limitierenden Effekt haben und das nachstehende als rein optional zu betrachten ist.

Der gleiche Klarheitseinwand gilt für den Gegenstand des Anspruchs 8 in Bezug auf den Ausdruck "Protein mit eigenem Pathogenitäts-bezogenem Potential".

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TRATADO DE COOPERACIÓN INTERNACIONAL EN MATERIA DE PATENTES

Remite: EL ORGANISMO DE BÚSQUEDA
INTERNACIONAL

PCT

A

LEDERER & KELLER
z.H. Keller, Günter
Prinzergentenstrasse 16
80538 München
ALEMANIA

NOTIFICACIÓN DE ENVÍO DEL INFORME DE
BÚSQUEDA INTERNACIONAL Y DEL INFORME
ESCRITO DEL ORGANISMO DE BÚSQUEDA
INTERNACIONAL O DE LA DECLARACIÓN

(regla PCT Nº 44.1)

Fecha de envío (día/mes/año)

26.10.2007

Referencia del Solicitante o Agente

ZEE20060628

PROCEDER

Ver abajo, puntos 1 y 4

Referencia Internacional

PCT/EP 2007/006190

Fecha de Presentación Internacional (día/mes/año)

12.07.2007

Solicitante

UNIVERSITÄTSKLINIKUM FREIBURG

1. ☒ Se notifica al solicitante de la confección del Informe de Búsqueda Internacional y de la opinión escrita del organismo de búsqueda internacional y de que se los envía adjunto a la presente.

Presentación de modificaciones y una declaración, según artículo 19:

El solicitante puede modificar por su propia voluntad las reivindicaciones de la solicitud internacional (ver regla 46):

¿Hasta cuando se pueden presentar las modificaciones?

El plazo para la presentación de tales modificaciones es usualmente de dos meses a partir del envío del Informe de Búsqueda Internacional; otros detalles se pueden obtener de las instrucciones de la hoja adjunta.

¿Dónde se deben presentar las modificaciones?

Directamente en la Oficina Internacional de la WIPO, 34, CHEMIN des Colombettes, CH-1211 Ginebra 20. Suiza, Telefax Nº: (41-22) 740.14.35

Instrucciones más detalladas se pueden obtener en las instrucciones de la hoja adjunta.

2. ☐ Se notifica al solicitante de que no se confecciona un Informe de Búsqueda Internacional y de que, según el artículo 17(2)a, se adjunta la declaración a la presente.
3. ☐ Acerca de la **objección** contra el pago de una tasa adicional (tasas adicionales) según la regla 40.2, se notifica al solicitante que
- ☐ la objeción y la resolución han sido enviadas desde aquí a la Oficina Internacional, junto con su solicitud de envío del texto, así como el requerimiento del solicitante a los organismos designados de impulsar la objeción y la resolución.
 - ☐ No existe todavía ninguna resolución acerca de la objeción; el solicitante será informado en cuanto se tome una resolución.

4. Recordatorios:

Brevemente tras el transcurso de **18 meses** a partir de la fecha de prioridad la Oficina Internacional publicará la solicitud internacional. Si el solicitante quiere impedir o postergar la publicación, debe presentar en la Oficina Internacional, según la regla 90^{bis}.1 a 90^{bis}.3, antes de concluir las preparaciones técnicas para la publicación internacional, una declaración acerca del desistimiento de la solicitud internacional o de la reivindicación de prioridad.

El solicitante puede presentar en la Oficina Internacional una posición informal acerca del informe escrito del Organismo de Búsqueda Internacional. La Oficina Internacional remite una copia de esta posición a todos los organismos designados en tanto no esté o recién hubiera sido confeccionado un Examen Preliminar Internacional. Tal posición también se haría pública, pero solo después de transcurrir 30 meses a partir de la fecha de prioridad.

Cuando el solicitante desea postergar en hasta **30 meses** (en algunos organismos hasta más) a partir de la fecha de prioridad el ingreso en la fase nacional, debe presentar una solicitud de Examen Preliminar Internacional dentro de los **19 meses** a partir de la fecha de prioridad; de otra manera, para ingresar en la etapa de la fase nacional, el solicitante debe llevar a cabo, dentro de los **20 meses** a partir de la fecha de prioridad, las gestiones prescriptas por estos organismos.

En otros organismos designados también es válido el plazo de **30 meses** (o algún plazo más largo) cuando no se presenta tal solicitud en el plazo de 19 meses.

Ver anexo del formulario PCT/IB/301. Indicaciones precisas de los plazos válidos en los organismos individuales se exhiben en *PCT Applicants Guide*, Volumen II, capítulo nacional, así como el sitio de Internet de WIPO.

Nombre y Dirección Postal del Organismo de Búsqueda
Internacional

Oficina de Patentes Europea, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040
Fax: (+31-70) 340-3016

Funcionario Apoderado

Katarina Faux

INSTRUCCIONES PARA EL FORMULARIO PCT/ISA/220

Estas instrucciones son indicaciones básicas para la presentación de modificaciones según el artículo 19. Estas observaciones se basan sobre los requisitos del Tratado de Cooperación Internacional en Materia de Patentes (PCT), del reglamento de aplicación y de las normas administrativas del mismo. Ante diferencias entre estas observaciones y los textos mencionados anteriormente son decisivos los últimos. Otros detalles se pueden obtener en el Manual PCT para solicitantes, una publicación de la WIPO.

Las expresiones "artículo", "regla" y "párrafo" empleados en estas instrucciones se refieren siempre a las disposiciones del Tratado PCT, al reglamento de aplicación PCT o a las normas administrativas PCT.

INSTRUCCIONES PARA MODIFICACIONES SEGÚN ARTÍCULO 19

Luego de recibir el Informe de Búsqueda Internacional, el solicitante tiene la posibilidad de modificar una vez las reivindicaciones de la solicitud internacional. Sin embargo, se debe hacer notar que, dado que todas las partes de la solicitud internacional (reivindicaciones, descripción y figuras) pueden ser modificados durante el Examen Preliminar Internacional, no existe usualmente la necesidad de presentar modificaciones de las reivindicaciones según el artículo 19, excepto cuando el solicitante desea la publicación de estas reivindicaciones o presenta algún otro motivo para la modificación de las reivindicaciones de su publicación internacional, por ejemplo, a los fines de una protección provisoria. También se debe tomar en consideración que una protección provisoria se puede obtener solo en algunos estados. (ver *PCT Applicants Guide*, Anexos A y B)

Se hace notar al solicitante del hecho de que no están permitidas enmiendas de las reivindicaciones según el artículo 19 cuando el Organismo de Búsqueda Internacional ha declarado, según el artículo 17(2), que no se confeccionó ningún Informe de Búsqueda Internacional (ver *PCT Applicants Guide*, Volumen I/A, párrafo 296).

¿Que partes de una solicitud internacional pueden ser modificadas?

En el marco del artículo 19 solo pueden ser modificadas las reivindicaciones.

En la fase internacional, según el artículo 34, las reivindicaciones también pueden ser modificadas (o nuevamente modificadas) ante el organismo encargado del examen preliminar internacional. Según el artículo 34, la descripción y las figuras solo pueden ser modificadas ante el organismo encargado del Examen Preliminar Internacional.

Al ingresar en la fase nacional, según el artículo 28 o, en casos dados, el artículo 41, pueden ser modificadas todas las partes de la solicitud internacional.

¿Hasta cuando se pueden presentar modificaciones?

Dentro de los dos meses a partir del envío del informe de búsqueda internacional o dentro de dieciséis meses a partir de la fecha de prioridad, según el plazo que venza más tarde. No obstante, las modificaciones son consideradas como válidas cuando son enviadas a la Oficina Internacional luego del plazo establecido pero antes de la conclusión de las preparaciones técnicas para la publicación internacional (regla 46.1).

¿Dónde no se deben presentar las modificaciones?

Las modificaciones solo pueden ser presentadas ante la Oficina Internacional y no en la Oficina de Ingreso o en el organismo de búsquedas internacionales (regla 46.2).

En caso de presentarse o haber presentado una solicitud de Examen Preliminar Internacional, ver abajo.

¿En que forma pueden ser llevadas a cabo modificaciones?

Una modificación puede proceder en la versión presentada mediante la eliminación de una o varias reivindicaciones, mediante el agregado de una o varias reivindicaciones nuevas o mediante modificaciones del texto de una o varias reivindicaciones.

Se debe presentar una hoja de reemplazo por cada hoja de reivindicaciones que, como consecuencia de una o varias modificaciones, se diferencia de la presentada originalmente.

Todas las reivindicaciones que aparecen una hoja de reemplazo deben estar numeradas con cifras arábigas. Así, cuando se elimina una reivindicación, las demás no deben ser numeradas nuevamente. En el caso de una nueva numeración, las reivindicaciones deben ser numeradas en forma sucesiva (normas administrativas, ítem 205b)).

Las modificaciones deben estar redactadas en el idioma en que fue publicada la solicitud internacional.

¿Qué documentos deben acompañar a las modificaciones?

Escrito adjunto (ítem 205b)):

Las modificaciones se deben presentar con un escrito adjunto.

El escrito adjunto no se publica junto con la solicitud internacional y las reivindicaciones modificadas. No se debe confundir con "declaración según artículo 19(1)" (Ver abajo, "declaración según artículo 19(1)").

El escrito adjunto puede estar redactado, a elección del solicitante, en idioma inglés o francés. En solicitudes internacionales vertidas en idioma inglés, el escrito adjunto debe estar redactado asimismo en idioma inglés y en las solicitudes internacionales redactadas en idioma francés en idioma francés.

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TRATADO DE COOPERACIÓN INTERNACIONAL
EN MATERIA DE PATENTES

PCT
Informe de Búsqueda Internacional

(Artículo PCT 18, así como reglas PCT 43 y 44)

Referencia del solicitante o del agente ZEE20060628	PROCEDER Ver notificación formulario PCT/ISA/220, así como, en tanto corresponda, el punto 5.abajo	
Referencia Internacional PCT/EP 2007/006190	Fecha de presentación Internacional (día/mes/año) 12.07.2007	Fecha de prioridad (la más antigua) (día/mes/año) 08.09.2006
Solicitante UNIVERSITÄTSKLINIKUM FREIBURG		

Este Informe de Búsqueda Internacional fue elaborado por el Organismo de Búsqueda Internacional y, de acuerdo con el artículo 18, es enviado al solicitante. Se envía una copia a la Oficina Internacional.

Este informe de búsqueda Internacional comprende, en total, 4 hojas.

- ☒ Además se adjunta una copia de cada uno de los antecedentes del estado de la técnica mencionados en este informe

1. Fundamentos del Informe

a. Con respecto al idioma, la búsqueda internacional fue llevada a cabo sobre la base de:

- ☒ la solicitud internacional en el idioma en que fue presentada
☐ una traducción de la solicitud internacional al _____, que es el idioma de una traducción suministrada a los fines de la búsqueda internacional (regla 23.1 (b)).

b. ☐ Con respecto a los **nucleótidos y/o aminoácidos** revelados en la solicitud internacional, ver campo N° 1

2. ☐ **Determinadas reivindicaciones no eran investigables** (ver campo II).

3. ☐ **La invención carece de unidad** (ver campo III).

4. Con respecto al **título**,

- ☒ se aprueba el texto presentado por el solicitante.
☐ el texto fue establecido por el organismo como sigue:

5. Con respecto al **resumen**,

- ☒ se aprueba el texto presentado por el solicitante.
☐ el texto fue establecido por el organismo, de acuerdo con la regla 38.2 b), en la versión indicada en el campo IV. El solicitante puede presentar una posición ante el organismo durante el transcurso de un mes a partir de la fecha de envío de este Informe de Búsqueda Internacional.

6. Con respecto a las **ilustraciones**

a. Con el resumen se debe publicar la siguiente figura de Las **ilustraciones**: figura N° 2 .

- ☒ sugerida por el solicitante.
☐ seleccionada por el organismo, dado que el mismo solicitante no sugiere ninguna figura.
☐ seleccionada por el organismo, dado que esta figura caracteriza mejor la invención.

b. ☐ con el resumen no se publica ninguna de las ilustraciones

INFORME DE BÚSQUEDA INTERNACIONAL

Referencia Internacional

PCT/EP 2007/006190

A. CLASIFICACIÓN DEL OBJETO DE LA SOLICITUD		
INV. C07K14/02 C12N15/62 ADD. A61K39/00 C12N7/04		
Según la clasificación internacional de patentes (IPC) o las clasificaciones nacional e IPC		
B. AMBITOS INVESTIGADOS		
Material mínimo investigado (Sistema de clasificación y símbolos de clasificación) C07K C12N		
Publicaciones investigadas pero no pertenecientes al material mínimo investigado, en tanto pertenezcan al ámbito investigado		
Banco electrónico de datos consultado durante la búsqueda internacional (nombre del banco de datos y expresiones de búsqueda eventualmente empleadas) EPO-Internal, WPI Data, EMBASE, BIOSIS		
C. ANTECEDENTES CONSIDERADOS RELEVANTES		
Categoría	Denominación de la publicación, así como, de ser requerido, indicación de los pasajes relevantes	Referente a la reivindicación N°
A	VOGEL MAREN ET AL: "Quaternary structure is critical for protein display on capsid-like particles (CLPs): Efficient generation of hepatitis B virus CLPs presenting monomeric but not dimeric and tetrameric fluorescent proteins" PROTEIN STRUCTURE FUNCTION AND BIOINFORMATICS, vol. 58, N° 2, 1 de febrero de 2005 (2005.02.01), pp. 478-488, XP009078277 todo el documento	
☒ Otras publicaciones están listadas en la continuación del campo C		
☒ Ver familia de patentes en el anexo		
"A"	Categorías particulares indicadas en publicaciones que define el estado general de la técnica pero no considerada como particularmente importante	"T" publicaciones posteriores a las difundidas en la fecha de presentación internacional o de la fecha de prioridad y que no colisionan con la solicitud, sino que solo están indicadas para mayor entendimiento del principio fundamental de la invención o de la teoría sobre la cual se basa
"B"	documento anterior pero publicado recién o después de la de la fecha de presentación internacional	"X" publicación de particular importancia: la invención reivindicada no puede ser considerada, solo como consecuencia de esta publicación, como novedosa o apoyarse en una actividad inventiva
"L"	publicación adecuada para hacer aparecer como dudosa una reivindicación de prioridad, o mediante la cual debe ser considerada la fecha de divulgación de otra publicación mencionada en el informe de búsqueda o que es indicada por otra razón particular (como realizada)	"Y" publicación de particular importancia: la invención reivindicada no puede ser considerada como apoyada en una actividad inventiva cuando puede ser relacionada con una o varias otras publicaciones de esta categoría y tal relación es notoria para un especialista
"O"	publicación que se refiere a una revelación oral, a una utilización, a una exposición o a otras disposiciones	"&" publicación que es miembro de la misma familia
"P"	publicación realizada antes de la fecha de presentación internacional pero luego de la fecha de prioridad reivindicada	
Fecha de finalización de la búsqueda internacional 8 de octubre 2007		Fecha de envío del Informe de Búsqueda Internacional 26.10.2007
Nombre y Código Postal de ISA European Patent Office – P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel.: (+31-70) 340-2040; Tx: 31 651 epo nl Fax: (+31-70) 340-3016		Funcionario Apoderado Schneider, Patrick

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INFORME DE BÚSQUEDA INTERNACIONAL

Referencia Internacional

PCT/EP 2007/006190

C. (continuación) ANTECEDENTES CONSIDERADOS RELEVANTES		
Categoría	Denominación de la publicación, así como, de ser requerido, indicación de los pasajes relevantes	Referente a la reivindicación N°
A	VOGEL ET AL: "In vitro assembly of mosaic hepatitis B virus capsid-like particles (CLPs): Rescue into CLPs of assembly-deficient core protein fusions and FRET-suited CLPs" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL vol. 579, N° 23, 26 de septiembre de 2005 (2005.09.26), pp. 5211-5216, XP006390469 ISSN: 0014-5793 todo el documento	
A	SKAMEL CLAUDIA ET AL: "Hepatitis B virus capsid-like particles can display the complete, dimeric outer surface protein C and stimulate production of protective antibody reponses against Borrelia burgdorferi infection" JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 281, N° 25, junio 2006 (2006.06), pp. 17474-17481, XP009078268 ISSN: 0021-9258 mencionado en la solicitud todo el documento	
A	O'SHEA E K ET AL: "PEPTIDE 'VELCRO' DESIGN OF A HETERODIMERIC COILED COIL" CURRENT BIOLOGY, CURRENT SCIENCE, GB, vol. 3, N° 10, 1993, pp. 658-667 XP000653001 ISSN: 0960-9822 mencionado en la solicitud todo el documento	
A	US 2003/175863 A1 (BIRKETT ASHLEY J [US]) 18 de septiembre de 2003 (2003.09.18) todo el documento	

Detalle de publicaciones que pertenecen a la misma familia de patentes

PCT/EP 2007/006190

Formulario PCT/ISA/210 (Anexo familia de patentes) (abril 2005)

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

Absender: INTERNATIONALE RECHERCHENBEHÖRDE

PCT

29.10.2007

An
LEDERER & KELLER
z.H. Keller, Günter
Prinzregentenstrasse 16
80538 München
ALLEMAGNE

MITTEILUNG ÜBER DIE ÜBERMITTLUNG DES
INTERNATIONALEN RECHERCHENBERICHTS
UND DES SCHRIFTLICHEN BESCHEIDS DER
INTERNATIONALEN RECHERCHENBEHÖRDE
ODER DER ERKLÄRUNG

(Regel 44.1 PCT)

Absendedatum
(Tag/Monat/Jahr) 26/10/2007

Aktenzeichen des Anmelders oder Anwalts

ZEE20060628

WEITERES VORGEHEN

siehe Punkte 1 und 4 unten

Internationales Aktenzeichen

PCT/EP2007/006190

Internationales Anmeldedatum

(Tag/Monat/Jahr) 12/07/2007

Anmelder

UNIVERSITÄTSKLINIKUM FREIBURG

1. ☒ Dem Anmelder wird mitgeteilt, daß der internationale Recherchenbericht und der schriftliche Bescheid der Internationalen Recherchenbehörde erstellt wurden und ihm hiermit übermittelt werden.

Einreichung von Änderungen und einer Erklärung nach Artikel 19:

Der Anmelder kann auf eigenen Wunsch die Ansprüche der internationalen Anmeldung ändern (siehe Regel 46):

Bis wann sind Änderungen einzureichen?

Die Frist zur Einreichung solcher Änderungen beträgt üblicherweise zwei Monate ab der Übermittlung des internationalen Recherchenberichts.

Wo sind Änderungen einzureichen?

Unmittelbar beim Internationalen Büro der WIPO, 34, chemin des Colombettes, CH-1211 Genf 20, Telefaxnr.: (41-22) 338.82.70

Nähere Hinweise sind den Anmerkungen auf dem Beiblatt zu entnehmen.

2. ☐ Dem Anmelder wird mitgeteilt, daß kein internationaler Recherchenbericht erstellt wird und daß ihm hiermit die Erklärung nach Artikel 17 (2) a) sowie der schriftliche Bescheid der Internationalen Recherchenbehörde übermittelt werden.
3. ☐ **Hinsichtlich des Widerspruchs** gegen die Entrichtung einer zusätzlichen Gebühr (zusätzlicher Gebühren) nach Regel 40.2 wird dem Anmelder mitgeteilt, daß
- ☐ der Widerspruch und die Entscheidung hierüber zusammen mit seinem Antrag auf Übermittlung des Wortlauts sowohl des Widerspruchs als auch der Entscheidung hierüber an die Bestimmungsbüro dem Internationalen Büro übermittelt worden sind.
- ☐ noch keine Entscheidung über den Widerspruch vorliegt; der Anmelder wird benachrichtigt, sobald eine Entscheidung getroffen wurde.

4. Zur Erinnerung:

Kurz nach Ablauf von **18 Monaten** seit dem Prioritätsdatum wird die internationale Anmeldung vom Internationalen Büro veröffentlicht. Will der Anmelder die Veröffentlichung verhindern oder auf einen späteren Zeitpunkt verschieben, so muß gemäß Regel 90^{bis} 1 bzw. 90^{bis} 3 vor Abschluß der technischen Vorbereitungen für die internationale Veröffentlichung eine Erklärung über die Zurücknahme der internationalen Anmeldung oder des Prioritätsanspruchs beim Internationalen Büro eingehen. Der Anmelder kann beim Internationalen Büro eine informelle Stellungnahme zum schriftlichen Bescheid der Internationalen Recherchenbehörde einreichen. Das Internationale Büro sendet allen Bestimmungsbüros eine Kopie dieser Stellungnahme, sofern nicht ein internationaler vorläufiger Prüfungsbericht erstellt worden ist bzw. gerade erstellt wird. Eine solche Stellungnahme würde auch der Öffentlichkeit zugänglich gemacht, allerdings erst nach Ablauf von 30 Monaten seit dem Prioritätsdatum. In bezug auf einige Bestimmungsbüros ist innerhalb von **19 Monaten** seit dem Prioritätsdatum ein Antrag auf internationale vorläufige Prüfung einzureichen, wenn der Anmelder den Eintritt in die nationale Phase verschieben und erst **30 Monaten** nach dem Prioritätsdatum (in manchen Ämtern sogar noch später) vornehmen möchte; ansonsten muß der Anmelder innerhalb von **20 Monaten** seit dem Prioritätsdatum die für den Eintritt in die nationale Phase vor diesen Bestimmungsbüros vorgeschriebenen Handlungen vornehmen. Bei anderen Bestimmungsbüros gilt die Frist von **30 Monaten** (oder eine etwaige längere Frist) auch dann, wenn innerhalb von 19 Monaten kein solcher Antrag eingereicht wird. Siehe Anhang zu Formblatt PCT/IB/301. Genaue Angaben zu den jeweils geltenden Fristen in den einzelnen Ämtern enthält der *PCT-Leitfaden für Anmelder*, Band II, Nationale Kapitel sowie die Website der WIPO.

Name und Postanschrift der Internationalen Recherchenbehörde



Europäisches Patentamt, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040
Fax: (+31-70) 340-3016

Bevollmächtigter Bediensteter

Katarina Faux

ANMERKUNGEN ZU FORMBLATT PCT/ISA/220

Diese Anmerkungen sollen grundlegende Hinweise zur Einreichung von Änderungen gemäß Artikel 19 geben. Diesen Anmerkungen liegen die Erfordernisse des Vertrags über die internationale Zusammenarbeit auf dem Gebiet des Patentwesens (PCT), der Ausführungsordnung und der Verwaltungsvorschriften zu diesem Vertrag zugrunde. Bei Abweichungen zwischen diesen Anmerkungen und obengenannten Texten sind letztere maßgebend. Nähere Einzelheiten sind dem *PCT-Leitfaden für Anmelder*, einer Veröffentlichung der WIPO, zu entnehmen.

Die in diesen Anmerkungen verwendeten Begriffe "Artikel", "Regel" und "Abschnitt" beziehen sich jeweils auf die Bestimmungen des PCT-Vertrags, der PCT-Ausführungsordnung bzw. der PCT-Verwaltungsvorschriften.

HINWEISE ZU ÄNDERUNGEN GEMÄSS ARTIKEL 19

Nach Erhalt des internationalen Recherchenberichts und des schriftlichen Bescheids der Internationalen Recherchenbehörde hat der Anmelder die Möglichkeit, einmal die Ansprüche der internationalen Anmeldung zu ändern. Es ist jedoch zu betonen, daß, da alle Teile der internationalen Anmeldung (Ansprüche, Beschreibung und Zeichnungen) während des internationalen vorläufigen Prüfungsverfahrens geändert werden können, normalerweise keine Notwendigkeit besteht, Änderungen der Ansprüche nach Artikel 19 einzureichen, außer wenn der Anmelder z. B. zum Zwecke eines vorläufigen Schutzes die Veröffentlichung dieser Ansprüche wünscht oder ein anderer Grund für eine Änderung der Ansprüche vor ihrer internationalen Veröffentlichung vorliegt. Weiterhin ist zu beachten, daß ein vorläufiger Schutz nur in einigen Staaten erhältlich ist (siehe *PCT-Leitfaden für Anmelder*, Band I/B, Anlagen B 1 und B 2).

Der Anmelder wird darauf hingewiesen, daß Änderungen nach Artikel 19 nicht zulässig sind, wenn die Internationale Recherchenbehörde nach Artikel 17 (2) erklärt hat, daß kein internationaler Recherchenbericht erstellt wird (siehe *PCT-Leitfaden für Anmelder*, Band I/A, Ziffer 296).

Welche Teile der internationalen Anmeldung können geändert werden?

Im Rahmen von Artikel 19 können nur die Ansprüche geändert werden.

In der internationalen Phase können die Ansprüche auch nach Artikel 34 vor der mit der internationalen vorläufigen Prüfung beauftragten Behörde geändert (oder nochmals geändert) werden. Die Beschreibung und die Zeichnungen können nur nach Artikel 34 vor der mit der internationalen vorläufigen Prüfung beauftragten Behörde geändert werden.

Beim Eintritt in die nationale Phase können alle Teile der internationalen Anmeldung nach Artikel 28 oder gegebenenfalls Artikel 41 geändert werden.

Bis wann sind Änderungen einzureichen?

Innerhalb von zwei Monaten ab der Übermittlung des internationalen Recherchenberichts oder innerhalb von sechzehn Monaten ab dem Prioritätsdatum, je nachdem, welche Frist später abläuft. Die Änderungen gelten jedoch als rechtzeitig eingereicht, wenn sie dem Internationalen Büro nach Ablauf der maßgebenden Frist, aber noch vor Abschluß der technischen Vorbereitungen für die internationale Veröffentlichung (Regel 46.1) zugehen.

Wo sind Änderungen nicht einzureichen?

Die Änderungen können nur beim Internationalen Büro, nicht aber beim Anmeldeamt oder der Internationalen Recherchenbehörde eingereicht werden (Regel 46.2).

Falls ein Antrag auf internationale vorläufige Prüfung eingereicht wurde/wird, siehe unten.

In welcher Form können Änderungen erfolgen?

Eine Änderung kann erfolgen durch Streichung eines oder mehrerer ganzer Ansprüche, durch Hinzufügung eines oder mehrerer neuer Ansprüche oder durch Änderung des Wortlauts eines oder mehrerer Ansprüche in der eingereichten Fassung.

Für jedes Anspruchsblatt, das sich aufgrund einer oder mehrerer Änderungen von dem ursprünglich eingereichten Blatt unterscheidet, ist ein Ersatzblatt einzureichen.

Alle Ansprüche, die auf einem Ersatzblatt erscheinen, sind mit arabischen Ziffern zu numerieren. Wird ein Anspruch gestrichen, so brauchen die anderen Ansprüche nicht neu numeriert zu werden. Im Fall einer Neunummerierung sind die Ansprüche fortlaufend zu numerieren (Verwaltungsvorschriften, Abschnitt 205 b)).

Die Änderungen sind in der Sprache abzufassen, in der die internationale Anmeldung veröffentlicht wird.

Welche Unterlagen sind den Änderungen beizufügen?

Begleitschreiben (Abschnitt 205 b)):

Die Änderungen sind mit einem Begleitschreiben einzureichen.

Das Begleitschreiben wird nicht zusammen mit der internationalen Anmeldung und den geänderten Ansprüchen veröffentlicht. Es ist nicht zu verwechseln mit der "Erklärung nach Artikel 19(1)" (siehe unten, "Erklärung nach Artikel 19 (1)").

Das Begleitschreiben ist nach Wahl des Anmelders in englischer oder französischer Sprache abzufassen. Bei englischsprachigen internationalen Anmeldungen ist das Begleitschreiben aber ebenfalls in englischer, bei französischsprachigen internationalen Anmeldungen in französischer Sprache abzufassen.

PATENT COOPERATION TREATY

PCT

INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

Aktenzeichen des Anmelders oder Anwalts ZEE20060628	WEITERES VORGEHEN siehe Formblatt PCT/ISA/220 sowie, soweit zutreffend, nachstehender Punkt 5	
Internationales Aktenzeichen PCT/EP2007/006190	Internationales Anmeldedatum (Tag/Monat/Jahr) 12/07/2007	(Frühestes) Prioritätsdatum (Tag/Monat/Jahr) 08/09/2006
Anmelder UNIVERSITÄTSKLINIKUM FREIBURG		

Dieser internationale Recherchenbericht wurde von der Internationalen Recherchenbehörde erstellt und wird dem Anmelder gemäß Artikel 18 übermittelt. Eine Kopie wird dem Internationalen Büro übermittelt.

Dieser internationale Recherchenbericht umfaßt insgesamt 4 Blätter.

☒ Darüber hinaus liegt ihm jeweils eine Kopie der in diesem Bericht genannten Unterlagen zum Stand der Technik bei.

1. Grundlage des Berichts

a. Hinsichtlich der **Sprache** beruht die internationale Recherche auf

- ☒ der internationalen Anmeldung in der Sprache, in der sie eingereicht wurde
☐ einer Übersetzung der internationalen Anmeldung in die folgende Sprache _____, bei der es sich um die Sprache der Übersetzung handelt, die für die Zwecke der internationalen Recherche eingereicht worden ist (Regeln 12.3 a) und 23.1 b)).

b. ☐ Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** siehe Feld Nr. I.

2. ☐ **Bestimmte Ansprüche haben sich als nicht recherchierbar erwiesen** (siehe Feld Nr. II).

3. ☐ **Mangelnde Einheitlichkeit der Erfindung** (siehe Feld Nr. III).

4. Hinsichtlich der **Bezeichnung der Erfindung**

- ☒ wird der vom Anmelder eingereichte Wortlaut genehmigt.
☐ wurde der Wortlaut von der Behörde wie folgt festgesetzt:

5. Hinsichtlich der **Zusammenfassung**

- ☒ wird der vom Anmelder eingereichte Wortlaut genehmigt.
☐ wurde der Wortlaut nach Regel 38.2b) in der in Feld Nr. IV angegebenen Fassung von der Behörde festgesetzt. Der Anmelder kann der Behörde innerhalb eines Monats nach dem Datum der Absendung dieses internationalen Recherchenberichts eine Stellungnahme vorlegen.

6. Hinsichtlich der **Zeichnungen**

- a. ist folgende Abbildung der **Zeichnungen** mit der Zusammenfassung zu veröffentlichen: Abb. Nr. 2
☒ wie vom Anmelder vorgeschlagen
☐ wie von der Behörde ausgewählt, weil der Anmelder selbst keine Abbildung vorgeschlagen hat.
☐ wie von der Behörde ausgewählt, weil diese Abbildung die Erfindung besser kennzeichnet.
b. ☐ wird keine der Abbildungen mit der Zusammenfassung veröffentlicht.

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES

INV. C07K14/02 C12N15/62

ADD. A61K39/00 C12N7/04

Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

C07K C12N

Recherchierte, aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

EPO-Internal, WPI Data, EMBASE, BIOSIS

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A	VOGEL MAREN ET AL: "Quaternary structure is critical for protein display on capsid-like particles (CLPs): Efficient generation of hepatitis B virus CLPs presenting monomeric but not dimeric and tetrameric fluorescent proteins" PROTEINS STRUCTURE FUNCTION AND BIOINFORMATICS, Bd. 58, Nr. 2, 1. Februar 2005 (2005-02-01), Seiten 478-488, XP009078277 das ganze Dokument ----- -/--	



Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen



Siehe Anhang Patentfamilie

- * Besondere Kategorien von angegebenen Veröffentlichungen :
- *A* Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist
- *E* älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist
- *L* Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)
- *O* Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht
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Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A	VOGEL ET AL: "In vitro assembly of mosaic hepatitis B virus capsid-like particles (CLPs): Rescue into CLPs of assembly-deficient core protein fusions and FRET-suited CLPs" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, Bd. 579, Nr. 23, 26. September 2005 (2005-09-26), Seiten 5211-5216, XP005390469 ISSN: 0014-5793 das ganze Dokument -----	
A	SKAMEL CLAUDIA ET AL: "Hepatitis B virus capsid-like particles can display the complete, dimeric outer surface protein C and stimulate production of protective antibody responses against Borrelia burgdorferi infection" JOURNAL OF BIOLOGICAL CHEMISTRY, Bd. 281, Nr. 25, Juni 2006 (2006-06), Seiten 17474-17481, XP009078268 ISSN: 0021-9258 in der Anmeldung erwähnt das ganze Dokument -----	
A	O'SHEA E K ET AL: "PEPTIDE 'VELCRO*': DESIGN OF A HETERODIMERIC COILED COIL" CURRENT BIOLOGY, CURRENT SCIENCE,, GB, Bd. 3, Nr. 10, 1993, Seiten 658-667, XP000653001 ISSN: 0960-9822 in der Anmeldung erwähnt das ganze Dokument -----	
A	US 2003/175863 A1 (BIRKETT ASHLEY J [US]) 18. September 2003 (2003-09-18) das ganze Dokument -----	

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Internationales Aktenzeichen

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Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
US 2003175863 A1	18-09-2003	US 2003185858 A1	02-10-2003

VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS

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08.09.2006

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INV. C07K14/02 C12N15/62
ADD. A61K39/00 C12N7/04

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- ☒ Feld Nr. I Grundlage des Bescheids
- ☐ Feld Nr. II Priorität
- ☐ Feld Nr. III Keine Erstellung eines Gutachtens über Neuheit, erfinderische Tätigkeit und gewerbliche Anwendbarkeit
- ☐ Feld Nr. IV Mangelnde Einheitlichkeit der Erfindung
- ☒ Feld Nr. V Begründete Feststellung nach Regel 43bis.1 a) i) hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung
- ☐ Feld Nr. VI Bestimmte angeführte Unterlagen
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Datum der Fertigstellung
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**SCHRIFTLICHER BESCHEID DER
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Feld Nr. I Grundlage des Bescheids

1. Hinsichtlich der **Sprache** beruht der Bescheid auf
 - ☒ der internationalen Anmeldung in der Sprache, in der sie eingereicht wurde
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 - a. Art des Materials:
 - ☐ Sequenzprotokoll
 - ☐ Tabelle(n) zum Sequenzprotokoll
 - b. Form des Materials:
 - ☐ in Papierform
 - ☐ in elektronischer Form
 - c. Zeitpunkt der Einreichung:
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 - ☐ zusammen mit der internationalen Anmeldung in elektronischer Form eingereicht
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**Feld Nr. V Begründete Feststellung nach Regel 43*bis*.1 a) i) hinsichtlich der Neuheit, der
erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur
Stützung dieser Feststellung**

1. Feststellung

Neuheit	Ja: Ansprüche <u>1-17</u> Nein: Ansprüche
Erfinderische Tätigkeit	Ja: Ansprüche <u>1-17</u> Nein: Ansprüche
Gewerbliche Anwendbarkeit	Ja: Ansprüche: <u>1-17</u> Nein: Ansprüche:

2. Unterlagen und Erklärungen:

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Zu Punkt V

Begründete Feststellung hinsichtlich der Neuheit, der erfinderischen Tätigkeit und der gewerblichen Anwendbarkeit; Unterlagen und Erklärungen zur Stützung dieser Feststellung

1 Neuheit, Erfinderischer Schritt (Art. 33(2) und (3) PCT)

Der Gegenstand der vorliegenden Anmeldung ein Trägersystem für Antigene Polypeptide basierend auf dem Hepatitis B Core-Protein, bei dem das Core-Protein in zwei Teile geteilt wird, wobei an eines oder beide Teile das antigene Fremdmolekül angehängt wird. Die beiden Teile des Core-Proteins sind weiterhin in der Lage, sich zu capsid-like-particles (CLP) zusammenzufinden, was für die Immunantwort gegen das Antigen essentiell ist. Ein derartiges Konzept der physischen Teilung des Core-Proteins zur vereinfachten Anlagerung eines Antigens ist im Stand der Technik weder beschrieben noch angedeutet noch nahegelegt worden, so dass der Gegenstand der vorliegenden Anmeldung neu und erfinderisch im Sinne der Art. 33(2) und (3) PCT ist.

2 Klarheit, Stützung, Offenbarung (Art. 5, 6 PCT)

Jeder Anspruch, der "Fremdmoleküle" aufweist, ist unklar nach Art. 6 PCT, da in der Anmeldung lediglich Polypeptide als "Fremdmoleküle" beschrieben. Der Begriff "Fremdmoleküle" ist jedoch wesentlich breiteren Umfangs, wobei dem Fachmann jedoch nicht klar ist, was darunter fällt und wie dies technisch zu bewerkstelligen ist. In diesem Zusammenhang sei darauf hingewiesen, dass Begriffe wie "insbesondere" keinen limitierenden Effekt haben und das nachstehende als rein optional zu betrachten ist.

Der gleiche Klarheitseinwand gilt für den Gegenstand des Anspruchs 8 in Bezug auf den Ausdruck "Protein mit eigenem Pathogenitäts-bezogenem Potential".

Quaternary Structure Is Critical for Protein Display on Capsid-Like Particles (CLPs): Efficient Generation of Hepatitis B Virus CLPs Presenting Monomeric but not Dimeric and Tetrameric Fluorescent Proteins

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ABSTRACT Self-organizing assemblies such as viral capsids may be used as symmetrical molecular platforms for the display of heterologous sequences, with applications ranging from vaccines to structural studies. The 183-amino-acid hepatitis B virus (HBV) core protein assembles spontaneously into icosahedral capsid-like particles (CLPs). The most exposed, and most immunogenic, substructure on the CLPs is a small loop that connects two long antiparallel α -helices which act as dimerization interface. Ninety (90) or 120 dimers multimerize into the capsid; the four-helix bundles formed by the dimers protrude as spikes from the surface. We recently demonstrated that the entire enhanced green fluorescent protein (eGFP) can be inserted into this loop, yielding CLPs that natively displayed eGFP on their surface. The central location of the insertion site requires that any insert be fixed to the carrier via both termini, with corresponding restrictions regarding insert size and structure. eGFP obviously satisfied these criteria but, surprisingly, all attempts to produce CLPs with the isostructural red fluorescent proteins DsRed1, DsRed2, and HcRed failed. Suspecting their oligomerization tendency to be responsible, we generated fusions containing instead monomeric yellow, cyan, and red fluorescent proteins (mYFP, mCFP and mRFP1). This strongly increased the yields of YFP and CFP-CLPs, and it allowed for the first time to efficiently generate red fluorescent CLPs. Hence insert quaternary structure is a highly critical factor for CLP assembly. These data have important implications for the rational design of self-assembling fusion proteins. *Proteins* 2005;58:478–488. © 2004 Wiley-Liss, Inc.

Key words: GFP; hepatitis B virus core protein; particle vaccine; virus-like particle; VLP

INTRODUCTION

The icosahedral nucleocapsids of hepatitis B virus (HBV), serologically defined as HBcAg, consist of 180 (triangulation number $T = 3$) or 240 subunits ($T = 4$) of a single 183-amino-acid, (aa) core protein. The first 140 aa are sufficient for particle assembly,^{1–3} the dispensable C-terminal region is rich in Arg residues and binds to nucleic acids.^{1,4,5} Capsid-like particles (CLPs) form spontaneously

upon expression of the core protein in a variety of systems, including *Escherichia coli*. The structure of recombinant CLPs lacking the nucleic acid binding domain [see Fig 1(A)] has been solved by X-ray crystallography,⁶ confirming an earlier model derived from biochemical and high resolution electron cryomicroscopy.^{7–10} The core protein fold is largely helical, with two long central α -helices from one monomer interacting with the corresponding helices from a second subunit to form a stable dimer. The resulting four-helix bundles protrude as 90 or 120 respectively, spikes from the particle surface. The tip of these spikes harbors the immunodominant B cell epitope of HBcAg,^{11,12} as it overlaps with one of two epitopes present on a second, nonassembling secreted form of the core protein, called HBeAg, it is usually referred to as c/e1 epitope.¹³

HBcAg is exceptionally immunogenic, largely because of its particulate nature that leads the major epitope to be repetitively displayed on the particle surface.^{14,15} These immune enhancing properties can be transferred to foreign sequences, provided their fusion to the core protein carrier does not compromise assembly competence (reviewed in Refs.^{16,17}). The small loop connecting the central helices overlapping the c/e1 epitope has been identified as a superior site for insertion of heterologous sequences; however, this requires the insert to be fixed via both of its termini to the core protein. While various short peptide inserts were tolerated, attempts to insert larger peptides frequently abolished CLP formation, suggesting the loop

Abbreviations: DsRed, *Discosoma spec.* red fluorescent protein; eBFP, enhanced blue emitting FP; eCFP, enhanced cyan emitting FP; eYFP, enhanced yellow emitting FP; eGFP, enhanced GFP; FP, fluorescent protein; GFP, *Aequorea victoria* green fluorescent protein; HBcAg, hepatitis B core antigen; HBV, hepatitis B virus; HcRed, *Heteractis crispa* derived red FP; mCFP, monomeric eCFP [A206K]; mRFP1, monomeric red FP; mYFP, monomeric eYFP [A206K]; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis

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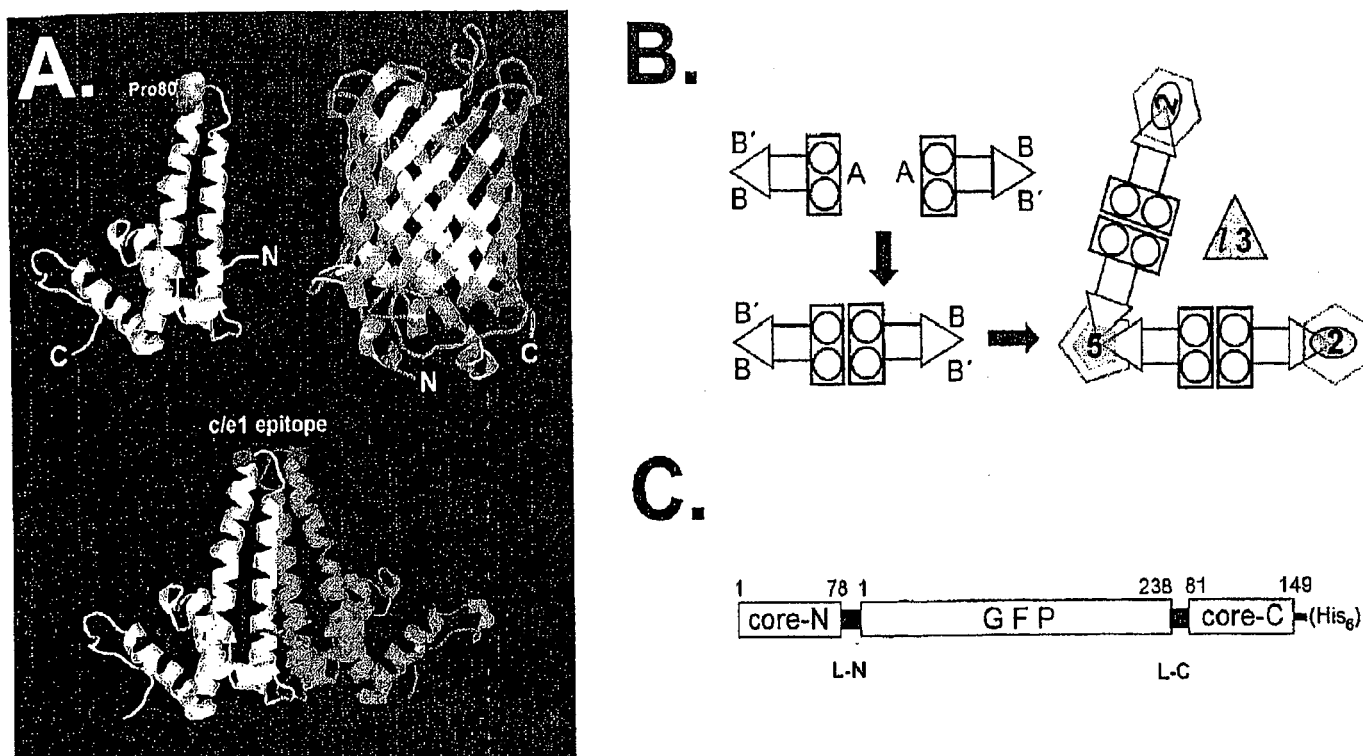


Fig. 1. Structural aspects of HBcAg-based fluorescent CLPs. A: Molecular structures of the HBV core protein and GFP. The fold of the core protein monomer (aa 1 to 143) is shown on the upper left. N and C refer to the amino- and carboxy-terminus. Pro80 which, together with Ala79 is replaced by the insert protein plus flanking linkers, is depicted as CPK model. The bottom is a side-view of the core protein dimer; the location of the surface-exposed c/e1 epitope is indicated. The upper right is representation of the β -can structure of GFP that is typical for all FPs used in this study. B: Scheme of wild-type HBV core protein assembly. Each monomer is trivalent; dimerization through the A interfaces, largely formed by the long central helices (symbolized as circles within the rectangular box), generates a tetraivalent dimer. Monomer folding may precede dimerization, or may be linked to dimerization. The two multimerization interfaces of each dimer (B, B'), located in the C terminal part of the assembly domains, interact to form the icosahedral CLP. Only two of the five dimers per each of the twelve five-fold symmetry axes (indicated as 5) per CLP are shown. 2 and 1/3 indicate the positions of two-fold and a local three-fold axis as present in a 120 dimer $T = 4$ CLP. C: Schematic representation of the fusion protein constructs used. The boxes represent the N terminal (core-N) and C terminal (core-C) parts with the bordering aa positions; L-N and L-C the flanking linkers, and GFP the inserted FP (eGFP, mGFP, eYFP, mYFP, DeRed1, DsRed2, HcRed, mRFP1); aa 238 refers to the C terminal aa of GFP; some of the other FPs are of slightly different length. (His₆) indicates the location of the His-tag present in most constructs.

has a naturally limited insertion capacity.^{16,17} However, we have demonstrated that the entire 238-aa green fluorescent protein (eGFP, i.e., F64L, S65T), when inserted between aa 78 and 80 of the core protein and flanked by Gly-rich linkers of about 10 aa on either side, allows for the formation of green fluorescent particles.¹⁸ Because GFP fluorescence depends on a correct three-dimensional structure, this also indicated native folding of at least about half the GFP subunits. By electron cryomicroscopy and image reconstruction these CLPs appeared as two-shelled particles, with the inner shell nearly identical to wild-type HBcAg and the outer electron density originating from the GFP domains.

At present, eGFP is the only complete foreign protein for which native surface display on HBcAg CLPs has unambiguously been demonstrated. Given various previous failures with other proteins it is essential for a broader application of the HBcAg platform to understand the factors that determine whether or not a corresponding fusion protein can assemble into CLPs. For wild-type HBcAg, assembly proceeds through dimerization of two monomers, largely via interactions between the long central helices, and multimerization of the dimers via interactions between the C terminal parts of the assembly domains;^{6,19,20} for symmetry reasons, each monomer per

dimer has to interact with two neighboring dimers [Fig. 1(B)]. For a corresponding fusion protein it is essential that all three domains of the fusion protein, that is, the N terminal (core-N) and C terminal (core-C) subdomains and the insert, must not interfere with folding and assembly of one another; hence sterical clashes between the two insert domains per dimer, and between the 180 or 240 inserts per CLP must be avoided. Obviously, eGFP satisfied all these criteria, plausibly because its compact 11-stranded β -barrel structure [β -can; see Fig. 1(A)] from which both termini protrude, in close proximity, from the same side^{21,22} does not interfere with folding of the core protein parts and easily accommodates into the predetermined geometry of the insertion site.

Surprisingly, however, a core protein fusion containing the enhanced yellow fluorescent protein (eYFP) instead of eGFP formed CLPs also less efficiently although eYFP, except for a few point mutations, is practically identical to eGFP in sequence and structure. More dramatically, all attempts to generate CLPs presenting any of the commercially available red FPs DsRed1, DsRed2, and HcRed failed completely, despite extensive variation of the culture conditions and work-up procedures. These reef-coral derived red FPs,²³ though highly divergent in primary sequence, fold into the GFP-typical β -can structure (re-

cently reviewed in Verkhusha and Lukyanov²⁴). Hence some other property than mere 3D structure was also important.

A fundamental difference to the GFP-derived FPs is the obligate tetrameric quaternary structure of the reef-coral proteins. This was first noted for DsRed1²⁵⁻²⁷ which, in addition, tends to aggregate and displays very slow chromophore formation kinetics. These limitations, but not tetramerization, were overcome by additional mutations, that is, in DsRed2²⁸ and DsRed.T1,²⁹ now commercially available (Clontech) as DsRed-Express. HcRed1, an engineered variant originally described as HcRed2A,³⁰ still forms dimers. This led us to suspect that an incompatible quaternary structure of the insert protein could cause the failure to form soluble CLPs. In particular, on the assembled CLPs, or multimeric assembly intermediates, the local concentration of the inserted proteins would be exceptionally high; hence even a relatively weak homoaffinity might cause them to undergo inter-FP interactions that compete with the HBcAg subunit interactions required for proper particle assembly. If so, CLP formation should be rescued if truly monomeric FPs were used in the fusion constructs.

Because inter-FP interactions are detrimental to a variety of FP applications³¹ Tsien and coworkers recently developed variants of eYFP and eCFP with an essentially abolished dimerization ability (monomeric YFP and CFP, termed mYFP and mCFP); for instance, the *K_d* for dimerization of eYFP was increased from 0.11 mM to an estimated 74 mM by a single mutation converting A206 at the crystallographic dimer interface into K.³² By a more complex procedure, involving 33 mutations, a monomeric variant of DsRed, called mRFP1³³ was developed.

These well-characterized monomeric FPs therefore provided an ideal set of related but distinctly different proteins to test for the influence of insert quaternary structure on CLP assembly. Below we show that using mYFP and mCFP instead of eYFP and eCFP markedly enhanced the yield of fluorescent, soluble HBcAg CLPs, and that formation of red fluorescent CLPs was exclusively, and efficiently, possible with mRFP1. These data define, in an structural context, the propensity of an inserted protein to undergo homomeric interactions as a factor as critical for CLP assembly as three-dimensional structure per se.

MATERIALS AND METHODS

Plasmid Constructs

The expression vector used throughout, pET28a2, is a derivative of pET28-a conferring ampicillin instead of kanamycin resistance. The gene encoding the original core eGFP fusion protein contained an enhanced version of GFP (eGFP, F64L + S65T;³⁴), flanked by linkers L-N = G₄SG₄T and L-C = G₄SG₄, inserted between aa 78 and 80 of a C-terminally truncated HBV core protein gene (encoding aa 1-149). It was transferred into pET28a2, yielding plasmid pET28a2-c149eGFP which served as acceptor for the subsequent constructs encoding the various FPs. The genes encoding eYFP, eCFP, DsRed1, DsRed2, and HcRed1 were amplified from the corresponding commercially avail-

able vectors (Clontech). Genes for monomeric mYFP and mCFP were obtained by PCR-mediated mutagenesis, introducing the A206K mutation.³² A plasmid encoding monomeric RFP1 (mRFP1) was kindly provided by Dr. R. Tsien.³³ In some constructs, a C terminal His6-tag was added, again via PCR mutagenesis. Plasmids were amplified in *E. coli* Top10 cells (Invitrogen) which do not express T7 RNA polymerase. All constructs were verified by DNA sequencing. For expression, the characterized plasmids were transformed into *E. coli* BL21 Codonplus cells (Stratagene) which carry a compatible chloramphenicol resistance conferring plasmid-encoding tRNAs that are rare in *E. coli*.

Expression and Enrichment of the Fusion Proteins

Single colonies of the BL21 transformants were grown at 37°C in LB medium containing 34 µg/ml of chloramphenicol and 50 µg/ml of ampicillin until an optical density at 600 nm of between 0.7 and 1.0 was reached. At this time, IPTG was added to a final concentration of 1 mM, and the flasks were further incubated on a shaking platform. The standard conditions used in most experiments were 14 to 16 h at room temperature (20° to 22°C) unless indicated otherwise. Bacteria were harvested by centrifugation, and the cell pellets were washed using TN buffer (25 mM Tris/HCl, pH 7.5; 50 mM NaCl). The pellets were kept frozen at -20°C until further use. For lysis, the frozen cells were suspended in TN buffer (4 to 5 ml for the cells from a 250 ml culture), incubated with lysozyme and benzonase, and sonicated.³⁵ Cellular debris and insoluble proteins were separated by centrifugation (SS 34 rotor; 10,000 rpm, 10 min at 4°C). The previously used ammonium sulphate precipitation step¹⁸ was omitted to avoid any effects of high ionic strength. Instead, the cleared supernatants (2 ml per tube) were directly subjected to sedimentation in 10% to 60% (wt/vol) sucrose step gradients (2-ml each of 10%, 20%, 30%, 40%, 50%, and 60% sucrose) as described, using a TST41.14 (Kontron) rotor (41,000 rpm, 2 h, 20°C; average RCF 200,000 g). Under these conditions, recombinant wild-type HBV capsids and regular fusion protein CLPs are typically recovered in the center of the gradient, that is, in fractions 7 to 9 or 10 from a total of 14 fractions. Fluorescent material was detected by visual inspection of the gradient tubes upon irradiation from a transilluminator or a hand-held UV lamp emitting light of 312 nm or 365 nm. Gradient fractions were analyzed by separating aliquots by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% acrylamide and 0.1% SDS in the Laemmli buffer system. Proteins were stained by Coomassie blue, or detected by Western blotting, using HBcAg-specific or FP specific antibodies followed by an appropriate secondary antibody conjugated to alkaline phosphatase (AP) or horse-radish peroxidase (PO). AP was detected using CDP-star reagent (Roche), PO using ECL plus (Amersham), either on X-ray film or on a Fuji LAS 3000 imager (Raytest). Fusion proteins contained in the insoluble fraction of the bacterial lysates were recovered by sequential extraction with increasing concentrations of urea (1 M, 2 M, 4 M, and 8 M).

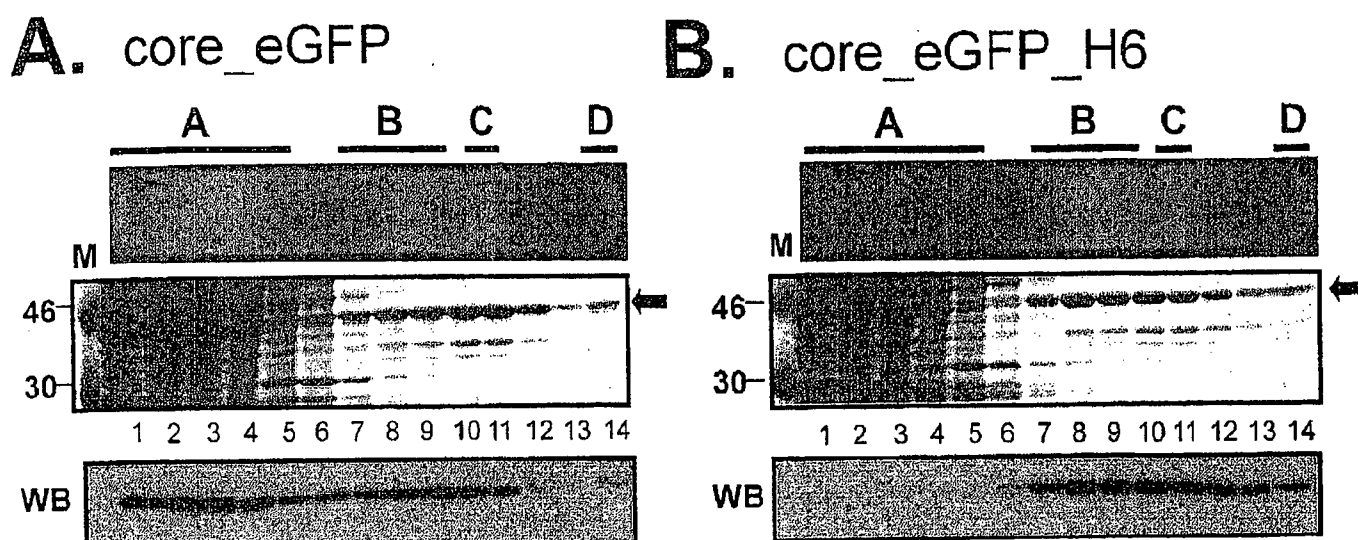


Fig. 2. Separation of different forms of core_eGFP fusion proteins by sedimentation in sucrose gradients. **A:** core_eGFP. The aa sequence of this protein is identical to the previously described coreGFP protein¹⁸ except that expression is controlled by a T7 RNA polymerase promoter. Cleared bacterial lysate was sedimented on a 10% to 60% (w/v) sucrose step gradient. The gradient tube was photographed with UV (365 nm) illumination (top panel); the four zones indicated on the top typically contain: A, nonparticulate fusion protein plus the bulk of bacterial proteins; B, ordered, nonaggregated CLPs; C, aggregated CLPs and probably complexes with remnants of the bacterial cell wall; D, insoluble aggregates. Aliquots from the 14 fractions were analyzed by SDS-PAGE and Coomassie blue staining (center panel), and by Western blotting using a core-specific antibody (bottom panel). The black arrow indicates the position of the fusion protein. The positions of marker proteins with their molecular masses in kDa are indicated on the left. **B:** core_eGFP_H6. The corresponding construct is identical to that in A, except it encodes a C-terminal His6 tag. Note that much less nonparticulate fusion protein is present in zone A, and more in zone B.

Approximate CLP yields were determined by densitometric scanning (AIDA software; Raytest) of the fusion protein band contained in the CLP fraction of the sucrose gradients (zone B, usually fractions 7 to 9) after SDS-PAGE and Coomassie blue staining, using a dilution series of bovine serum albumin of known concentration as standard; in independent repeat determinations, a variation of at most 10% was observed. For normalization, the total amount of soluble protein present in the cleared bacterial lysates was measured by the Bradford assay; relative CLP yields were then expressed as the fraction of specific product per 100 mg of total soluble protein.

Native Agarose Gel Electrophoresis

Aliquots from the gradient fractions were subjected to electrophoresis in 1% agarose gels in $1/2 \times$ TBE buffer containing 0.5 μ g/ml of ethidium bromide as previously described.^{19,36} FPs were detected by illumination with UV light (312 nm, or 365 nm) and by staining the agarose gels with Coomassie blue. Alternatively, the fusion-protein specific bands were detected by immunoblotting as described.¹⁹

UV/VIS Spectroscopy

Chromophore formation was assessed by measuring the absorbance spectra from 320 to 700 nm (Ultrospec 3000, Pharmacia) of the material contained in the center of the CLP fraction (usually fraction 8). The approximate concentration of fusion protein in these fractions was determined densitometrically as described above, so as to exclude contributions to the total protein content from contaminating *E. coli* proteins. The molar extinction coefficients obtained in this way were related to the published extinc-

tion coefficients of the non-fused FPs for an approximate estimate of the fraction of natively folded FP domains on the CLPs.

Electron Microscopy

Electron micrographs were obtained on a Zeiss EM 902 at 80 kV with a nominal magnification of 140,000-fold. Samples from the sucrose gradients were directly applied to the carbon grids, washed three times with 50 mM Tris/Cl⁻ buffer, pH 7.9, and stained with 2% methylamine tungstate, pH 7.0.

RESULTS

Experimental Design

Since our original report on core_eGFP CLPs¹⁸ we have introduced several modifications in the expression system, including the use of a T7 promoter controlled IPTG inducible pET vector in *E. coli* BL21(DE3) cells instead of the pL promoter vector in GI 698 cells.³⁷ The T7 system turned out to be more robust but it did not significantly affect the properties of the fusion protein as such, as shown below. The major expected products were the unassembled subunits, complete CLPs, plus possibly assembly intermediates and misfolded and/or mis-assembled aggregated forms. Sedimentation is an established means of separating these different forms. Because sedimentation in 10% to 60% sucrose step gradients¹⁹ was the basic assay in all subsequent experiments, a representative separation, using the same core_eGFP fusion as previously described but expressed from the T7 system, is shown in Figure 2.

After centrifugation four zones containing fluorescent material were routinely distinguished which, according to SDS-PAGE and Western blotting of aliquots from gradient

TABLE 1. Summary of the Experimental Characteristics of the Core_FP Fusions Analyzed in this Study

Inserted FP ^a	His6 tag ^b	L-N ^c	L-C ^d	Total soluble protein ^e [mg]	CLP yield total ^f [mg]	CLP yield ^g [% of soluble protein]	Extinction coefficient of parental FP ^h [M ⁻¹ cm ⁻¹]	Experimental extinction coefficient of CLP ⁱ [M ⁻¹ cm ⁻¹]	Chromophore yield in CLP ^j [%]
eGFP	NO	G ₄ SG ₄ T	G ₄ SG ₄	125	0.91	0.73	55,000 (489 nm)	29,400	53.5
eGFP	+	G ₄ S	G ₄	154	1.01	0.66	55,000 (489 nm)	47,950	89.0
eYFP	+	G ₄ SG ₄ T	G ₄	120	0.24	0.20	84,000 (514 nm)	90,800	108
mYFP	+	G ₄ SG ₄ T	G ₄	122	2.72	2.23	79,000 (514 nm)	77,700	98.4
mYFP	NO	G ₄ SG ₄ T	G ₄	123	2.55	2.07	79,000 (514 nm)	85,000	107.6
eCFP	+	G ₄ SG ₄ T	G ₄	91	1.31	1.44	26,000 (434 nm)	26,000	100
mCFP	+	G ₄ SG ₄ T	G ₄	94	6.57	6.99	26,000 (434 nm)	21,450	82.5
mCFP	NO	G ₄ SG ₄ T	G ₄	116	1.43	1.23	26,000 (434 nm)	31,700	121.9
DsRed1	+	G ₄ SG ₄ T	G ₄ SG ₄	91	n.d.	n.d.	52,000 (558 nm)	n.d.	n.d.
DsRed2	+	G ₄ T	G ₄	124	n.d.	n.d.	43,800 (561 nm)	n.d.	n.d.
HcRed1	+	G ₄ T	G ₄	81	n.d.	n.d.	80,000 (590 nm)	n.d.	n.d.
mRFP1	+	G ₄ SG ₄ T	G ₄	96	2.21	2.30	44,000 (583 nm)	36,640	83.3

^aAll FPs were inserted between aa D78 and S81 of the HBV core protein.
^bHis6 tag fused to core protein aa 149.
^cSequence of the N terminal linker [one-letter-code; cf. Fig. 1(C)].
^dSequence of the C terminal linker.
^eTotal protein content in cleared bacterial lysates from 200 ml cultures as determined by the Bradford assay.
^fApproximate total amount of fusion protein present in gradient fractions 7 to 9, i.e., zone B, as determined by densitometry of the fusion protein bands upon SDS-PAGE.
^gApproximate relative CLP yield normalized to total soluble protein.
^hPublished molar extinction coefficients at the maximal absorbance wavelength for the parental FPs.
ⁱCalculated from the absorbance in gradient fraction 8 at the maximum absorbance wavelength relative to the specific protein concentration determined by densitometry.
^jRatio of experimentally determined molar extinction coefficients in CLP preparation versus published extinction coefficients of non-fused FP × 100.
n.d. = not detectable.

fractions, contained the fusion protein (Fig. 2; an uncropped version showing the entire molecular weight range covered by the SDS-PAGE is available as Supplementary Fig. 1). As with the previous pL promoter construct,¹⁸ about one half of the fusion protein remained in the top fractions together with the bulk of soluble *E. coli* proteins (fractions 1 to 6, subsequently termed zone A), therefore representing nonparticulate forms. A second portion sedimented into fractions 7 to 9 (zone B) which is typical for regular CLPs; during native agarose gel electrophoresis they migrate as distinct bands (see below). In the experiments described below, the amount of fusion protein in zone B was therefore considered as the critical indicator for the efficiency of CLP formation. Some of the protein accumulated in fraction 11 (zone C), together with mostly nonproteinaceous material likely originating from the bacterial cell wall; zone C, recognizable by a slight turbidity, also appeared when lysate from nontransformed bacteria was sedimented under these conditions. Finally, small amounts of fusion protein were present at the bottom of the centrifuge tube (zone D), indicating they represent insoluble, macromolecular aggregates.

Intending to facilitate isolation of the nonassembled fusion protein from zone A, we generated a core_eGFP fusion with a His6-tag at the C terminal aa V149 (core_eGFP_H6). Unexpectedly, this modification led to a marked reduction in the amounts of nonparticulate forms, and the fluorescence intensity in the CLP-typical zone B increased [Fig. 2(B)]. The total yield of fusion protein in zone B was

similar for the nontagged and the tagged version (0.73 mg vs. 0.66 mg per 100 mg of total soluble protein; see Table I); however, the molar extinction coefficient at 488 nm, the absorbance maximum of GFP, was much closer to the published value for eGFP (55,000 M⁻¹cm⁻¹) with the tagged (47,950 M⁻¹cm⁻¹) than with the untagged protein (29,400 M⁻¹cm⁻¹), suggesting about 90% vs. only 50% of the GFP domains being properly folded (see Table I). This suggested that addition of a His6-tag would improve CLP formation also with other core protein fusions. Most of the fusion constructs with different FPs described below therefore contained this tag, and for those able to form CLPs, very little if any nonparticulate material was found in zone A. By contrast, for control constructs with eYFP, eCFP, and eBFP lacking the His-tag about half (core-eCFP) or most of the fusion proteins remained in zone A (Supplementary Fig. 2).

Regarding the flanking linkers (termed L-N for the N terminal, and L-C for the C terminal linker in Table I), their systematic shortening in the context of the core_eGFP fusion had shown that all combinations including a single glycine on each side were tolerated; however, cryo EM analysis of the latter construct revealed substantial distortions in the structure of the HBcAg derived CLP part (M. Vogel, M. Ploss, B. Böttcher and M. Nassal, unpublished data). Hence all constructs used below contained the L-N/L-C combinations G₄SG₄T/G₄, or G₄S/G₄ (see Table I for a summary).

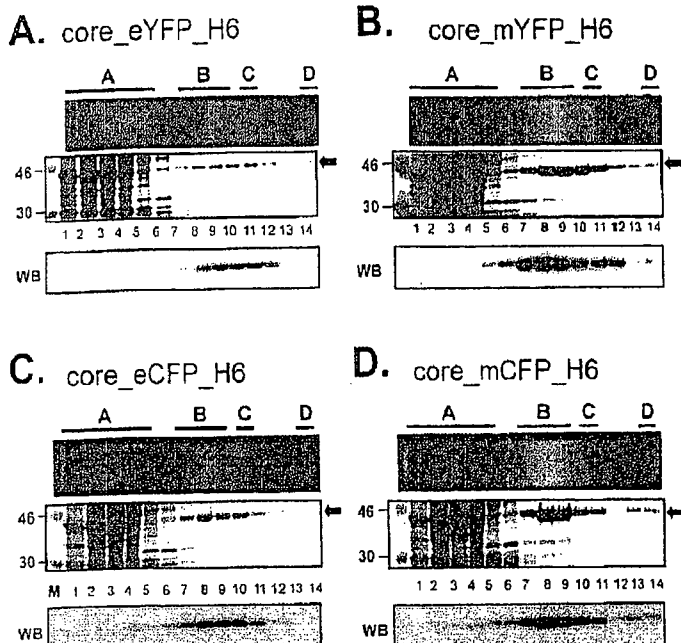


Fig. 3. Replacement of the FP moieties in core_eYFP_H6 and core_eCFP_H6 by monomeric mYFP and mCFP greatly increases formation of soluble, fluorescent CLPs. Cleared lysates from bacteria transformed with pET vectors encoding the indicated fusion proteins and induced for 14 h at room temperature were separated by sucrose gradient centrifugation and analyzed as described in the legend to Figure 2. Note the increased fluorescence in zone B of the monomeric FP samples and the concomitantly increased intensities of the Coomassie blue-stained and immuno-stained bands representing the fusion proteins.

Finally, preliminary experiments had indicated that for all FP fusions expression at room temperature was superior with respect to chromophore formation over expression at 37°C [see Fig. 5(B) for an example]. Hence the bacterial cultures were induced at room temperature for 14 to 15 h unless specifically indicated otherwise. To exclude variations from nonidentical culture conditions, that is, in oxygen tension or the exact composition of the LB media, all comparative experiments were performed in parallel.

A Single aa Exchange in eCFP and eYFP that Prevents Dimerization Strongly Enhances Formation of Fluorescent Core_CFP and Core_YFP CLPs

eYFP and eCFP are point mutants of eGFP with shifted spectral characteristics but essentially identical three-dimensional structure. However, the core_eYFP_H6 construct produced less fluorescent fusion protein in the particle-typical zone B (0.2 mg/100 mg of total soluble protein) than the eGFP_H6 construct although essentially no nonparticulate fraction was seen [Fig. 3(A)]. The calculated molar extinction coefficient ($E_{514} = 90,800 \text{ M}^{-1}\text{cm}^{-1}$ vs. a published value of 84,000 for non-fused eYFP) indicated that most if not all eYFP domains were properly folded. The corresponding eCFP_H6 fusion formed CLPs somewhat more efficiently [1.44 mg per 100 mg total soluble protein in zone B; Fig. 3(C)]. The calculated molar extinction coefficient ($E_{434} = 26,000 \text{ M}^{-1}\text{cm}^{-1}$ vs. 26,000 $\text{M}^{-1}\text{cm}^{-1}$ for the non-fused eCFP protein) indicated again efficient folding.

Based on the failure to obtain CLPs with the red FPs (next paragraph), and on the low but detectable dimerization potential of eYFP and eCFP and its practical elimination by a single A206K mutation³² we next replaced eYFP and eCFP in the constructs by their monomeric variants mYFP and mCFP. This modification drastically increased the yields of fusion protein, and fluorescence, in zone B of the gradients for both FPs [Fig. 3(B,D)]. Quantitation showed an approximately 11-fold higher CLP yield for the mYFP_H6 vs eYFP_H6 construct (2.72 mg CLPs per 100 mg of total soluble protein), and an approximately five-fold increase for the mCFP_H6 vs. eCFP_H6 construct (6.99 mg CLPs per 100 mg of total soluble protein). The molar extinction coefficients, being approximately 83% and 98% of the published values for the non-fused FPs, again indicated efficient folding.

We also analyzed the corresponding mYFP and mCFP constructs without a His-tag. The CLP yield of the mYFP construct was similar, that of the mCFP construct lower than that of the tagged version (see Table I); because these two experiments were performed separately, the absolute amounts may not be directly comparable. However, inspection of the gradient fractions by SDS-PAGE, and particularly by Western blotting (Supplementary Fig. 3), revealed that about half of each fusion protein had remained in zone A, in accord with the data obtained with the other non-tagged fusions. Hence, the monomeric FPs themselves did not detectably affect the ratio of nonassembled versus assembled forms.

Together these data demonstrated that abolishing the dimerization potential of YFP and CFP by a single point mutation drastically increased the yields of CLPs without compromising proper folding and chromophore formation.

Red-Reef-Coral-Derived FPs: Efficient Fluorescent CLP Formation Exclusively From a Monomeric Red FP

Both DsRed1 and DsRed2 are obligate tetramers, HcRed is an engineered dimer.³⁰ The corresponding fusion constructs core_DsRed1_H6, core-DsRed2_H6, and core_HcRed1_H6 were expressed as described above for the GFP-derived proteins. Bacterial colonies, and the crude lysates of induced cultures, showed the typical red fluorescence, indicating successful expression and chromophore formation. However, in all three cases the colored material was exclusively visible in the insoluble fraction (Supplementary Fig. 4). To test for the potential presence in the cleared lysates of small amounts of fusion proteins, or molecules lacking a mature chromophore (see below), the lysates were analyzed by sucrose gradient sedimentation and SDS-PAGE and Western blotting. No prominent bands at the expected positions around 46 kDa were visible by Coomassie blue staining, and only trace amounts were detectable on Western blots after very long exposure (Fig. 4). The DsRed1 fusion was confined to the upper part of the gradient (zone A); the DsRed2 fusion was mainly present in zone A, with a small proportion extending into zone B; the HcRed1 construct showed some material in zone A plus some accumulation at the lower edge of zone B, possibly

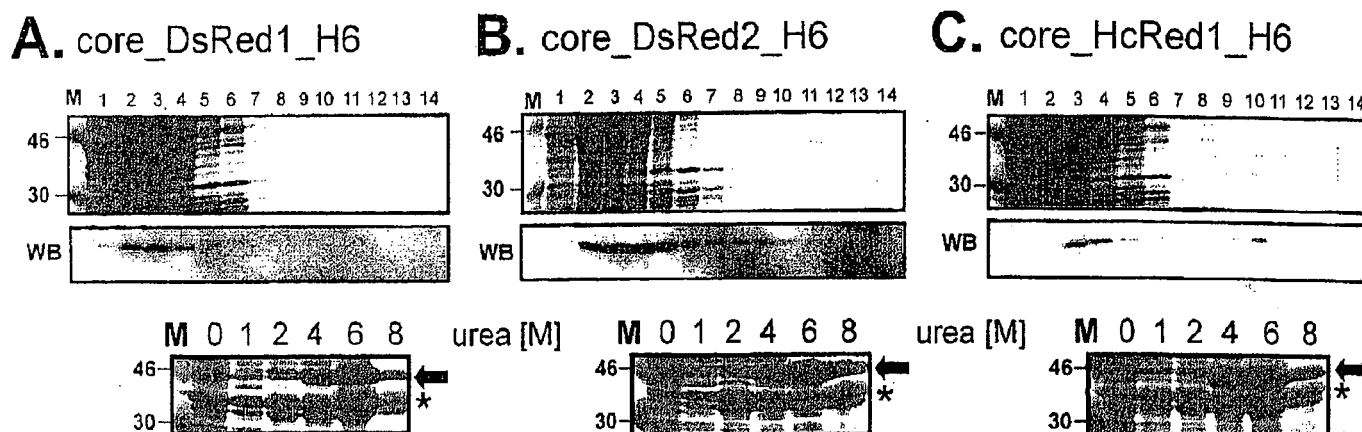


Fig. 4. Failure of core fusion proteins containing DsRed1, DsRed2, and HcRed1 to form soluble fluorescent CLPs. **A:** Sucrose-gradient separation of the cleared bacterial lysates. Bacterial cultures were induced and worked-up as described for the YFP and CFP derivatives. No bands that could be clearly assigned to the red FP fusion proteins were visible by Coomassie blue staining. On the Western blots, trace amounts of the fusion proteins became detectable after very long exposure. **B:** Urea extraction of the insoluble lysate fractions. The colored pellets remaining after clearing the bacterial lysates (see Supplementary Fig. 4) were sequentially extracted with buffer containing increasing concentrations of urea, as indicated. All pellets contained significant amounts of the corresponding fusion proteins. The band indicated by an asterisk corresponds to *E. coli* outer membrane protein C (OmpC), a major constituent of the insoluble lysate fraction.

indicating a tiny amount of particles (signal in fraction 10). To estimate the overall amount of fusion protein produced, the insoluble lysate fraction was sequentially extracted with buffer containing increasing concentrations of urea. Substantial amounts of each fusion protein were solubilized starting at 4-M urea (Fig. 4, lower panels) with the highest concentrations found in the 6-M urea fraction. Quantitation of the band intensities after Coomassie blue staining showed that all three fusion proteins had been produced in amounts of more than 2 mg per 200 ml bacterial culture, that is, at a level comparable to that seen with the other constructs. Though we did not determine the specific extinction coefficients of the insoluble lysate fractions, their vivid red colors (Supplementary Fig. 4) indicated that a significant fraction of all three fusions had formed the typical chromophores. Apparently, therefore, CLP assembly rather than folding of the red FP domains per se was compromised.

To explicitly test for a detrimental effect of the quaternary structures of the red FPs, we next generated a core protein fusion (core_mRFP1_H6) carrying the monomeric mRFP1.³³ When expressed at room temperature under the same conditions as the other constructs, the soluble fraction of the bacterial lysate displayed a strong red color. Upon sedimentation, most of the red color, and of the red fluorescence, was concentrated in zone B [Fig. 5(A)], with a smaller fraction accumulating in zone C. Western blotting confirmed the presence of intact fusion protein. The CLP yield was about 2.3 mg per 100 mg of total soluble protein, with a calculated molar extinction coefficient close to that reported for free mRFP1 ($E_{583} = 36,650 \text{ M}^{-1}\text{cm}^{-1}$ vs. $E_{583} = 44,000 \text{ M}^{-1}\text{cm}^{-1}$).

For comparison, a second aliquot of the same bacterial preculture was induced at 37°C for 3 h; under these conditions, nearly no color developed. Nonetheless, substantial amounts of the fusion protein were present in the soluble fraction fraction (2.68 mg per 100 mg of total soluble protein). However, the bulk of the protein sedimented clearly faster, that is into fractions 10 and 11

[compare Fig. 5 (A and B)]. We were unable to detect any fusion-protein-related material in the gradient that migrated as a distinct band upon native agarose gel electrophoresis (see next paragraph). Hence, most if not all the core-mRFP1_H6 protein expressed at 37°C was unable to form ordered CLPs, suggesting lacking chromophore formation in the FP domain correlated with aggregate formation of the fusion protein.

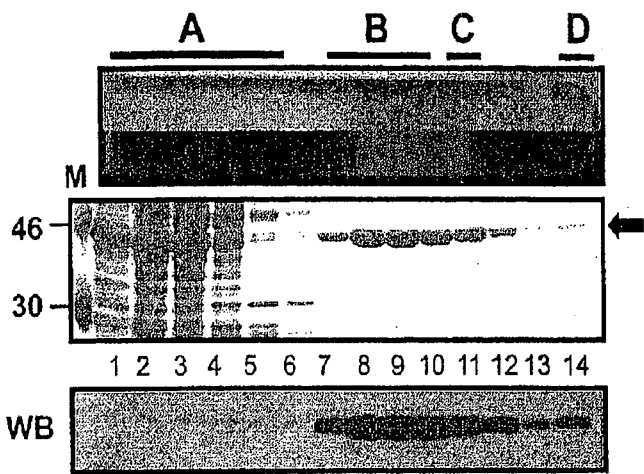
Together, these data demonstrated that exclusively a monomeric red FP but neither tetrameric nor dimeric variants can natively be presented on HBcAg derived CLPs.

Direct Evidence for Ordered CLP Formation by the Differently Colored FP Fusions

The sedimentation analyses were strongly suggestive of successful ordered CLP by several of the fusion proteins. However, the relatively broad distribution of some of the fusion proteins, and the presence in fraction 7 to 9 of part of the 37°C expressed nonfluorescent mRFP1_H6 fusion [Fig. 5(B)], indicated some overlap in the sedimentation behavior of ordered CLPs versus less regular or disordered structures. We therefore used fraction 8, that is, the center of the CLP-typical zone B, from the above described preparations for a more direct analysis. Native agarose gel electrophoresis is able to separate single particles from other forms of the constituent protein(s) that differ in size, shape, multimerization state or surface charge. In addition, compared to nonassembled subunits, CLPs have a much lower diffusion coefficient such that they produce relatively sharp-edged bands.³⁸ All preparations for which sedimentation had suggested the presence of CLPs did indeed produce the expected bands [Fig. 6(A)]. These were fluorescent, and the same bands contained the bulk of protein as shown by Coomassie blue staining of the same gel. The presence of the specific fusion protein in the same bands was corroborated by Western blotting using two different core-protein-specific antibodies.

Finally, fraction-8 aliquots from all fusions with monomeric FPs were subjected to EM analysis by negative

A. core_mRFP1_H6 / r.t.



B. core_mRFP1_H6 / 37°C

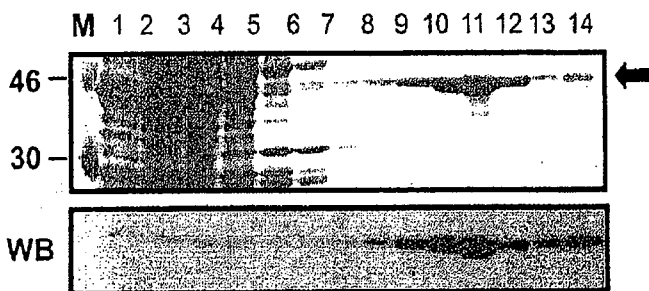
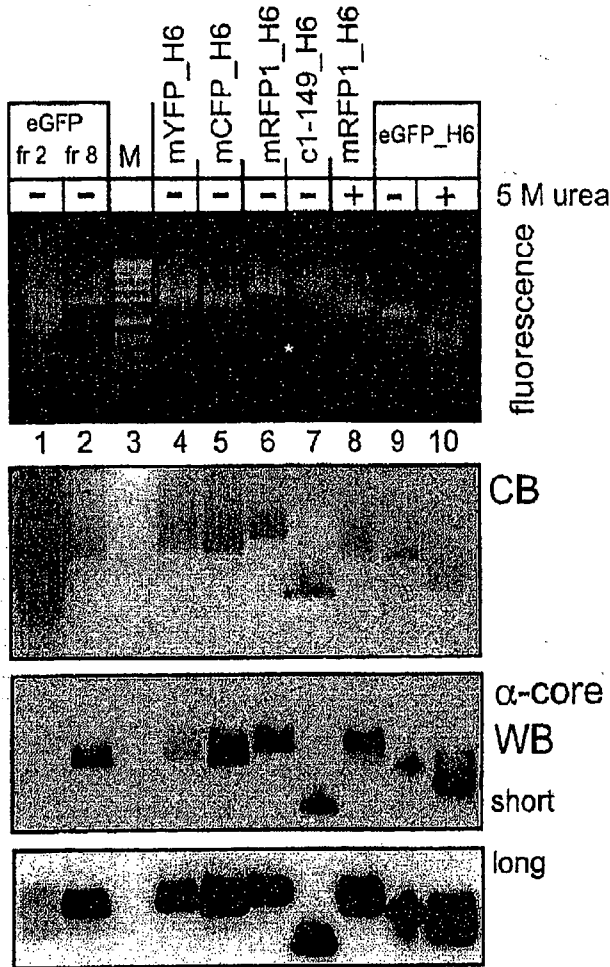


Fig. 5. Efficient formation of red fluorescent CLPs by core_mRFP1_H6. A culture of bacteria transformed with a pET construct encoding core_mRFP_H6 was split, and one half was induced at room temperature for 15 h (A), the other half for 3 h at 37°C (B). On the top of A, a composite picture of the gradient tube observed at daylight, or under illumination by 365 nm UV light is shown; no color or fluorescence was detectable for the 37°C expressed preparation yet substantial amounts of the fusion protein were found by Coomassie blue staining and Western blotting of the gradient fractions. Note that the bulk of this material is shifted downwards, almost completely overlapping with zone C.

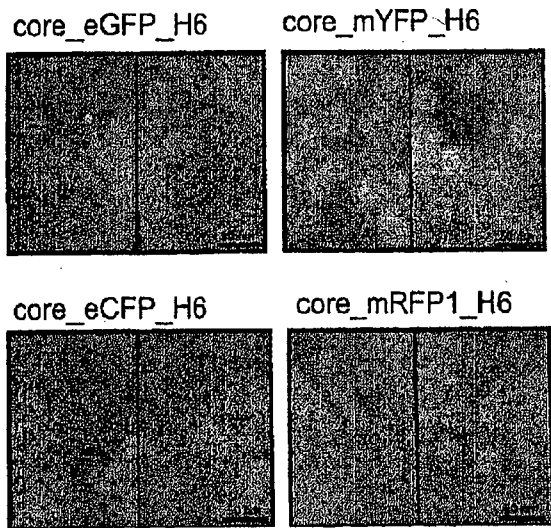
Fig. 6. Independent evidence for fluorescent CLP formation. A: Native agarose gel electrophoresis. Aliquots from fractions 8 of the gradients shown in the previous figures were run side-by-side with a DNA size marker, and a sample of CLPs derived from wild-type core protein 1-149 with a C terminal His₆ tag (c1-149_H6; lane 7). To the latter samples ethidium bromide was added to visualize the DNA, and the bacterial RNA present, in small amounts, in recombinant HBcAg lacking the C terminal basic region;† from the others ethidium bromide was omitted to not obscure the FP-specific fluorescence. To illustrate the different mobilities of assembled versus nonassembled forms, fraction 2 from the core_eGFP gradient (Fig. 2) was also loaded on the gel (lane 1); aliquots from fraction 8 of the core_mRFP1_H6 and eGFP_H6 gradients were incubated in 5 M urea for 15 min to dissociate the CLPs (lanes 8, 10) prior to loading. The gel was illuminated on a 365 nm transilluminator and photographed through a 515-nm long pass filter. The fuzzy stain near the top of lane 7 originates from unincorporated ethidium bromide, the weakly fluorescent band (asterisk) comigrated exactly with the Coomassie blue stained band of c1-149_H6 capsids (asterisk) and therefore represents encapsidated RNA. The colored fusion-protein bands migrated with the bulk of protein (CB, Coomassie blue stain; middle panel) which was also recognized by core-specific antibodies (WB; lower panel); the bottom panel shows a longer exposure to reveal the nonassembled core_eGFP protein in lane 1. B: Electron microscopy. Samples from fractions 8 of the indicated gradients were analyzed by negative staining. The bar corresponds to 50 nm. Two independent fields from each preparation are shown.

staining. The micrographs revealed abundant regular particles [Fig. 6(B)]. By contrast, no regular structures were seen upon inspection of gradient fractions 10 and 11 from the core_mRFP1_H6 preparation obtained after induction at 37°C although the fusion protein as such was present at similar concentration (not shown).

A.



B.



DISCUSSION

Viral capsids may be considered as naturally occurring nanoparticles formed by one or a few different protein species. This simple composition and their often highly symmetric architecture provides distinct advantages for structural studies; in fact, the HBV core protein was the first polypeptide whose fold was determined, at about 7.5 Å resolution, by electron cryomicroscopy and image reconstruction of CLPs.^{9,10} Forcing heterologous, non-self-assembling proteins into a symmetrical arrangement by fixation to such CLPs may thus be exploited for structural investigations.¹⁸ A more applied aspect is the presentation of proteins as antigens on CLPs or virus-like particles (VLPs) because multiple presentation on a particle can drastically increase immunogenicity.³⁹ HBV CLPs are considered an especially promising carrier whose immune enhancing properties have been documented for several small peptides and a few intermediately sized protein fragments.^{16,17,40,41} Extending this approach to complete and natively folded protein antigens offers obvious advantages over single peptide epitopes, which may adopt many irrelevant conformations if fused via one end to the carrier and, if inserted via both ends, may be forced into nonauthentic structures. Thus far, however, eGFP was the only complete protein for which native surface presentation on HBcAg CLPs has unambiguously been demonstrated.¹⁸ Though this proved that insert structure rather than mere sequence length is important, other factors affecting CLP formation, and therefore crucial for the rational design of HBcAg CLP-based fusion proteins, are largely unexplored. Our results prove insert quaternary structure as an additional critical factor; importantly, they also demonstrate how this potential limitation can be overcome.

Homomeric Insert Interactions Affect CLP Assembly

Each of the steps eventually yielding the icosahedral HBcAg CLP, i.e., folding of the monomeric subunits, dimerization, and multimerization, is potentially influenced by the inserted extra domain. Interactions between insert and core protein parts may lead to global misfolding of the monomer. If all authentic secondary structure elements form properly, a bulky insert could still prevent the central helices of the core protein to come into close juxtaposition; conversely, successful intra-core protein interactions could prevent native folding of the insert such that either the insert, or the core protein parts, but not the entire fusion protein chain adopts the correct structure. Additional constraints are imposed by the spatial proximity of the insertion sites in the core protein dimer, and by the high density of foreign domains on the CLP structure (see Fig. 1). Hence any foreign domain that is too bulky, or has an otherwise unsuitable structure, would be detrimental to CLP assembly; conversely, given that eGFP met all requirements for successful CLP formation, it was surprising that isostructural inserts should be incompatible with particle assembly. Reducing, or abolishing, inter-insert interactions entirely alleviated this obstacle, strongly suggesting that insert quaternary structure is as critical as three-dimensional structure itself.

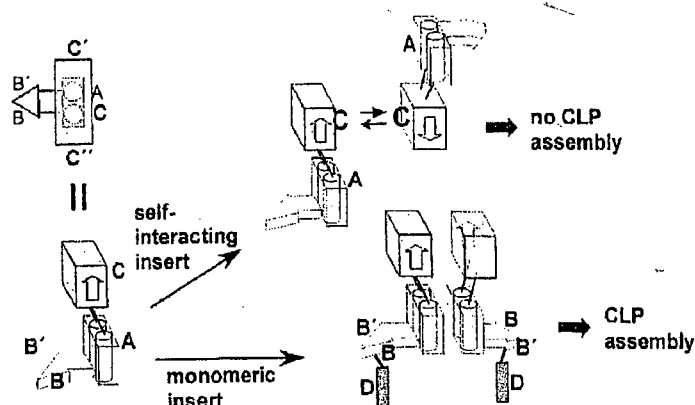


Fig. 7. Model for the influence of insert quaternary structure on CLP assembly. As in Figure 1(B), a schematic model of the core protein monomer with its A, B, and B' interfaces is shown except that it contains an inserted protein symbolized by the rectangle over the dimer interface. A highly schematic three-dimensional view is shown below with the cubic object representing the insert. If the insert contains no interaction sites on its own, (lower pathway) assembly to CLPs can proceed as with wild-type core protein dimers, given the sterical requirements for folding, dimerization and multimerization are met. If, in contrast, the insert contains inter-insert interaction sites (symbolized by C, C', and C'') these may, depending on their number, strength, and specific location relative to the neighboring inserts, compete with the interactions mediating CLP formation. Note that an antiparallel inter-insert interaction, as is likely to occur with the nonmonomeric GFP derivatives, as well as tetramerization, as with the DsRed proteins, is incompatible with HBcAg CLP assembly. Conversely, a strategically positioned interaction facilitator such as the C terminal His-tag used in this study provides an additional interface (D) that promotes, rather than competes with, CLP formation.

A model illustrating this point is shown in Figure 7. In the wild-type core protein, dimerization saturates the intra-dimer interface (termed A), and only the multimerization interfaces (termed B and B') are left for further interactions, that is, particle assembly. An inert foreign insert does not alter this setting, as long as its structure is compatible with dimerization and multimerization of the core protein parts. However, if an insert, by itself, provides one or more additional interaction sites (termed C, C', C'') CLP assembly is no longer the only route by which the free valencies can be saturated; instead competing pathways become accessible which may lead to various regular, or irregular aggregates of fusion protein subunits. Though simplistic this model accounts for all the experimental results from this study. One prediction is that the stronger the inter-insert interaction, the more likely it is to affect CLP assembly. A quantitative treatment is currently impossible but the published homo-affinities of several of the FPs used in this study clearly confirm this trend. GFP has been reported to be weakly dimerizing (approximate K_d 0.1 mM⁴²), and a similar value (0.11 mM) has been determined for eYFP.³² The estimated dimerization K_d of 74 mM for the 30 kDa mYFP translates into a concentration of 2.2 g/ml; the protein molecules in 1 L of a 33-mM solution would occupy 1 L. This emphasizes the practically monomeric character of mYFP and, most likely, mCFP. Even though the homoaaffinities of unmodified eYFP and eCFP are weak, their replacement by the monomeric versions strongly enhanced soluble CLP formation. We ascribe this to the very high local concentration of the FP domains when present on a core protein dimer, or as-

sembled CLP, where diffusion is totally restricted. A further prediction is that the yields of eGFP CLPs should also be increased by introducing the monomerizing A206K mutation,³² corresponding experiments are underway.

An exact value for the homoaffinity of HcRed1 has not been published, however the protein behaved as a dimer during gel filtration under all concentration conditions tested,³⁰ for DsRed1 the K_d for oligomerization is below 10^{-9} M.²⁷ Plausibly such strong homomeric interactions compete effectively with the core-protein interactions required for assembly. Notably, although the core-protein dimer appears highly stable, the interdimer interactions in the capsid are only weak.²⁰ Whereas a tetrameric insert structure is incompatible with the clustered dimer arrangement of subunits in the HBcAg shell, dimerization per se is not, as is obvious from the dimeric nature of the core protein itself (Fig. 1). However, to fit onto the two parallel monomers, the subunits of a dimeric insert would have to adopt a parallel, or near parallel, orientation. The DsRed tetramer consists of two antiparallel, skewed dimers, that is, the four monomers point alternately up and down.^{25,26} Tetrameric HcRed is likely to adopt a similar arrangement, and the antiparallel orientation is most likely preserved in the engineered dimeric HcRed1; furthermore, in various crystals, GFP and its direct homologs also adopt antiparallel orientations.⁴³ Hence not dimerization per se, but rather the unfavorable orientation of the subunits in the dimer is the major reason that the red FPs, except mRFP1, are incompatible with HBcAg dimerization and CLP formation. Conversely, a dimeric insert with parallel subunits might be able to support assembly. An example is the outer surface protein C (OspC) from *Borrelia burgdorferi*. This protein is an obligate dimer, but the two monomers have a parallel orientation and, in addition, all four termini of the dimer protrude from one side.^{44,45} Indeed, we have generated core_OspC fusions that efficiently assemble into CLPs (C. Skamel, T. Stehle, M.M. Simon, M. Nassal; manuscript in preparation).

Implications for CLP Design

Successful generation of CLPs that present foreign epitopes has relied to a large extent on trial and error. The structure elucidation of various self-assembling moieties, such as HBcAg and other virus capsids defined the fundamental steric requirements on the carrier side. Knowing the structure of the foreign protein will significantly increase the reliability of predictions on whether or not this protein will support CLP assembly. Even then, however, most stable insert quaternary structures are likely to interfere with particle formation. Therefore, testing for higher order structures of a candidate insert, e.g., by gel filtration, is worthwhile. This also holds for the mass of protein antigens for which no high resolution structure is available but which appear attractive on the basis of their immunological properties. With whole pathogen genomes now being explored as a source for detecting the most potent antigens⁴⁶ this may soon become a standard situation. Even though reducing the oligomerization potential by point mutations will rarely be feasible, removing known and/or predictable interaction domains should be highly beneficial.

A fortuitous finding was that, in the setting of the HBcAg carrier system, the addition of a C terminal His6 tag substantially increased the proportion of assembled versus nonparticulate forms. The exact mechanism remains to be established but structural studies suggest some clues. Fused to aa-149 of the core protein, the tag must reside at, or close to, the multimerization interfaces in the C terminal region of the assembly domain.⁶ A cysteine residue added to aa-149 was explicitly shown to reside in the particle interior close to the interdimer contacts.⁴⁷ In line, CLPs from wild-type core protein with a His-tag fused to aa-149 do not bind to a matrix carrying immobilized Ni^{2+} ions unless they are dissociated into dimers (M. Vogel and M. Nassal, unpublished data). Conceivably, a tag that can interact, for example, by metal coordination, with a similar tag on a different dimer will promote assembly by providing an additional valency (D in Fig. 7) that now favors, rather than competes with, proper assembly. Adding an appropriately designed and strategically placed interaction facilitator should therefore be a general way of enhancing particle assembly also with other carrier systems.

Applications of Fluorescent CLPs

The main purpose of using GFP and its variants in this study was to exploit their dependence on proper folding for chromophore formation as an indicator for native folding. In addition, the new fluorescent CLPs may also be of immediate use. One is the generation of FP-specific antibodies, as with core_eGFP.¹⁸ Another is the definition of FPs that are suitable as reporters to monitor intracellular trafficking and assembly of the wild-type HBV core protein by fluorescence microscopy.⁴⁸ Evidently, the use of DsRed or HcRed fused to the core protein would lead to completely misleading results because they are unable to support capsid assembly but still mature to fluorescent species in the fusion-protein context; the monomeric derivatives, by contrast, would be well suited. Furthermore, the successful generation of fluorescent CLPs carrying FPs that are established fluorescence resonance energy transfer (FRET) pairs, such as CFP and YFP or improved versions,^{43,49} opens the way to analyze, by FRET and related techniques, capsid assembly as well as its inhibition by compounds targeting the core protein. Such compounds are beginning to be explored both as basic tools⁵⁰ and as potential novel drugs against chronic hepatitis B.⁵¹

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In vitro assembly of mosaic hepatitis B virus capsid-like particles (CLPs): Rescue into CLPs of assembly-deficient core protein fusions and FRET-suited CLPs

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Abstract Hepatitis B virus core protein self-assembles into icosahedral, highly immunogenic capsid-like particles (CLPs) that can serve as molecular platforms for heterologous proteins. Insertion into the centrally located c/e1 epitope leads to surface display, fusion to the C terminus to internal disposition of the foreign domains. However, symmetry-defined space restrictions on the surface and particularly inside the CLPs limit the size of usable heterologous fusion partners. Further, CLPs carrying differing foreign domains are desirable for applications such as multivalent vaccines, and for structure probing by distance sensitive interactions like fluorescence resonance energy transfer (FRET). Here, we report an *in vitro* co-assembly system for such mosaic-CLPs allowing successful CLP formation with a *per se* assembly-deficient fusion protein, and of CLPs from two different fluoroprotein-carrying fusions that exert FRET in an assembly-status dependent way.

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Keywords: Capsid-like particles; Capsid structure; Fluorescence resonance energy transfer; Hepatitis B virus; Hepatitis B virus capsid protein

1. Introduction

The 183 amino acid core protein forms the protein shell of the icosahedral hepatitis B virus (HBV) nucleocapsid (core particle). Heterologously expressed core protein self-assembles into morphologically similar capsid-like particles (CLPs). The first about 140 amino acids form the assembly domain, i.e., they are sufficient for assembly [1,2]; the basic C terminus, in CLPs mostly located in the particle lumen [3,4], acts as a nucleic acid binding domain [5,6]. Due to its exceptional immunogenicity the core particle, serologically defined as hepatitis

B core antigen (HBcAg), is of major interest as a particulate vaccine carrier for heterologous sequences [7,8]. The predominant particle form (triangulation number of $T=4$) is composed of 120 and a minor form ($T=3$) of 90 core protein dimers [3,9–11]. The most surface-exposed part is a short loop around amino acid 80 [12], overlapping with the immunodominant c/e1 B cell epitope [13], that connects two long helices forming the core protein's dimer interface [14,15]. As exemplified by the green fluorescent protein (GFP), even whole protein domains of at least 238 amino acids can be inserted into this loop without compromising particle formation [16]. However, specific properties of the inserted protein, such as an unfavorable molecular shape [17] or the propensity to homo-oligomerize [18], can severely restrain particle assembly. A universal restriction is the limited space, defined by the particle symmetry, on the surface and in particular inside the CLP. CLPs with internally disposed heterologous domains can be obtained by their fusion to the C terminus of the assembly domain [19,20]. According to X-ray data of recombinant HBcAg CLPs ending with amino acid 149 [15] the available inner volume in a $T=4$ CLP is about 9000 nm³, leaving roughly 38 nm³ for each of the 240 added foreign domains. Indeed, a core fusion protein with the 17 kDa *Staphylococcus aureus* nuclease, with an approximate volume of 36 nm³, fused to aa 155 formed rather unstable particles [19]. Hence larger heterologous proteins are expected to prevent assembly (Fig. 1A).

Both surface and internal crowding should be reduced if, in one particle, some of the fusion protein subunits are replaced by sterically less demanding subunits, e.g., non-fused wild-type protein. Thus far, this mosaic-CLP concept has been addressed by co-expression of the two different protein subunits, either from two compatible plasmids [19] or by limited translational read-through [21]. However, these *in vivo* approaches offer only limited controllability of the CLP composition, and little flexibility regarding the nature and number of the assembly partners. Here, we present an alternative *in vitro* co-assembly approach largely overcoming these limitations. We demonstrate its principal feasibility by co-assembly of wild-type core protein subunits with an assembly-competent and an assembly-deficient core-GFP fusion (enhanced green fluorescent protein, eGFP inserted into c/e1, and fused to aa 148, respectively), and by mosaic-CLP formation from two core fusions with different GFP derivatives that are suitable for fluorescence resonance energy transfer (FRET).

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Abbreviations: CLP, capsid-like particles; eGFP, enhanced green fluorescent protein; FP, fluorescent protein; FRET, fluorescence resonance energy transfer; HBV, hepatitis B virus; mCFP, monomeric cyan FP; mYFP, monomeric yellow FP; NTA, nitrilotriacetic acid; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis

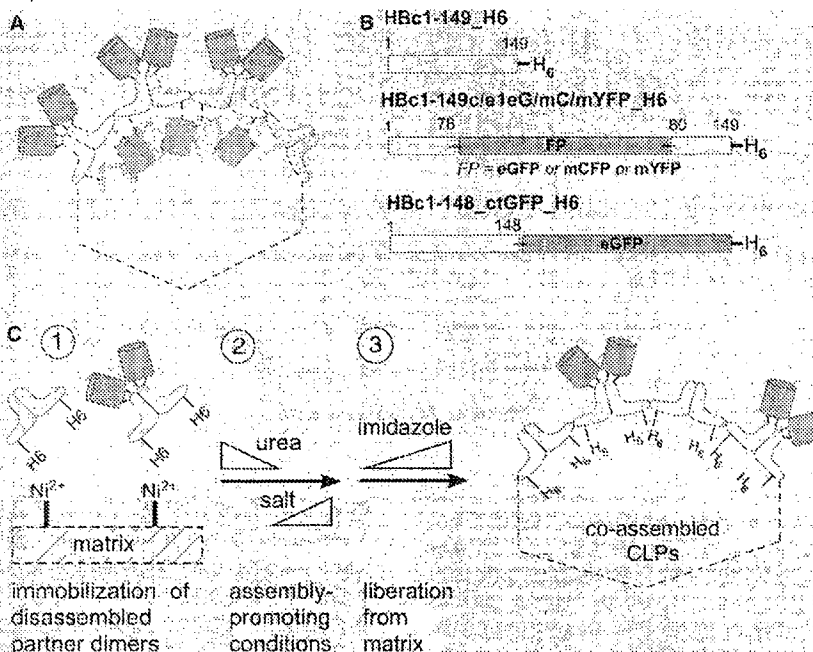


Fig. 1. Concept of mosaic HBV CLPs, and core proteins used. **(A) Structural aspects.** HBV core protein (shown as L-shaped object) forms dimers that assemble into icosahedral CLPs. The dimer interface is formed by two antiparallel helices per monomer, connected by a short loop overlapping with the c/e1 epitope around aa 80; the four-helix bundles protrude as spikes from the CLP surface. Foreign proteins as large as GFP (barrel structures) can be inserted into the loop without compromising assembly; fusion to the C terminus of the core protein's assembly domain leads instead to an internal disposition (transparent barrel structures), with increased steric crowding. This is relieved if some of the fusion protein subunits are replaced by non-fused core protein subunits (darker shading), i.e., by mosaic-CLP formation. **(B) Schematic representation of the core protein derivatives used.** All proteins contained C terminal His₆ tags. HBc1-149_H6 is the wild-type core protein without the basic C terminus; HBc1-149c/e1G/mCFP/mYFP_H6 denotes three related fusion proteins, containing between core protein aa 78 and 80 either eGFP, mCFP, or mYFP; in HBc1-148_ctGFP_H6, eGFP is fused to aa 148 of the core protein. **(C) Outline of the *in vitro* co-assembly procedure.** In step 1, a mixture of the assembly partner proteins is immobilized via their His-tags to a Ni²⁺-matrix in the presence of urea; in step 2, conditions are shifted, on column, towards promoting assembly by decreasing the urea and increasing the salt concentration; in step 3, the proteins are eluted from the column by increasing imidazole concentration. During this process, co-assembly into mosaic-CLPs can occur.

2. Materials and methods

2.1. Plasmid constructs

The T7 promoter containing expression plasmids encoding C terminally His₆-tagged proteins HBc1-149_H6 (wild-type core protein from aa 1 to 149) and HBc1-149c/e1eGFP/mCFP/mYFP_H6 (carrying either eGFP, or monomeric CFP, or monomeric YFP [22] inserted between aa 78 and 80) have previously been described [18]. pET28a2-HBc148-ctGFP_H6 encodes core protein 1–148 with eGFP, plus a C-terminal His₆-tag, fused to amino acid 148 via a linker with the sequence A(G₃S)₃G₄T. A schematic representation of the proteins is shown in Fig. 1B.

2.2. Expression and enrichment of core proteins variants

All proteins were expressed using *Escherichia coli* BL21(DE3) Codonplus RIL cells (Stratagene). Cell lysis and core protein enrichment by sucrose gradient centrifugation were performed as previously described [18]. Protein HBc1-148ctGFP_H6, not forming regular CLPs, was purified by immobilized metal ion affinity chromatography (IMAC) on Ni²⁺-nitrilotriacetic acid (NTA) agarose (Qiagen) under native conditions as suggested by the manufacturer. Proteins were analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% acrylamide and 0.1% SDS in the Laemmli buffer system. For immunoblotting, HBV core protein specific monoclonal antibodies mc312, mc158 or mc275 [23,24], or a monoclonal antibody mixture against GFP (Roche) were used. Detection was performed using secondary antibody-peroxidase conjugates and a chemiluminescent substrate (ECL+, Amersham). Gradient or IMAC fractions containing the desired proteins were combined, dialyzed against TN-300 buffer (25 mM Tris/HCl, pH 7.5, 300 mM NaCl) and concentrated by ultra filtration (Amicon Ultra devices, 30 kDa cut-off; Millipore). Protein concentrations were determined using the Bradford assay (Bio-Rad).

2.3. *In vitro* co-assembly

Pre-purified core protein derivatives, at about 1–2 mg/ml, were adjusted to dissociation buffer (6 M urea, 10 mM imidazole, 50 mM sodium phosphate buffer, pH 8.0, and 50 mM NaCl), and incubated at room temperature for 15 min, followed by another 15 min at 37 °C; all subsequent steps were performed at room temperature. The solutions were mixed at the desired molar ratios, and bound to a Ni²⁺-NTA column pre-equilibrated in dissociation buffer (usually 1–2 ml protein solution for a 2 ml gel bed column). The urea concentration was decreased by washing with 2 ml each of washing buffer (20 mM imidazole, 50 mM sodium phosphate, pH 8.0, and 50 mM NaCl) containing 5, 4, 3, 2, 1 and 0.5 M urea, followed by pure washing buffer, then washing buffer containing 300 mM NaCl. Finally, twice the gel bed volume of elution buffer (250 mM imidazole, 50 mM sodium phosphate buffer, pH 8.0, and 300 mM NaCl) was added and, after a 30 min incubation, the proteins were eluted by gravity flow. To separate CLPs from non-assembled forms, dialyzed and concentrated samples were subjected to sedimentation in 10–60% (wt/vol) sucrose step gradients. Fluorescent mosaic CLPs were detected by visual inspection upon irradiation with a UV lamp emitting light of 312 or 365 nm.

2.4. Native agarose gel electrophoresis

Aliquots from individual gradient fractions were subjected to electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris-acetate, 0.1 mM EDTA, pH 8.0) containing 0.5 µg/ml of ethidium bromide as previously described [18], and detected directly via their fluorescence or immunologically after blotting to PVDF membranes.

2.5. Electron microscopy

Electron micrographs were obtained on a Zeiss EM 902 at 80 kV with nominal magnification of 85000–140000-fold. Samples from appropriate sucrose gradient fractions were stained with 2% methylamine tungstate, pH 7.0, as previously described [18].

2.6. Fluorescence spectroscopy

Fluorescence measurements were performed at 20 °C using a Perkin-Elmer LS 45 spectrometer in quartz cuvettes (5 mm light path, Suprasil; Hellma). All spectra were corrected for lamp intensity and detector sensitivity. Proteins were used at concentrations of 0.18–0.9 µg/µl in 25 mM Tris/HCl, pH 7.5, 150 mM NaCl. Data were recorded with an integration time of 1000 ms. Emission spectra were recorded at 410 nm as excitation maximum for monomeric cyan FP (mCFP) fusion proteins and FRET samples, and at 483 nm for monomeric yellow FP (mYFP) fusion proteins. The spectra were normalized to their maxima.

3. Results

3.1. Experimental design

Due to the highly stable fold of the core protein and its dimer, the inter-dimer contacts in HBcAg CLPs can be dissociated, without denaturation of the subunits, by partially denaturing conditions [1,25], for instance, depending on the protein concentration and the ionic strength of the solution [25], by 2–6 M urea. This property can be exploited to expose a C terminal His-tag fused to aa 149, which is buried inside intact CLPs, and to immobilize the dimers on a Ni²⁺-NTA matrix. As outlined in Fig. 1C, the first step of our method consists in this urea-assisted immobilization of the two proteins at a preselected ratio on the same Ni²⁺-matrix. Non-assembling His-tagged fusion proteins may directly be immobilized on the matrix. In the second step, similar to on-column refolding of denatured proteins (for review, see [26]), the buffer composition is gradually altered towards assembly-promoting conditions via decreasing the urea and increasing the salt concentration; finally the proteins are eluted by increasing the imidazole concentration. This general scheme was applied to the different protein mixtures reported below. To detect mosaic-CLP formation, the eluates were dialyzed against high salt buffer, concentrated, and analyzed by sedimentation in sucrose gradients, by native agarose gel electrophoresis and Western blotting, and by electron microscopy (EM).

3.2. Mosaic-CLPs from wild-type core protein 1–149 and an assembly-competent coreGFP fusion

For proof of concept, His-tagged wild-type core protein HBc1-149_H6 and the assembly-competent coreGFP fusion HBc1-149_c/e1GFP_H6 [18] were bound, at an approximate 1:1 molar ratio, to Ni²⁺-NTA beads in the presence of urea; without urea, essentially no binding occurred, consistent with the C terminal His-tag being inaccessible in intact CLPs. Employing the above described reassembly procedure the two proteins co-eluted from the column at 250 mM imidazole concentration. Upon sedimentation in 10–60% sucrose gradients approximately one third of the fusion protein, and two thirds of the wild-type accumulated in the particle-typical central fractions [18]; the remainder stayed in the top fractions (Fig. 2A), suggesting the wild-type protein assembles somewhat more efficiently than the fusion protein. To distinguish true mosaic-CLPs from a mixture of two separate CLP populations, an aliquot from gradient fraction 7 was subjected to native agarose gel electrophoresis, in parallel with pure HBc1-149_H6 and HBc1-149_c/e1GFP_H6 CLPs. After blotting, the membrane was probed with anti-core and anti-GFP antibodies (Fig. 2B). The unmodified CLPs migrated substantially faster than the pure coreGFP CLPs whereas, as expected

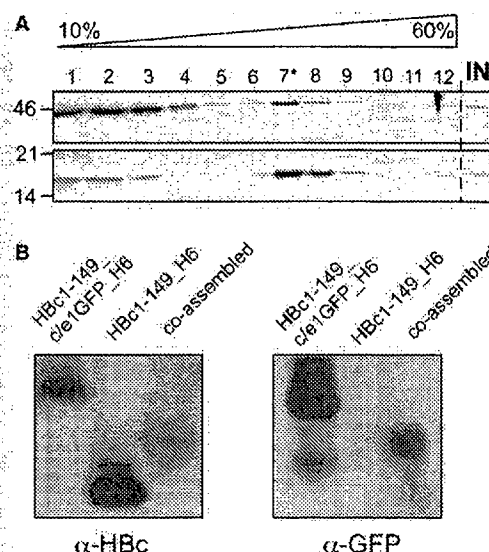


Fig. 2 Mosaic-CLPs from an assembly-competent coreGFP fusion and wild-type core protein. HBc1-149_c/e1GFP_H6 and HBc1-149_H6 CLPs were subjected to the procedure outlined in Fig. 1C. The eluate from the Ni-NTA column was sedimented through a 10–60% sucrose gradient. (A) SDS-PAGE. Aliquots from gradient fractions 1–12 were analyzed by SDS-PAGE and Coomassie Blue staining. M, protein size marker; IN, input before sedimentation. (B) Native agarose gel electrophoresis. Pure HBc1-149_c/e1GFP_H6 and HBc1-149_H6 CLPs as isolated from the bacteria, and an aliquot of gradient fraction 7 from the co-assembly reaction were run on an agarose gel, blotted, and one sample set was probed with anti-core antibody m6158 (α-HBc), the other with anti-GFP (α-GFP) antibodies, then visualized using a chemiluminescent substrate.

for mosaic-CLPs, the co-assembled particles produced a band of intermediate mobility which was detected by both antibodies; the HBc1-149_H6 CLPs, by contrast, were recognized only by the anti-core antibody. These data attested to the successful formation of mosaic-CLPs.

3.3. Rescue of an assembly-deficient core fusion protein by *in vitro* co-assembly with wild-type core protein

As a more stringent test for the potential of the method we next addressed *in vitro* co-assembly of the HBc1-149_H6 protein with a *per se* assembly-deficient core protein fusion. In this protein, HBc1-148ctGFP_H6, cGFP was fused to core protein aa 148 (Fig. 1B). This should lead to a luminal disposition of the GFP domains but the larger volume of GFP (about 47 nm³) was expected to interfere with particle formation. The protein was well expressed and showed strong green fluorescence, indicating the GFP part was natively folded. However, on sucrose gradients it displayed a broad distribution from the top region to the center (Fig. 3A, top panel). This suggested the presence of at most a small fraction of regular CLPs and, congruently, mostly irregularly shaped aggregates were detected by negative staining EM (Fig. 3C). In addition, the protein bound well to Ni²⁺-NTA beads in the absence of urea, indicating the C terminal His tags were solvent accessible.

Next the fusion protein was mixed with HBc1-149_H6 at molar ratios of 5:1, 1:1, and 1:5, then the three samples were subjected to the protocol described above. In all cases, both proteins co-eluted at 250 mM imidazole. After sucrose gradient

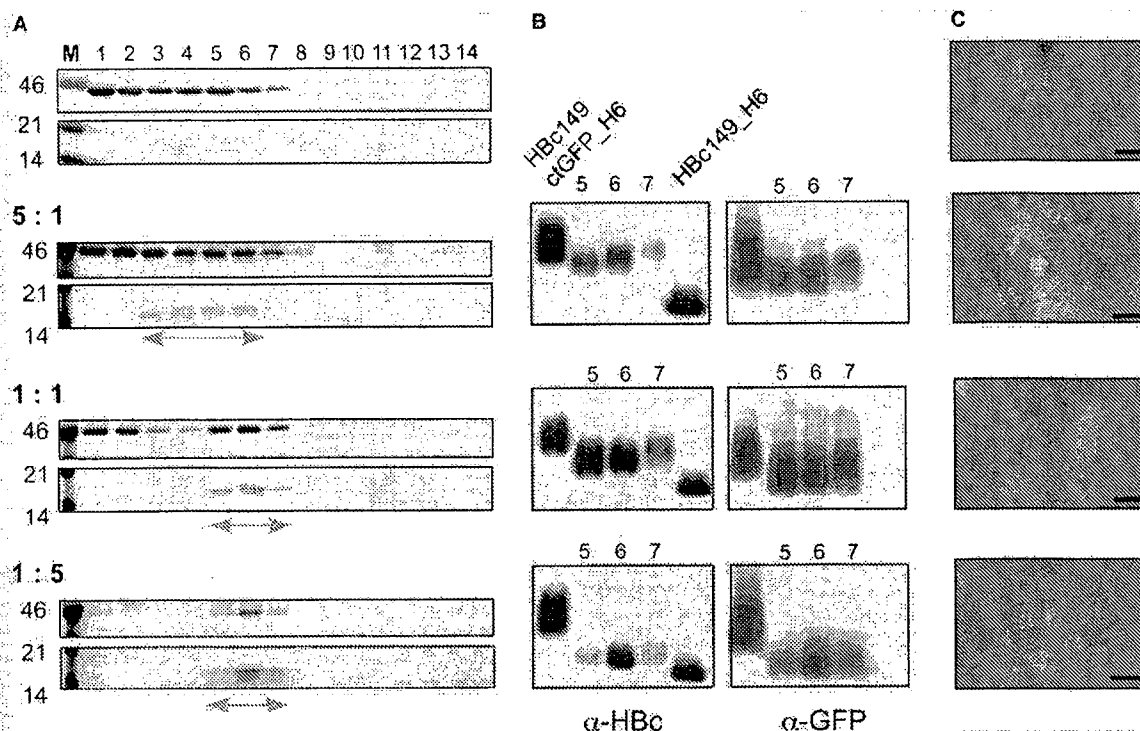


Fig. 3. Mosaic-CLPs from an assembly-deficient coreGFP fusion and wild-type core protein. Proteins HBcl-149ctGFP_H6 and HBcl-149_H6 were subjected to the procedure outlined in Fig. 1C at the indicated molar ratios, then the reaction products were sedimented through sucrose gradients as in Fig. 2A. (A) *SDS-PAGE*. Aliquots from gradient fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The top panel shows the fusion protein alone; the following panels are derived from co-assembly reactions at the indicated fusion protein to wild-type protein ratios. The arrows indicate the distribution of the wild-type core protein. (B) *Native agarose gel electrophoresis*. Aliquots from gradient fractions 5–7 of the co-assembly reactions were analyzed by agarose gel electrophoresis and immunoblotting as in Fig. 2B. Note the gradual shift of the co-assembly products towards the position of wild-type CLPs. (C) *Electron microscopy*. Samples from gradient fraction 6 were analyzed by negative staining EM. The bar corresponds to 50 nm. Note the gradual increase in the fraction of regular CLPs.

sedimentation, SDS-PAGE analysis revealed that with the fusion protein in excess, the distribution of the fusion protein was similar as in the absence of wild-type protein; notably, even the wild-type protein was shifted towards the top of the gradient (Fig. 3A, 5:1), suggesting a negative effect on assembly by the fusion protein. By contrast, at a 1:1 ratio a distinct peak for both proteins was seen in fractions 5–7; about one half of the fusion protein remained in the top fractions (Fig. 3A, 1:1). With excess wild-type protein finally, both proteins were confined to fractions 5–7 (Fig. 3A, 1:5). To confirm the presence of mosaic-CLPs, gradient fractions 5–7 from the three assembly reactions were analyzed by native agarose gel electrophoresis and subsequent immuno-blotting with anti-core and anti-GFP antibodies; pure HBcl-149_H6 CLPs and HBcl-149ctGFP_H6 proteins served as references. The fusion protein itself, recognized by both anti-core and anti-GFP antibodies, migrated as a relatively broad band with distinctly lower mobility than pure HBcl-149_H6 CLPs (Fig. 3B). With increasing proportion of wild-type protein in the assembly reaction, the mobility increased to nearly that of pure wild-type CLPs, as expected for internally disposed heterologous domains. Consistently, negative staining EM confirmed a decreased proportion of irregular particles and a concomitant increase of regular CLPs with approximately wild-type CLP diameters of, on average, less than 30 nm (Fig. 3C). Hence in vitro co-assembly with the wild-type core protein rescued an assembly-deficient fusion protein into regular CLPs.

3.4. Formation of mosaic CLPs from two different fusion proteins and proof of their mosaic nature by FRET

A further application for co-assembly would be the generation of mosaic-CLPs from two or more different fusion proteins. An attractive model combination are two different GFP derivatives whose spectral properties allow for FRET. Because FRET depends on spatial proximity between donor and acceptor, mosaic-CLPs, but not a mixture of two separate CLP populations, should give rise to FRET. The cyan (CFP) and yellow (YFP) fluorescent proteins are an established FRET donor-acceptor pair [27], and their monomeric variants, mCFP and mYFP [22], can individually be inserted into the core c/e1 epitope yielding fluorescent CLPs [18]. The distance between two neighboring spikes on wild-type CLPs is about 5 nm [15], close to the Foerster radius (distance at which energy transfer efficiency is 50 %) for CFP-YFP and hence ideally suited for FRET. Therefore, HBcl-149-c/e1-mCFP-H6 and HBcl-149-c/e1-mYFP-H6, at an equimolar ratio, were subjected to the in vitro co-assembly procedure. Both proteins co-eluted from the Ni^{2+} -NTA column, and about half of both proteins co-sedimented into the particle-typical gradient fractions. Native agarose gel electrophoresis showed a distinct band with nearly the same mobility as that of the pure mCFP- and mYFP-containing CLPs (Fig. 4A). The similar mobilities and the lack of antibodies specifically recognizing mCFP but not mYFP or vice versa prevented a conventional assessment of mosaic-CLP formation. Instead, the CLPs were analyzed

by fluorescence spectroscopy. The excitation and emission spectra of the individual CLPs were very similar to those of the unfused fluorescent proteins, with emission maxima at 474 nm (plus a sideband around 500 nm) for the mCFP- and

at 530 nm for the mYFP-containing CLPs (Fig. 4B, dashed blue and orange lines). When irradiated at 410 nm for specific excitation of mCFP with minimal mYFP excitation, the emission spectrum of a 1:1 mixture of preformed CLPs (green line) was very similar to that of pure mCFP-containing CLPs. By contrast, the co-assembled CLPs (red line) yielded a strong peak at the 530 nm mYFP emission maximum while the mCFP emission at 474 nm was reduced, exactly as expected for efficient FRET. To prove that dynamic changes in the capsid status can be monitored, the co-assembled CLPs were incubated with 6 M urea. Upon this treatment, the emission spectrum showed a strong reduction in the 530 nm peak, and a simultaneous fluorescence increase at the 474 nm mCFP peak (Fig. 4C, black line), fully consistent with urea-induced dissociation of the mosaic-CLPs. The spectra of the individual fluoroprotein-presenting CLPs and their mixture (Fig. 4C, green line) remained unaffected, confirming the fluorophores themselves were stable under these conditions.

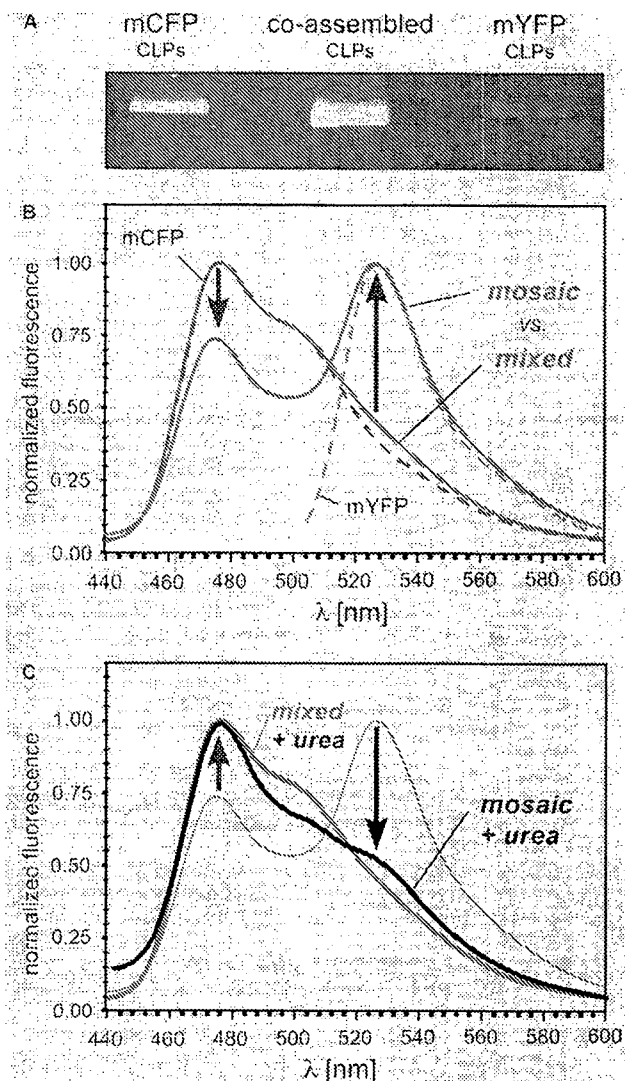


Fig. 4. Co-assembly of FRET-suited coreGFP-fusion variants. Core protein fusions HBc1-149_c1mCFP_H6 and HBc1-149_c1mYFP_H6 were co-assembled as outlined in Fig. 1C, and the reaction products were separated by sucrose gradient sedimentation as in Fig. 2A. (A) Native agarose gel electrophoresis. An aliquot from gradient fraction 7 was analyzed in parallel with pure mCFP- and mYFP-containing CLPs, and detected via fluorescence. (B) Mosaic-CLPs but not mixed CLPs exert FRET. The emission spectra of pure mCFP- and mYFP-containing CLPs (dashed blue and orange lines), or a 1:1 mixture, or the co-assembly product were recorded by fluorescence spectroscopy. At 410 nm excitation, the mixture (green line) displayed a nearly identical spectrum as pure mCFP-CLPs whereas the co-assembled sample (red line) showed a strong peak at the 530 nm mYFP emission maximum, and a reduced emission at the 474 nm mCFP maximum. (C) FRET-monitoring of urea-mediated CLP dissociation. The co-assembled and the mixed sample were incubated with urea, and the emission spectra were recorded as in (B). Compared to the co-assembled sample prior to urea treatment (thin red line with shaded area underneath), the 530 nm peak was strongly reduced whereas the 474 nm emission increased (thick black line). The signal from the mixed CLPs remained essentially unchanged (thick green line).

4. Discussion

The here described in vitro co-assembly method overcomes several of the limitations of previous in vivo co-expression approaches for mosaic-CLP generation: the procedure is straightforward; using prepurified components, the input amounts of each subunit are easily controlled, allowing, to a much larger extent, the proportion of the individual subunits per particle to be adjusted; most likely, more than two different subunits could be co-assembled in this way; not the least, the method allowed for the first time to monitor HBV CLP formation, and disassembly, by the sensitive FRET technique.

Several lines of evidence support the formation of true mosaic-CLPs. Co-assembly of HBc1-149_H6 with the assembly-competent HBc1-149c/e1-eGFP_H6 gave rise to particles which displayed an intermediate electrophoretic mobility on native agarose gels. A fusion of eGFP to the C terminus of the core protein assembly domain formed essentially no stable CLPs on its own but did so upon co-assembly with HBc1-149_H6, fully consistent with reduced luminal crowding. Hence the co-assembly approach with wild-type core protein enables particle formation for fusions that are too bulky to assemble into closed shells on their own, and heterologous proteins considerably larger than the 238 aa GFP should be amenable to presentation on, or packaging into, HBV CLPs.

The co-assembly data for fusions with mCFP and mYFP demonstrate the feasibility of generating CLPs containing two, and probably more, different heterologous domains per particle. In particular, they provide a novel means for spectroscopic analyses of HBV capsid assembly and disassembly. Our data clearly confirm mosaic CLP formation by the unique FRET signal observed for co-assembled but not mixed CLPs. Moreover, the intensity of the FRET signal strongly suggests a mCFP to mYFP distance close to the Förster radius for this FRET pair, congruent with a random distribution of the differently labeled subunits on the particles. Mosaic CLPs containing these, or other suitable chromophores, should therefore become valuable tools to quantitatively assess HBV nucleocapsid assembly and disassembly but also to screen for capsid-targeting antivirals such as the recently described heteroarylpyrimidines

[28]. Finally, using core proteins containing the arginine-rich C terminal domain the approach may be adapted for the in vitro encapsidation of selected nucleic acids.

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Hepatitis B Virus Capsid-like Particles Can Display the Complete, Dimeric Outer Surface Protein C and Stimulate Production of Protective Antibody Responses against *Borrelia burgdorferi* Infection^{*[S]}

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Hepatitis B virus capsid-like particles (CLPs), icosahedral assemblies formed by 90 or 120 core protein dimers, hold promise as immune-enhancing vaccine carriers for heterologous antigens. Insertions into the immunodominant c/e1 B cell epitope, a surface-exposed loop, are especially immunogenic. However, display of whole proteins, desirable to induce multispecific and possibly neutralizing antibody responses, can be restrained by an unsuitable structure of the foreign protein and by its propensity to undergo homomeric interactions. Here we analyzed CLP formation by core fusions with two distinct variants of the dimeric outer surface lipoprotein C (OspC) of the Lyme disease agent *Borrelia burgdorferi*. Although the topology of the termini in the OspC dimer does not match that of the insertion sites in the carrier dimer, both fusions, coreOspC_α and coreOspC_β, efficiently formed stable CLPs. Electron cryomicroscopy clearly revealed the surface disposition of the OspC domains, possibly with OspC dimerization occurring across different core protein dimers. In mice, both CLP preparations induced high-titered antibody responses against the homologous OspC variant, but with substantial cross-reactivity against the other variant. Importantly, both conferred protection to mice challenged with *B. burgdorferi*. These data show the principal applicability of hepatitis B virus CLPs for the display of dimeric proteins, demonstrate the presence in OspC of hitherto uncharacterized epitopes, and suggest that OspC, despite its genetic variability, may be a valid vaccine candidate.

The icosahedral nucleocapsid of hepatitis B virus (HBV)² is formed by multiple dimeric copies of the 183-residue core protein. The particle, serologically termed hepatitis B core antigen (HBcAg), is exceptionally immunogenic and can act as a T cell-dependent and as a T cell-inde-

pendent antigen (1), most likely due to the repetitive presentation of the epitopes on its surface (2). This property, shared by some other viral particles (3), has been exploited to design recombinant HBV capsid-like particles (CLPs) as an immune-enhancing carrier for heterologous peptides (reviewed in Refs. 4, 5) and recently for whole chain polypeptides (6–9). Fusions into the most exposed region on the capsid surface (10–12), encompassing the immunodominant c/e1 B cell epitope of HBcAg (13), are particularly immunogenic for B cells. However, the need to conserve the particulate structure for high immunogenicity together with the central location in the primary sequence of the c/e1 epitope imposes restraints on the nature of the heterologous sequence that can be inserted.

The core protein consists of an N-terminal assembly domain encompassing the first ~140 aa (14, 15) and an Arg-rich C-terminal nucleic acid binding domain (16, 17). The structure of HBV CLPs is known from electron cryomicroscopy (10, 18–21) and x-ray crystallography (22). The capsids occur in two size classes (triangulation numbers T 3 and T 4) consisting of 90 and 120, respectively, core protein dimers (Fig. 1A). Dimerization involves a four-helix bundle to which each monomer contributes two long antiparallel α -helices (α 3 and α 4) (22); these bundles protrude as spikes from the capsid surface, with the c/e1 epitope at their tips (11, 12, 23).

The c/e1 loop lends itself as an insertion site; however, both the N and C termini of an insert must fit to the geometry of the core acceptor sites (Fig. 1B), a requirement not easily met by many natively folded proteins. In the green fluorescent protein (GFP), the first complete protein displayed on HBcAg CLPs (6), the N and C termini are closely juxtaposed (24). By contrast, the termini of the outer surface protein A (OspA) of the Lyme disease agent *Borrelia burgdorferi* (see below) are at opposite ends of the elongated structure (25), such that generation of a multimerization-competent fusion required very long connecting linkers (9). Less obvious but equally critical is the propensity of a candidate insert to undergo homomeric interactions. Dimerizing and tetramerizing color variants of GFP, though isostructural to GFP, failed to form CLPs; the key role of insert quaternary structure was demonstrated by successful CLP formation when engineered monomeric variants were used instead (8). However, monomerization may involve the exchange of many amino acids (e.g. 33 in the case of the monomeric red fluorescent protein) (26) and thus not be suited for a protein to be used as a vaccine. Here we explored the possibility of generating HBcAg CLPs displaying a naturally dimeric whole protein antigen.

The outer surface protein C (OspC) of *B. burgdorferi* appeared as an ideal model insert. OspCs are ~210 aa in length, including an 18-aa

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^[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental text and supplemental Figs. S1 and S2.

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² The abbreviations used are: HBV, hepatitis B virus; aa, amino acid; Ab, antibody; mAb, monoclonal Ab; CLP, capsid-like particle; EM, electron microscopy; GFP, green fluorescent protein; HBcAg, hepatitis B core antigen; IS, immune serum; Osp, outer surface protein; PBS, phosphate-buffered saline; recGST-OspC_β, recombinant glutathione S-transferase-OspC_β fusion protein; ELISA, enzyme-linked immunosorbent assay.

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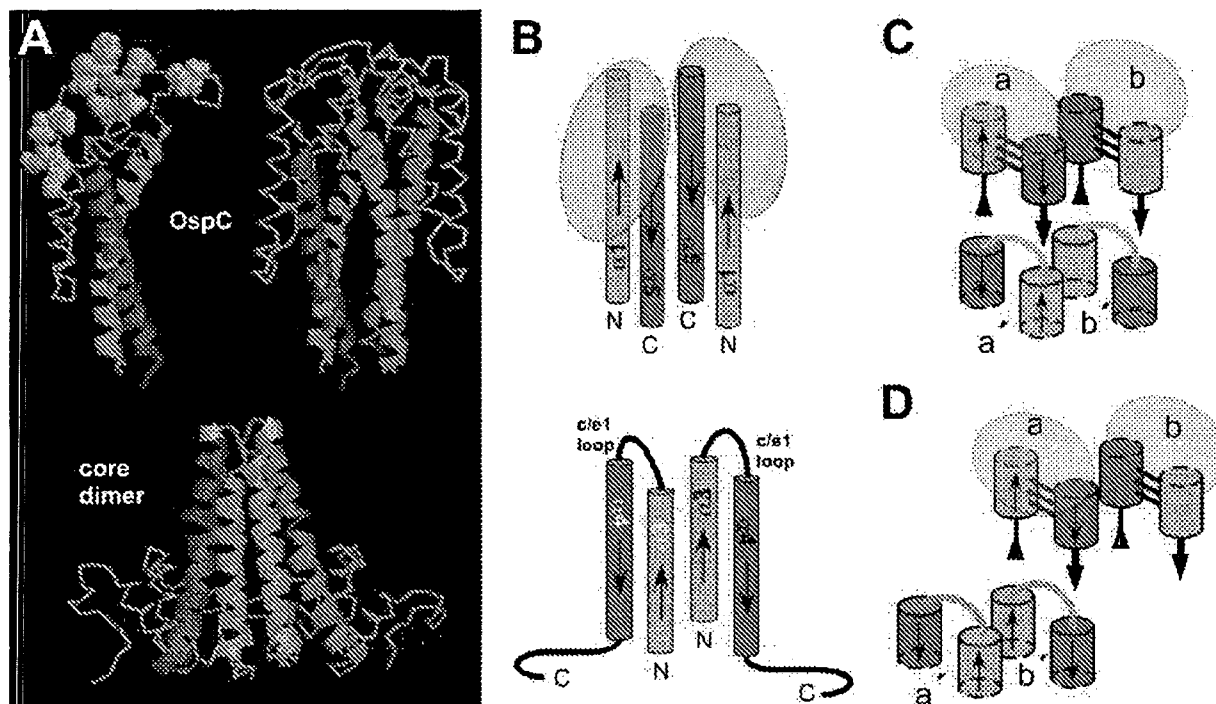


FIGURE 1. Structural aspects. A, three-dimensional structures of OspC and core protein. Both proteins dimerize through formation of four-helix bundles; the involved helices ($\alpha 1$ and $\alpha 5$ in OspC and $\alpha 3$ and $\alpha 4$ in the core protein) are shown as *ribbons*, the remaining sequences as a *backbone trace*. Upward pointing helices are drawn in *green*, downward pointing helices in *red*. Residues differing between OspC_a and OspC_b are highlighted in the OspC monomer (top left) by *space-filled* representations. B–D, schematic representation of the topology of the termini of the OspC dimer and the acceptor sites in the core protein dimer. Helix $\alpha 3$ of the core protein must be fused to $\alpha 1$ of OspC and $\alpha 5$ of OspC to $\alpha 4$ of the core protein. B, the helices of the four-helix bundles are represented by *straight cylinders* with their directions indicated by *arrows*. C and D, close-ups of the connection sites. If arranged as in *panel C*, the termini of OspC dimer subunits *a* and *b* do not match to those of core dimer subunits *a'* and *b'*. D, the ends of OspC subunit *a* fit to those of core subunit *b'*, but OspC subunit *b* would be dislodged. Possible outcomes include that (i) OspC dimerization prevents core dimerization and particle assembly, (ii) core dimerizes and assembles but prevents OspC dimerization, (iii) both proteins dimerize. This would require massive distortion of the connecting sequences such that in *panel D* OspC subunit *b* would fit to core subunit *a*. Alternatively, OspC subunit *b* could be contributed by a neighboring fusion protein dimer in the particle.

signal peptide, cleavage of which exposes an N-terminal Cys residue for subsequent lipidation. The solved structures of three OspC variants (27, 28) revealed, for the aa sequence encompassing ~positions 40–200, a common mushroom-shaped dimeric structure with each monomer contributing half the stalk and half the head (Fig. 1A) and with all four termini protruding from the foot of the stalk at the start and the end, respectively, of long helices ($\alpha 1$ and $\alpha 5$). Though reminiscent of the four acceptor sites in the core protein dimer, the topology of the termini in the two dimers is different (Fig. 1, B and C). Hence, within one fusion protein dimer either the core parts or the OspC parts, but not both, could interact as in the unfused proteins. Successful CLP formation would therefore require substantial flexibility in either the carrier or the insert or not be possible at all.

Second, OspC is of medical interest as a major surface antigen of Lyme disease-causing *Borreliae*. Lyme borreliosis, the most common vector-borne disease in Europe and the United States (for reviews see Refs. 29–31), is a progressive inflammatory disorder with dermal, cardiac, musculoskeletal, and neurological manifestations. It is transmitted to humans by *Ixodes* ticks from animals, mostly rodents, infected with various strains of three species of *Borrelia*, in the U.S. mainly by *B. burgdorferi sensu stricto* and in Europe by *B. afzelii* and *B. garinii* as well. Of the various outer surface proteins, OspA probably mediates colonization of the tick gut (32), whereas OspC is crucial for infection of the mammalian host (33). Hence, despite the substantial inter-strain and intra-strain variability of OspC (34–36), which has questioned its usefulness as a vaccine, the different OspC variants might have common interaction partners, e.g. the immunosuppressive Salp15 protein from tick saliva (37, 38), and consequently shared epitopes. Importantly, mouse models of Lyme disease are established that allow us to assess the

capacity of a given vaccine formulation to induce neutralizing antibodies, which play the major role in clearing infection (39). For instance, a recombinant glutathione *S*-transferase, OspC fusion protein (recGST-OspC), but not the same OspC protein after removal of the GST part (rec-OspC), induced antibodies with prophylactic, and upon passive transfer but not active immunization, also therapeutic potential (40, 41).

Here we generated fusions of the HBV core protein with two variants of OspC, termed OspC_a and OspC_b, that according to molecular modeling form the typical mushroom-like dimer structures but differ at 15 surface-exposed aa positions (Figs. 1A and 2B). We addressed their ability to form CLPs, looked into the structural consequences of the nonmatching dimeric insert structure by electron microscopy and image reconstruction, and monitored their immunogenicity and protective potential in the mouse model.

EXPERIMENTAL PROCEDURES

Plasmids—The parental expression vector pET28a2-HBc149_H6 encodes the HBV core protein from aa 1 to 149 with a C-terminal His₆ tag; in the context of similar fusions with color variants of GFP the tag increased the assembly efficiency (8). The genes for OspC_a and OspC_b were isolated from a single *B. burgdorferi* ZS7-infected tick and cloned into the pGEX vector.³ The GST fusion protein encoded by pGEX-OspC_b, recGST-OspC_b, served as control antigen in the immunization experiments; recombinant OspC_b without the GST part was obtained by thrombin cleavage (41). Using PCR primers OspC(+) GGAGCTC-GAG.GAT.gga.ggg.ggt.ggt.acc.GGA.AAA.GAT.GGG.AAT.GCA and

³ R. Wallich, T. Stehle, C. Brenner, A. Siebers, L. Gern, and M. Simon, unpublished data.

HBV Capsids Displaying Complete OspC

OspC(-), complementary to GTG.GCA.GAA.AGT.CCA.AAA.AAA.-CCT.ggt.ggc.TCT.AGA.GTGTG (codons separated by dots, XhoI and XbaI cloning sites in underlined italics; linker codons Gly₄-Thr, and Gly₂ in lowercase), the genes for OspC_a and OspC_b, starting with codon Gly-23 and ending with the authentic C-terminal codon for Pro-211 (Gly-5 to Pro-193 in the mature protein) were amplified from the pGEX vectors and cloned into the parental HBcAg vector such that Pro-79 and Ala-80 were replaced (Fig. 2A). The new vectors were termed pET28a2-c149_OspC_a-H6 and pET28a2-c149_OspC_b-H6.

Fusion Protein Expression and Purification—The pET vectors were transformed into *Escherichia coli* BL21<DE> Codonplus RIL cells (Stratagene). Fusion protein expression was induced by isopropyl-1-thio-β-D-galactopyranoside addition, and after shaking for 10 h at room temperature the cultures were harvested and cleared lysates were prepared as previously described (8). To enrich for particles, the lysates were sedimented through 10–60% sucrose step gradients (8). Aliquots from the gradient fractions were analyzed by SDS-PAGE using the Laemmli buffer system. Proteins were stained by Coomassie Blue, or the fusion proteins were detected by Western blotting using a monoclonal antibody (mAb) against HBcAg (mAb158, recognizing a linear epitope around aa 130 of the core protein) (42) or a mAb against OspC (LA97.1) (43). According to densitometric scanning of the Coomassie Blue-stained gel lanes, the fusion proteins in the center gradient fractions amounted to more than 90% of the total protein present, which was sufficiently pure for electron microscopy. For immunizations, the preparations were dialyzed (Slide-a-lyzer cassettes, 30-kDa molecular mass cutoff; Pierce), concentrated (Amicon Ultra devices, 30-kDa cutoff; Millipore), and subjected to a second round of sucrose gradient sedimentation. The center gradient fractions were pooled, dialyzed against phosphate-buffered saline (PBS), and concentrated to ~0.75 mg/ml; purity as estimated by densitometric scanning was ~94%.

Electron Cryomicroscopy and Image Reconstruction—Samples were frozen as described (44). Micrographs were taken under low dose conditions at a nominal magnification of 52,000 at a Philips CM 120 Biotwin operating at 100 kV and equipped with a LaB6 filament. For cryomicroscopy a Gatan cryoholder maintaining a temperature of 94 K was used. Micrographs were recorded on Kodak SO 163 film and scanned with a Zeiss Scai scanner with a step size of 21 μm/pixel corresponding to 4 Å at specimen level. From 5 micrographs a total of 407 small particle images were selected. Image processing was done with the IMAGIC 5 software package (45, 46). For two-dimensional reconstructions, single particle images were normalized in their gray value distribution, inverted in contrast, and centered by translational alignment to a rotationally averaged sum of all particles. Each particle was rotated by increments of 45° (0–315°), and the rotated particles were added to the data set enabling comparison. For accessing the local organization of OspC a segment of the outer rim of the particles (140 by 150 Å, including the edge of the HBc core shell and the peripheral density of the OspC layer) was analyzed where OspC is seen in side view projection and, due to the curvature, overlaps only a little with other features. The variation in density distribution in this segment was analyzed by multivariate statistical analysis, and particles were grouped according to their similarity. Particles in the same group were averaged, showing side view projections of OspC bound to the spikes. A three-dimensional icosahedral reconstruction approach is described in the supplemental material.

Immunization of Mice—For generation of immune sera (IS), four adult female BALB/c mice (H-2d; bred under specific pathogen-free conditions at the Max-Planck-Institut für Immunbiologie, Freiburg, Germany) per construct were repeatedly immunized into the base of the tail with initially 30 μg and subsequently 10 μg of the respective recom-

binant proteins (coreOspC_a, coreOsp_b, and recGST-OspC_b) in the presence of 100 μl of MPL-TDM adjuvant (composed of monophosphoryl Lipid A and synthetic trehalose dicorynomycolate; Sigma) and bled on the indicated days post-immunization. As control, IS was generated against wild-type core protein 1–149 following the same protocol. Prior to further analyses, the IS from each group of animals immunized with the same construct were pooled. OspC- and core protein-specific Ab in IS were quantified by a solid-phase ELISA as described (43) using 1 μg/ml of either recombinant OspC_b or wild-type core protein 1–149 CLPs as substrate and mAb 97.1 or the core-specific mAb 312 (42) as reference antibodies. Western blot analysis using recGST-OspC_b as antigen (250 ng/lane) was done as described (47). Pooled preimmune sera from the same animals served as negative controls.

Disease Model and Protection Experiments—Pools of IS to be used in protection experiments were collected from immunized BALB/c mice (groups of 3–4 mice) every 7–10 days for up to 93 days post-immunization starting after the third challenge. For passive immunization, three C.B-17 SCID mice (bred under specific pathogen-free conditions) were injected intraperitoneally with IS containing the equivalent of 10 μg of antibody specific for the individual recombinant protein or with the OspA-specific mAb LA-2 (5 μg/mouse) (43) and challenged experimentally (subcutaneously) with 10³ spirochetes (strain ZS7)/mouse 2 h later. Control mice received either anti-wild-type core IS (30 μg of specific antibody), normal mouse serum, or PBS (300 μl each). Development of clinical arthritis in the tibiotarsal joints was monitored in the right and left tibiotarsal joint and scored under double-blind conditions (40). Testing for the outgrowth of viable spirochetes from cultured ear biopsies was done on day 42 post inoculation as described (41).

RESULTS

Generation of and Efficient CLP Formation by CoreOspC_a and CoreOspC_b Fusion Proteins—As carrier we used a HBV core protein truncated after aa 149 with a C-terminal His₆ tag that efficiently forms CLPs in *E. coli* (48). The inserts (Fig. 2) encompassed the nearly full-length mature proteins from aa residues 23 to 211 of OspC_a and OspC_b. In the OspC crystal structures, residues preceding aa 40 and following aa 204 were not resolved (27, 28), suggesting these sequences are flexible; therefore, only short flanking linkers were added (G₄T and G₂).

Both the OspC_a and the OspC_b fusions were well expressed and largely soluble. Upon sedimentation in sucrose gradients of the cleared bacterial lysates, both proteins formed a sharp peak in the center of the gradient (shown in Fig. 3A for coreOspC_a), as is typical for ordered HBcAg CLPs (8). A band of identical mobility was detected by immunoblotting using an anti-HBcAg mAb (Fig. 3A) as well as an anti-OspC mAb (not shown). Direct confirmation of efficient CLP formation was obtained by negative staining electron microscopy (EM) that revealed abundant particles exhibiting, compared with wild-type CLPs, a rough surface that on some particles appeared to consist of distinct spikes (Fig. 3B).

Structural Analysis of CoreOspC_a CLPs—For a more detailed analysis, the coreOspC_a sample was subjected to electron cryomicroscopy (cryo-EM) and image processing. Cryoelectron micrographs confirmed the presence of abundant spherical, though sometimes distorted, shells of different size classes (Fig. 4A). Compared with CLPs from unmodified His-tagged core protein 1–149, the fraction of small particles was substantially increased, as was also found for other, including smaller, c/e1 insertions (49). All showed distinct, large, outward protruding spikes, indicating that the OspC domains are indeed surface exposed.

For an approximate comparison of the size of the outer spikes with the expected dimensions of OspC, a two-dimensional image-processing approach was used. Particle images were centered, and then segments of

FIGURE 2. Scheme of the constructs used. A, arrangement of the core protein and OSpC parts in the coreOspC fusion proteins. The carrier part, HBc1-149_H6, encompasses the assembly domain of the core protein, with the C-terminal basic domain (clusters of Arg residues symbolized by + signs) replaced by a His₆ tag. Amino acids Pro-79 and Ala-80 were replaced by OSpC_a or OSpC_b, starting with aa Gly-23 and ending with Pro-211 (Gly-5 and Pro-193 in the mature protein), flanked by G₄T and G₂ linkers. B, sequence comparison between OSpC_a and OSpC_b. The 18-aa signal peptide is omitted. Numbering starts with the lipidated cysteine Cys-19. The complete sequence is that of OSpC_a; residues exchanged in OSpC_b, are indicated in the line below. The known three-dimensional structures (27, 28) encompass residues Pro-40 to Pro-202. Helices $\alpha 1$ to $\alpha 5$ are indicated by bars; the bracketed region denoted loop 5 contains recently identified type-specific linear epitopes (36).

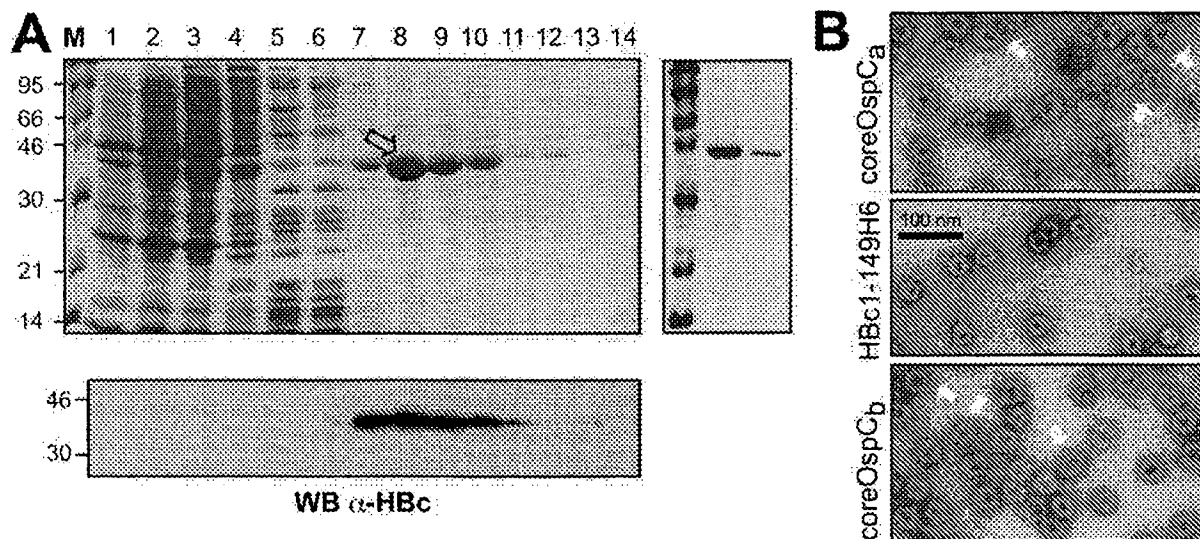
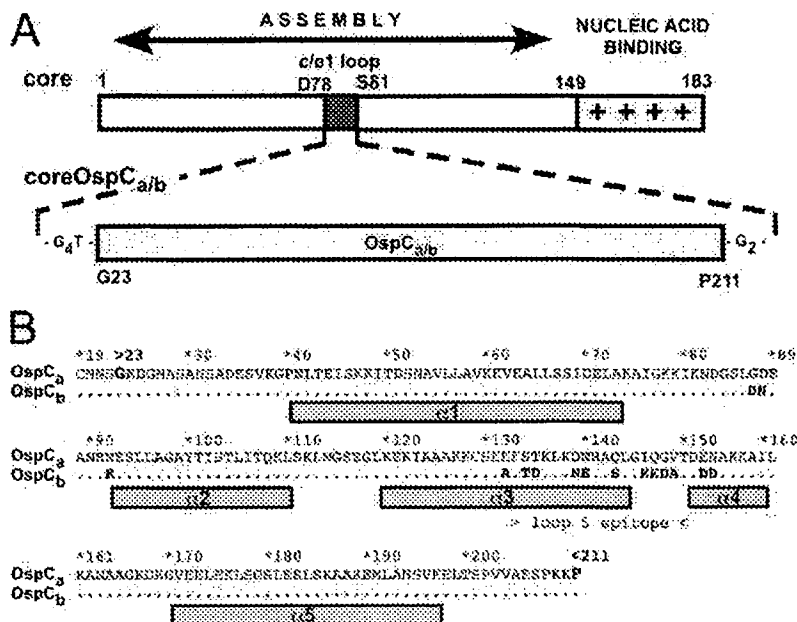


FIGURE 3. Expression of and CLP formation by the coreOspC fusion proteins. A, sucrose gradient sedimentation. Aliquots from fractions (top, fraction 1; bottom, fraction 14) of a sucrose gradient loaded with cleared bacterial lysate containing the coreOspC_a fusion protein were analyzed by SDS-PAGE. The fusion protein (arrow) accumulated in fractions 7–10; a separate gel was used for immunoblotting with a mAb against the core protein (bottom panel). For immunization, the pooled fractions 7 to 10 were concentrated and resubjected to gradient sedimentation. 10 and 3 μ l of this material were analyzed by SDS-PAGE (right panel). B, electron microscopy. Negative staining EM of coreOspC_a (top) and coreOspC_b (bottom) as obtained from the first sucrose gradient revealed abundant particles with a much more elaborate surface structure than seen with CLPs from the unmodified carrier protein HBc1-149H6 (middle). Arrows point to CLPs with well visible surface protrusions.

the outer rim showing the outer densities in side view were compared. Particles with similar peripheral density distributions were grouped, and those within one group were averaged. The corresponding projections from several such groups are shown in Fig. 4B. All displayed regularly arranged densities at the positions expected for the core dimers constituting the inner shell, plus elongated and slightly kinked outward reaching protrusions. The largest of these protrusions were on average ~ 6.5 nm long and ~ 4 nm wide, in good agreement with the dimensions of an OSpC dimer (~ 6.2 by 4.2 nm when viewed from the broadside); others had a similar length but appeared narrower. This would be compatible with edgewise-viewed OSpC dimers or with monomers (Fig. 4C).

Obtaining three-dimensional icosahedral reconstructions was hampered by the variability in the arrangement of the OSpC layer between individual particles and by difficulty in accurately assigning particle ori-

entations. Nonetheless, a plausible model could be derived by combining a reconstruction based on a known reference map of wild-type T 3 particles that emphasizes the inner core shell with a single particle-based reconstruction emphasizing the outer OSpC shell (see supplemental Fig. S1 and legend). Although little detail of the core dimer spikes was visible, indicating that the model is of limited accuracy, it strongly suggests that the packing of the core dimers and the location of the spikes in the inner shell are comparable with wild-type CLPs. Furthermore, because the OSpC densities were not centered above the core dimer spikes (supplemental Fig. S2), OSpC dimerization is unlikely to occur between the two OSpC moieties present on one core dimer. The data are, however, compatible with dimerization between OSpCs present on neighboring core dimers. Irrespective of how the OSpC moieties are arranged in detail, these data provide strong evidence for the ability of the HBcAg carrier to accommo-

HBV Capsids Displaying Complete OspC

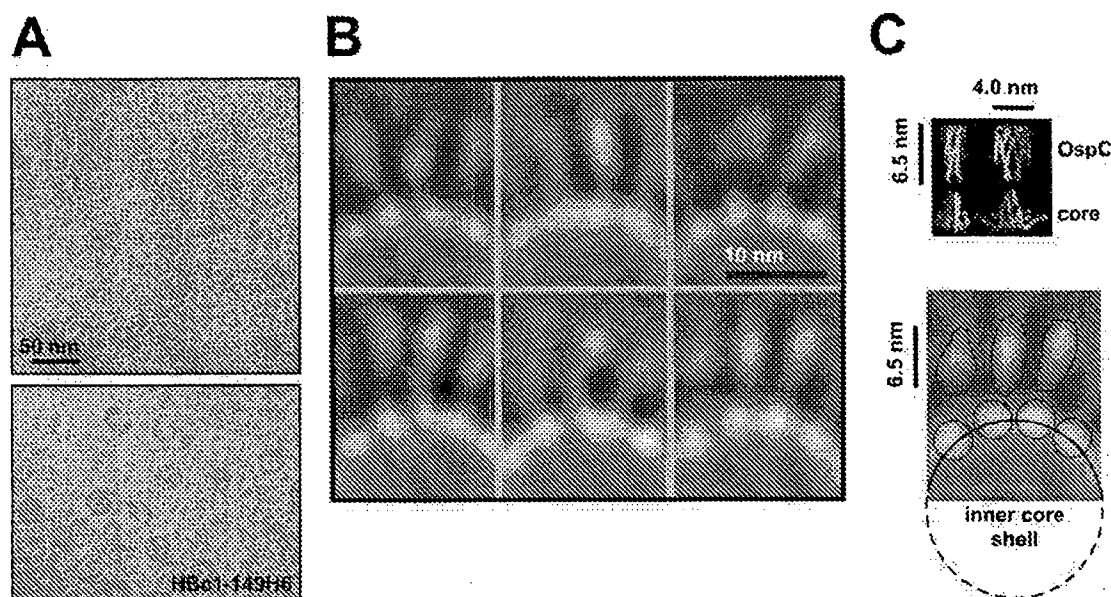


FIGURE 4. Cryo-EM analysis of coreOspC_a CLPs. A, cryoelectron micrographs. A sample of the gradient-enriched coreOspC_a CLPs was visualized under near-native conditions in vitrified ice. Note the corona of extended spikes around each particle that is absent from the wild-type HBc1-149H6 CLPs shown below. B, two-dimensional image reconstructions. Images from individual CLPs were centered and grouped according to congruent peripheral density features. Averaged projections from representative groups are shown. Light shading corresponds to high electron density. C, correlation of density features with atomic models of the OspC and core dimer. In the projection from the lower right section in panel B, distinct density features in the inner and outer shell are outlined. The models in the upper panel are depicted at approximately the same scale. The dimers are shown once viewed versus their narrow and once versus their broad side together with their approximate dimensions.

date the insertion of a dimerization-competent whole protein antigen and to present it on an intact CLP surface.

Immunological Characterization of CoreOspC_a and CoreOspC_b CLPs—Next, coreOspC_a and coreOspC_b were compared with recGST-OspC_b in their potential to induce specific antibodies (Ab) in BALB/c mice. When tested at day 7 after the first immunization, IS from recipients immunized with coreOspC_a and coreOspC_b, but not with recGST-OspC_b, contained specific Ab as revealed by Western blot analysis using recGST-OspC_b as target (Fig. 5A). At day 15 following the second challenge with antigen, all three IS contained specific Ab (Fig. 5A). For semiquantitation of OspC- and core-specific Ab, IS were taken at different time points following repeated immunization and analyzed by ELISA using either rec-OspC_b (lacking the GST part) or wild-type HBcAg as target protein (Fig. 5B). ELISA readings were related to those obtained with known amounts of mAbs against OspC and core. Assuming the same average affinity for the polyclonal antibody pools in the IS and the mAbs, IS to coreOspC_b contained high titers (up to 450 µg/ml) of OspC-specific antibodies, which peaked after three immunizations and then declined but again increased after a further antigen challenge. Similar kinetics of OspC Ab were seen with IS to coreOspC_a and recGST-OspC_b, though at ~5-fold lower levels. Yet the Ab levels in IS to coreOspC_a were still as high as those in IS to recGST-OspC_b, although the test antigen was OspC_b. As expected, Ab to HBcAg were seen only in IS of mice immunized with the core fusions, however at titers much lower compared with OspC Ab (Fig. 5B).

For a more detailed analysis of the respective IS, 2-fold serial dilutions obtained after the fourth immunization were tested by ELISA using either coreOspC_a, coreOspC_b, or rec-OspC_b as targets (Fig. 5C). All three antigen preparations were recognized by the OspC-specific mAb LA97.1 (43), confirming the expression of authentic epitopes in the two coreOspC fusion proteins. When comparing IS to coreOspC_a and coreOspC_b, higher Ab titers (~4–15-fold) were always found when tested on the homologous OspC antigen, yet the response to the other OspC type was still substantial, indicating the existence of both distinct and common epitopes. In addition, titers of OspC Ab in IS to coreOspC_b

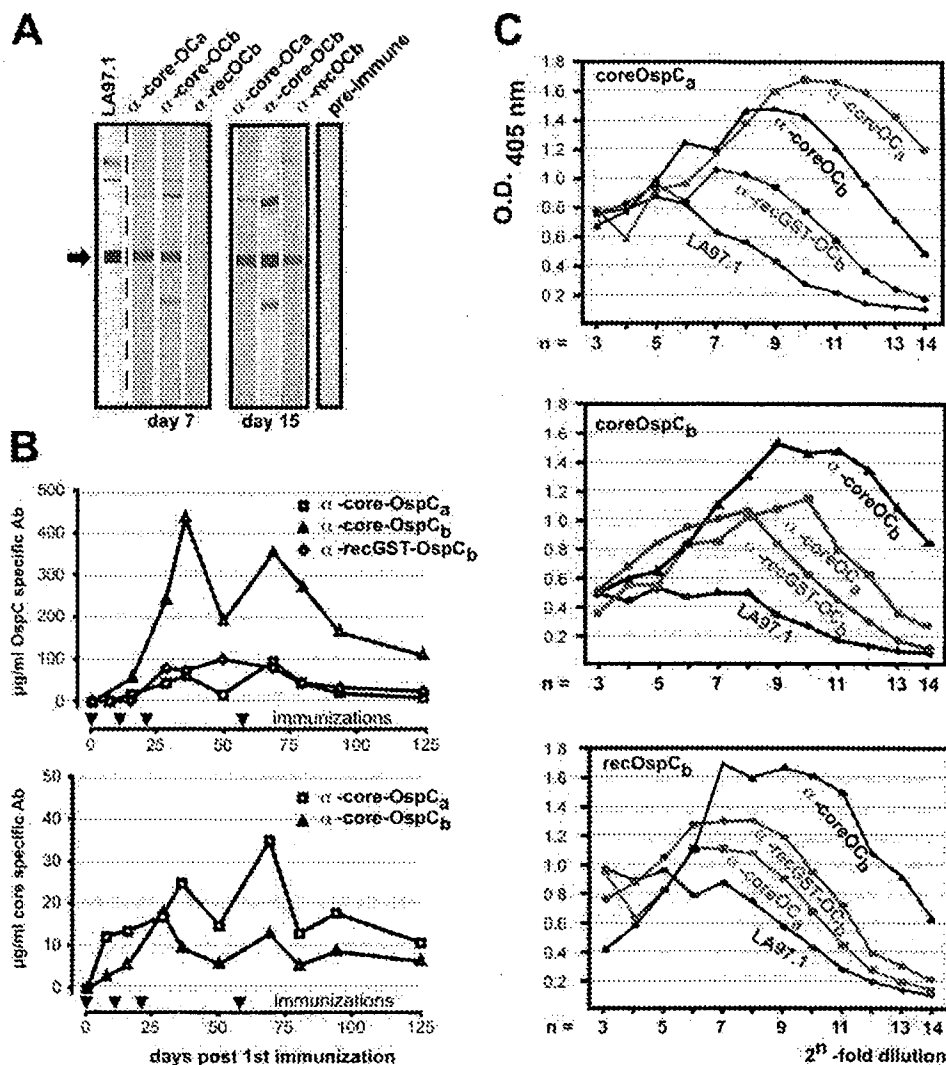
exceeded those in IS to recGST-OspC_b by up to 8-fold when tested on either of the three antigen preparations, demonstrating a prominent adjuvant activity of the CLP carrier.

Passive Immunization with CoreOspC_a and CoreOspC_b Immune Sera Protects against Challenge with *B. burgdorferi*—In light of the previous findings that recGST-OspC_b can confer active and passive protection against the homologous *B. burgdorferi* strain in both immunocompetent and immunodeficient mice (40, 41) IS to coreOspC_a, coreOspC_b, and recGST-OspC_b were analyzed for neutralizing Ab *in vivo*. Accordingly, IS were passively transferred to C.B-17 SCID mice, known to develop chronic *B. burgdorferi* infection and disease, including arthritis and carditis (50), and the course of infection and disease (clinical arthritis) was monitored following challenge with the pathogen. The established neutralizing anti-OspA mAb LA-2 served as positive control (51), while IS to the core protein only, normal mouse serum, and PBS were used as negative controls. As shown in Table 1, transfer of IS to either coreOspC_a, coreOspC_b, or recGST-OspC_b into C.B-17 SCID mice (3 mice/group) 2 h prior to challenge led to complete protection against infection, as monitored by reculturing of spirochetes from ear biopsies, and disease (development of clinical arthritis) except in one coreOspC_b IS-treated animal. In comparison, 3/3 mice pretreated with LA-2 but none of the mice pretreated with either IS to the core protein only, normal mouse serum, or PBS was protected against infection and disease.

DISCUSSION

The immune-enhancing property of HBV CLPs presenting short heterologous peptides or other small molecules is well established (4, 5, 52). Surface display of whole protein antigens, desirable for a vaccine addressing multiple epitopes and therefore less vulnerable to genetic variability, is structurally much more demanding, and as yet only few protein-chemical and immunological data are available (6–9, 48). The present study demonstrates that two distinct variants of a complex dimeric lipoprotein of *B. burgdorferi*, OspC_a and OspC_b, can successfully be presented on such CLPs; that the OspC polypeptide chains are exposed on the particle surface, possibly in a patchwork of OspC dimers

FIGURE 5. Immunological characterization of immune sera induced by coreOspC_a and coreOspC_b CLPs. A, immunoblot. The ability of IS induced by the coreOspC preparations, or the recGST-OspC_b fusion, to recognize recGST-OspC_b was tested on day 7 after the first and on day 4 after the second immunization; the OspC-specific mAb LA 97.1 served as positive control. No signal was observed with preimmune sera (strip labeled pre-immune). B, kinetics of OspC- and core-specific antibody induction. ELISA plates were coated with either rec-OspC_b (top) or wild-type core protein (bottom) and incubated with the indicated IS. ELISA readings were normalized using known amounts of OspC- (mAb LA97.1) or core-specific (mAb 312) antibody. Assuming comparable affinities, values from the IS samples are given as $\mu\text{g/ml}$ of OspC- or core-specific antibody. Note the different scales for the OspC- versus the core-specific response. C, specificity and cross-reactivity of IS against the homologous and heterologous OspC antigens. ELISA plates were coated with the indicated antigen, and binding of the different IS and mAb LA97.1 was monitored using 2-fold dilution series; the first dilution was 10-fold for the IS and 70-fold for LA97.1 (100 ng/well). The y-axis shows the absorbance at 405 nm. In panels A and C, OspC_a and OspC_b are abbreviated as OC_a and OC_b.



across different carrier dimers; that these CLPs are highly immunogenic in mice, inducing IS that are specific for their cognate OspC but cross-reactive with the other; and that both IS are able to passively protect mice from experimental challenge with *B. burgdorferi*.

Structural Implications—Both the HBV core protein dimer (22) and the OspC dimer (27, 28, 53) are stabilized through large, mostly hydrophobic interfaces, keeping the constituent monomers in a defined arrangement. Because of the non-matching topology of the termini to be joined in the fusion protein (Fig. 1, B and C), it was possible that the dimerization tendency of one of the partners overrides that of the other, such that no regular particles would form. However, both coreOspC fusions efficiently formed particles, as demonstrated by the absence of non-assembled fusion protein in the gradients and by the abundant presence of particles upon EM inspection (Fig. 3). Furthermore, the solubility of the fusion proteins argues for proper OspC folding and dimerization because solvent exposure of the hydrophobic dimer interface would probably lead to aggregation. It is likely therefore that both partners, despite the non-matching topology, were able to dimerize.

Surface exposure of the OspC chains was clearly corroborated by cryoelectron microscopy (Fig. 4A) showing the CLPs as spheres of wild-type-like appearance but surrounded by a corona of prominent outward-pointing spikes. Two-dimensional image reconstructions (Fig. 4B) showed that the protrusions had approximately the length of 6.5 nm

expected for OspC; the width varied between ~3 and 4 nm, with the latter corresponding to that of an OspC dimer viewed toward its broad-side. The smaller values are compatible with both an edgewise-viewed dimer or a monomer. The original micrographs and the two-dimensional reconstructions indicated that the OspC domains are not rigidly fixed at topologically equivalent positions, similar to what was seen with CLPs displaying GFP flanked by long connecting linkers (6) or a 120-aa segment of a hantavirus nucleocapsid protein (7). The terminal OspC sequences flanking the structured part would provide for sufficient flexibility such that each OspC domain can occupy alternative positions at the CLP surface. The partial disorder in the OspC layer and possible deviations from strict icosahedral symmetry plus the difficult assignment of the exact particle orientations complicated a three-dimensional icosahedral image reconstruction. By combining an inner shell-biased reconstruction with another one based on a single particle approach emphasizing the outer OspC densities, we arrived at a model that allowed us to locate the approximate positions of the core dimer spikes of the inner shell (supplemental Fig. S1). Notably, except for weak densities above the 2-fold axes, no evidence for an accumulation of OspC densities directly above the core dimer spikes was seen as would be expected if the corresponding two OspC chains had dimerized. Instead, ~300 rather than 180 OspC-related densities (60 dimer-like densities each around the 5-fold and 3-fold axes, plus two OspC monomer-like

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TABLE 1

Prophylactic effect of different immune sera specific for OspC on *B. burgdorferi* Z57 infection in C.B-17 SCID mice

IS specificity ^a	Mouse	Clinical arthritis ^b							Recultivation ^c
		8	15	22	28	46	69	81	
	Number	days p.i.							
mAb LA2	1	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—
recGST-OspC _b	1	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—
CoreOspC _a	1	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—
CoreOspC _b	1	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—
	3	—	(±)/±	+/+	++/++	++/++	++/++	++/++	+
Wild-type core	1	—	(±)/(±)	+/±	++/++	++/++	++/++	++/++	ND ^d
	2	—	±/±	++/++	++/++	++/++	++/++	++/++	ND
	3	—	(±)/(±)	+/+	++/++	++/++	++/++	++/++	ND
Normal serum	1	—	(±)/(±)	+/+	++/++	++/++	++/++	++/++	+
	2	—	(±)/(±)	++/++	++/++	++/++	++/++	++/++	+
	3	—	±/(±)	++/++	++/++	++/++	++/++	++/++	+
PBS	1	—	±/-	±/±	+/+	++/++	++/++	++/++	ND
	2	—	±/(±)	+/+	++/++	++/++	++/++	++/++	ND
	3	—	(±)/(±)	++/++	++/++	++/++	++/++	++/++	ND

^a Mice were given either 5 µg of mAb LA2 or the equivalent of 10 µg of recOspC_b, coreOspC_a, coreOspC_b, or 25 µg of wild-type core-specific antibodies or 300 µl of BALB/c normal mouse serum or 300 µl of PBS, intraperitoneally, 2 h before *B. burgdorferi* Z57 infection (10³ spirochetes/mouse).

^b Scoring of arthritis symptoms for left and right tibiotarsal joint: —, none; (±), reddening without swelling; ±, mild; +, moderately severe; ++, severe swelling.

^c Recultivation of *B. burgdorferi* Z57 from ear biopsy was done on day 42 post inoculation.

^d ND, not done.

densities per each of the 30 2-fold axes) were observed, with approximately half the density as that in the inner shell. Hence these densities likely reflect averaged probability volumes for OspC; in an individual real particle only about half of the densities would be occupied (supplemental Fig. S2).

A plausible model compatible with all the data, including the solubility of the CLPs that likely would be compromised by a significant portion of monomeric OspC moieties, is that OspC dimerization occurs between OspC chains from different, neighboring core dimers. The N atom of Pro-79 of the core protein is roughly at the center of the OspC insertion site. The distance to the Pro-79 N atom within the same core dimer is 10.9 Å, and that to the closer of the two Pro-79 N atoms in a neighboring core dimer is 43.7 Å. Assuming that in an extended conformation each aa residue contributes close to 3 Å in length, spanning that distance requires ~15 residues total. The N-terminal connector sequence between core and OspC consists of 5 linker aa plus 19 OspC residues not ordered in the x-ray structure; at the C-terminal junction, there are 16 non-ordered OspC residues plus 2 extra glycines. Hence two OspC chains could well meet halfway between neighboring core dimers. The presence of two closest neighbors for each of the 90 core protein dimers per T 3 particle allows for an enormous number of possible inter-core-dimer OspC dimers, such that each single CLP could have a unique OspC-dimer pattern. Regardless of how exactly the OspC chains are arranged, the important conclusion for the use of HBcAg as carrier for foreign protein antigens is that even a dimerization-competent protein with non-matching topology of its termini can successfully be presented on HBcAg CLPs.

Enhanced OspC Type-specific yet Cross-reactive Immunogenicity of CoreOspC CLPs—Previous studies demonstrated that CLPs displaying truncated or full-length protein antigens, including a Hantavirus N protein segment (7) and *B. burgdorferi* OspA (9), are highly immunogenic and readily induce Ab to the foreign polypeptide chains. In line with these results, both coreOspC_a and coreOspC_b CLPs induced strong OspC-specific antibody responses in mice and in rabbits (not shown). The Ab titers obtained with the two CLP preparations significantly

exceeded those obtained with recGST-OspC_b (Fig. 5 and Refs. 40, 41), further substantiating the adjuvant potential of the HBV capsid. Furthermore, the Ab levels against the HBcAg carrier were at least one log lower compared with the OspC-specific responses, a desired feature for the use of CLPs as vaccine candidates (54, 55) and probably a consequence of breaking up the immunodominant authentic c/e1 epitope by the insertions.

The anti-coreOspC responses were specific for OspC_a and OspC_b in that the highest antibody titers were always evoked against the homologous OspC. Hence, whereas nearly all previous work focused on epitopes in the conserved stalk region (56, 57), this demonstrates the presence of immunogenic epitopes in the more variable head region of OspC. These data are fully in line with a very recent study that defined linear type-specific epitopes in a surface-exposed loop preceding helix α5 ("loop 5") (36), exactly where OspC_a and OspC_b differ most in sequence (Fig. 2B). According to the typing system used there, the corresponding region in OspC_a belongs to type B and that in OspC_b to type Q. However, the anti-coreOspC IS also displayed substantial cross-reactivity, indicating that the CLP preparations were able to induce a multispecific response directed against different, namely conserved and variant, parts of the two displayed antigens, illustrating the distinct advantage of CLPs displaying a whole foreign protein rather than a small peptide segment.

Most remarkably, both coreOspC-CLP preparations induced protective Ab that, upon passive transfer, prevented experimental infection and disease in *B. burgdorferi*-challenged C.B-17 mice. Given that a denatured OspC, though not of identical sequence and in a different mouse strain, induced OspC-specific yet non-protective antibodies (58, 59), the coreOspC CLPs are highly likely to express authentic native OspC epitopes, a prerequisite for the induction of protective immunity, on their surface.

The exact nature of the protective OspC epitopes is not clear. The stalk has been suggested to contain a dominant neutralizing epitope (60). It is presently unclear how this region, containing the lipid anchor attaching OspC to the spirochete membrane, induces and is targeted by

antibodies; similarly, this particular region is buried in the CLPs between the outer OspC and inner HBcAg shell. One interpretation is that, despite these steric constraints, antibodies against the conserved stalk part are formed that are neutralizing, similar to the mAb B5 directed against a conformational epitope in this region (57). However, it is equally likely that other, more accessible regions on the coreOspC CLPs contribute to the neutralizing activity.

At any rate, HBV CLPs displaying two OspC variants, which with respect to their loop 5 epitopes (36) belong to distant OspC types, both induced protective immunity. This may give rise to cautious optimism that a coreOspC-based vaccine encompassing a confined number of OspC variants could protect from infection by more than one strain of *B. burgdorferi*.

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Peptide 'Velcro*': design of a heterodimeric coiled coil

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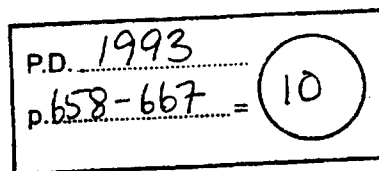
Background: The leucine zipper is a protein structural motif involved in the dimerization of a number of transcription factors. We have previously shown that peptides corresponding to the leucine zipper region of the Fos and Jun oncoproteins preferentially form heterodimeric coiled coils, and that simple principles involving electrostatic interactions are likely to determine the pairing specificity of coiled coils. A critical test of these principles is to use them as guidelines to design peptides with desired properties.

Results: Based on studies of the Fos, Jun and GCN4 leucine zippers, we have designed two peptides that are predominantly unfolded in isolation but which, when mixed, associate preferentially to form a stable, parallel, coiled-coil heterodimer. To favor heterodimer formation, we chose peptide sequences that would be predicted to give destabilizing electrostatic interactions in the homodimers that would be relieved in

the heterodimer. The peptides have at least a 10⁵-fold preference for heterodimer formation, and the dissociation constant of the heterodimer in phosphate-buffered saline is approximately 30 nM at pH 7 and 20°C. Studies of the pH and ionic strength dependence of stability confirm that heterodimer formation is favored largely as a result of electrostatic destabilization of the homodimers.

Conclusions: Our successful design strategy supports previous conclusions about the mechanism of interaction between the Fos and Jun oncoproteins. These results have implications for protein design, as they show that it is possible to design peptides with simple sequences that have a very high preference to pair with one another. Finally, these sequences with 'Velcro'-like properties may have practical applications, including use as an affinity reagent, in lieu of an epitope tag, or as a way of bringing together two molecules in a cell.

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Background

Structural and energetic studies of leucine zipper peptides [1-4] and analyses of coiled-coil sequences [5-8] suggest that simple principles may determine the specificity of pairing for coiled coils. A critical test of these principles is to use them as guidelines in the design of peptides with desired properties.

One strategy to promote preferential pairing between two peptides is to design their sequences so that the heterodimer has specific stabilizing interactions. For example, sequences could be designed to pack preferentially at the hydrophobic interface in a heterodimer. However, attempts to design heterodimeric coiled coils with leucine-alanine interactions at the dimer interface did not result in heterodimers that are more stable than both corresponding homodimers [9].

Sequences that result in favorable electrostatic interactions between the helices in a heterodimer could also be designed. Circular dichroism (CD) studies of the leucine zipper of the yeast transcription factor GCN4,

however, suggest that it may be difficult to stabilize a leucine zipper with interhelical ionic interactions. Studies of the GCN4 leucine zipper have shown its thermal stability does not depend strongly on pH or ionic strength (the melting temperature, T_m , in solution in PBS is 51°C, 57°C and 49°C at pH 2.7 and 11, respectively, and the T_m is 57°C $\pm$ 2°C from 15 mM to 1.5 M NaCl at pH 7). This suggests that ion pairs observed in the crystal structure of this peptide [3] may not be significantly stabilizing.

A less obvious strategy to promote heterodimer formation is illustrated by the Fos/Jun leucine zipper heterodimer, in which preferential pairing of the Fos and Jun peptides is driven primarily by the destabilization of the Fos homodimer [1,4]. Unfavorable interhelical electrostatic interactions substantially destabilize the Fos homodimer, thereby favoring heterodimer formation. We chose to design a heterodimeric coiled coil consisting of two peptides that would have many unfavorable interhelical electrostatic interactions in the homodimeric states.

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Results and discussion

Peptide design

The coiled-coil structure consists of two right-handed α -helices wrapped around one another with a slight left-handed superhelical twist [10–12]. This structural motif is found in many proteins, including tropomyosin, keratin, bacterial surface proteins, leucine-zipper proteins and tumor suppressor gene products [12–16]. The sequences of coiled-coil proteins consist of heptad repeats, where positions of the heptad are labeled with the letters **a–g** [5,17]. Analysis of these sequences has shown that coiled-coil proteins have a characteristic 4–3 hydrophobic repeat, with hydrophobic amino acids spaced every four and then every three residues [5,17]. The hydrophobic residues occur at positions **a** and **d** of the heptad repeat, whereas residues at positions **e** and **g** are predominantly charged (Fig. 1a).

These sequence preferences were rationalized by McLachlan and Stewart [5] with a three-dimensional model for the coiled coil. Experimental support for this model is provided by the X-ray structure of the GCN4 leucine-zipper homodimer [3], a two-stranded, parallel coiled coil. The structure of the coiled coil can be represented as a ladder, in which the helix backbones are the sides of the ladder and the rungs are comprised of residues at the dimer interface (Fig. 1a). There are two types of rung, each containing four residues, which alternate along the superhelical axis. One type of rung consists of residues from positions **a**, **a'**, **g**, **g'**, and the second type of rung has residues from positions **d**, **d'**, **e**, **e'**. Residues at positions **e** and **g** can also participate in interhelical ionic interactions (Fig. 1a).

The stability of homodimers containing residues of like charge at positions **e** and **g** is strongly pH-dependent [1,4], which can be explained by reference to the crystal structure of the GCN4 leucine zipper [3]. Side-chain methylene groups of residues at positions **e** and **g** pack against the predominantly hydrophobic residues at positions **a** and **d**, completing the dimer interface (Fig. 1b) and bringing together charged groups of side chains from opposing subunits (Fig. 1a). Thus, if positions **e** and **g** are occupied by residues of like charge, destabilization of the dimer is expected to result from electrostatic repulsion as well as loss of hydrophobic contacts.

The two peptides that we have designed differ only at positions **e** and **g** (Fig. 2). Glutamic acid was chosen for these positions in one peptide, designated ACID-p1, whereas lysine was chosen in a second peptide, designated BASE-p1. The ACID-p1 and BASE-p1 homodimers are expected to be destabilized at neutral pH by unfavorable electrostatic interactions. Leucine was chosen for positions **a** and **d** because it is the most common amino acid at these positions in coiled-coil proteins [6–8] and because Hodges [18,19] and DeGrado [20] have placed leucine at these positions

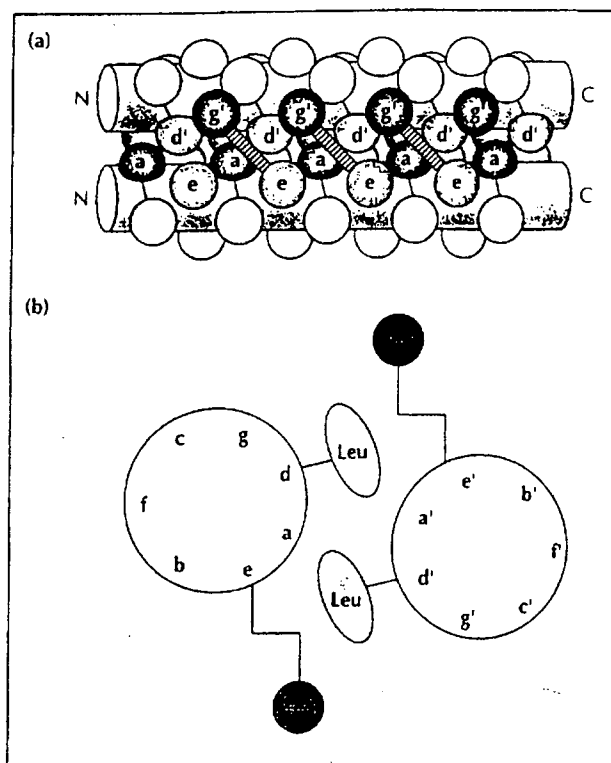


Fig. 1. Schematic representations of the coiled-coil structure illustrating the interactions between residues at the dimer interface. (a) The coiled-coil structure can be thought of as a ladder with sides formed by the helix backbones and alternating rungs composed of four residues. A side view of a coiled coil is shown: the positions of the heptad of one subunit are labeled with the letters **a–g**; residues **a'–g'** are on the other subunit. One set of rungs (red) consists of side chains from positions **a**, **a'**, **g**, **g'**, whereas the second set (blue) consists of side chains from positions **d**, **d'**, **e**, **e'** (only **a**, **g'**, **d** and **e'** are marked). Residues at positions **e** and **g** pack against positions **a** and **d**, as well as participating in interhelical electrostatic interactions, which are indicated with bridges. For simplicity, the supercoiling of the helices is not depicted. (b) Schematic cross section through the dimer, illustrating the interactions between four residues in a rung. The large circles represent the helical backbone and line segments represent bonds between carbon atoms. As an example, a rung consisting of leucine at position **d** and glutamate at position **e** is shown. Residues at positions **d** and **d'** make side-to-side interactions, as in a handshake. Additionally, methylene groups from residues at the charged positions **e** and **e'** make contacts with the hydrophobic residues at positions **d** and **d'**. These types of interaction are also seen in the other type of rung, in which methylene groups from residues at positions **g** and **g'** make contacts with residues at positions **a** and **a'**.

in simple designed homomeric coiled coils. A single asparagine residue was placed at position **a** in the second heptad to favor the parallel orientation of helices. Replacing a hydrophobic residue (at position **a**) with an asparagine in a leucine zipper that has little intrinsic preference for helix orientation results in a marked preference for the parallel orientation (R Rutkowski, EKO and PSK, unpublished observations).

A polar residue is often found at an **a** position in the sequences of leucine-zipper proteins, including GCN4 [21]. In the crystal structure of the GCN4 leucine

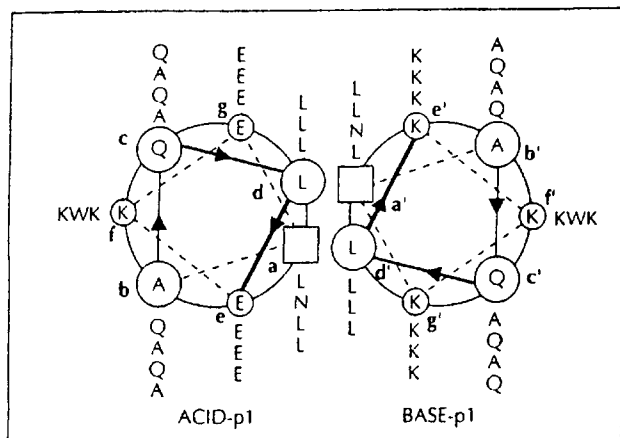


Fig. 2. Helical wheel representation [5,17] of the ACID-p1/BASE-p1 heterodimer. ACID-p1 differs from BASE-p1 only at positions **e** and **g**, where ACID-p1 contains glutamate and BASE-p1 contains lysine. The homodimers of these peptides are expected to be destabilized by unfavorable intrahelical and interhelical electrostatic interactions arising from residues at positions **e** and **g**. This destabilization is expected to be relieved in the heterodimer. The view shown is from the amino termini, looking down the superhelical axis. The peptide sequences begin at position **b** and end at position **c**. The sequence of peptide ACID-p1 is: Ac-AQLEKELQALEKENAQLWEWLQALEKELAQ-NH₂; the sequence of BASE-p1 is Ac-AQLKKKLQALKKKNAQLKWK-LQALKKKLAQ-NH₂.

zipper, in which the helices have a parallel orientation, an asparagine is part of the dimer interface and forms a buried hydrogen bond with the corresponding asparagine from the opposing subunit [3]. An asparagine at position **a** is expected to disfavor an antiparallel orientation of helices, because each asparagine (at position **a**) would be paired with a leucine (position **d**) from the opposing subunit. Based on similar reasoning, an asparagine at position **a** is expected also to favor an unstaggered arrangement of the helices [3].

The peptides contain alternating alanine and glutamine residues at positions **b** and **c**. Uncharged residues were desired at these positions in order to avoid substantial electrostatic interactions with adjacent residues at positions **e** and **g** (Fig. 2). In addition, alanine and glutamine residues promote helix formation [20,22,23] and the polar glutamine side chain is expected to increase solubility [24]. Lysine was chosen for the residues on the outside of the dimer at position **f** to increase the solubility of the peptides and to discourage aggregation. One residue at position **f** was chosen to be a tryptophan in order to facilitate concentration-determination by absorbance [25].

The ACID-p1/BASE-p1 heterodimer is helical and stable
CD spectra indicate that the individual ACID-p1 and BASE-p1 peptides are predominantly unfolded at 37 °C and pH 7 in phosphate-buffered saline (PBS) (Fig. 3a). In contrast, the CD spectrum of an equimolar mixture of ACID-p1 and BASE-p1 indicates that the mixture is highly helical (Fig. 3a). This helical structure is stable: at a heterodimer concentration of 5 μ M, the T_m is 65 °C

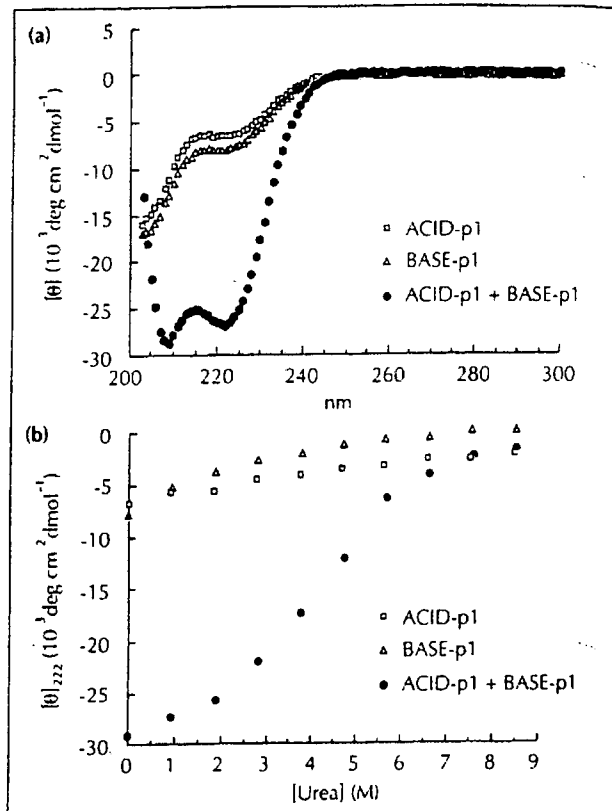


Fig. 3. The ACID-p1 and BASE-p1 peptides show little evidence of structure in isolation, but when mixed form a stable, helical structure. **(a)** The ACID-p1 and BASE-p1 peptides are predominantly unfolded in PBS (pH 7.0, 37 °C), but a mixture of the peptides forms a highly helical structure characterized by CD minima at 222 nm and 208 nm. **(b)** The structure of the ACID-p1 plus BASE-p1 mixture is stable and undergoes a cooperative unfolding transition with a midpoint at approximately 4.5 M urea (PBS, pH 7.0, 37 °C). In contrast, isolated ACID-p1 and BASE-p1 peptides show no evidence for significant folding.

in PBS at pH 7. In addition, whereas a urea-induced unfolding transition is observed for the heterodimer, the isolated peptides show no evidence for cooperative unfolding (Fig. 3b). Sedimentation equilibrium studies of an ACID-p1 plus BASE-p1 mixture at 20 °C in PBS, pH 7 indicate definitively that it has the molecular weight expected for the heterodimer: the observed molecular weight is 7200 D (the expected molecular weight is 7123 D). Thus, the ACID-p1 and BASE-p1 peptides associate preferentially and fold as a stable, helical heterodimer.

The amide proton exchange behavior of the ACID-p1/BASE-p1 heterodimer was characterized by nuclear magnetic resonance (NMR) spectroscopy. A coiled-coil dimer with a well-packed hydrophobic core is expected to have some protons protected from exchange by factors approaching the equilibrium constant for folding of the molecule. For example, several amide protons in a GCN4 leucine-zipper peptide have protection factors of 10^5 – 10^6 , comparable to the equilibrium constant for folding [26]. In contrast, slowly exchanging protons in the designed four-helix bundle

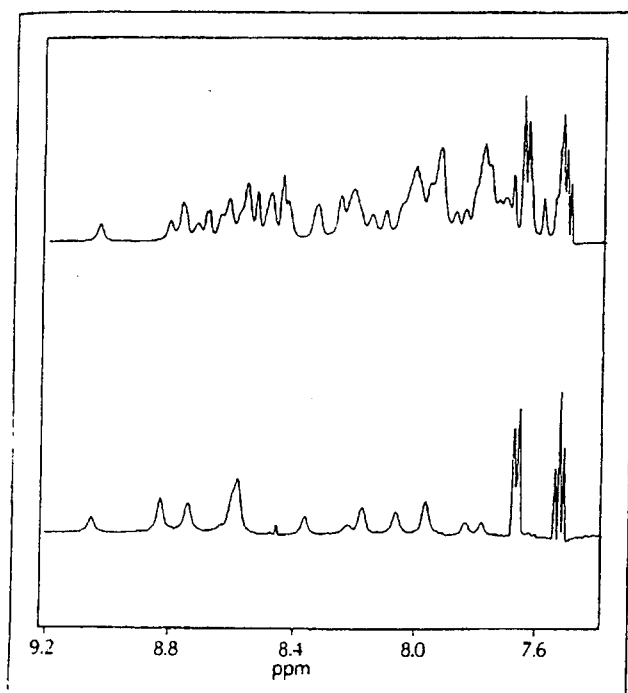


Fig. 4. Several amide protons are protected from exchange by a factor of at least 10^4 , suggesting that the interface between helices in the ACID-p1/BASE-p1 heterodimer has a well-defined structure. A region of the one-dimensional ^1H -NMR spectrum containing resonances arising from the amide and aromatic protons is shown. The top spectrum is taken in H_2O (0 hours) and the bottom spectrum is taken 70 hours after the sample was dissolved in D_2O (pH 4.7, 20°C). The dissociation constant measured by urea denaturation (see later, Fig. 8a) indicates that a 1 M solution of peptide would have a free energy of unfolding at 20°C , pH 7.0 of $-10.1 \text{ kcal mol}^{-1}$. The concentration dependence of stability is given by $\Delta G^0 = \Delta G + RT \ln c$, where ΔG^0 is the standard state free energy and c is the concentration of peptide. Thus, a 3 mM peptide solution at 20°C , pH 7.0 is expected to have a free energy of unfolding of $-6.7 \text{ kcal mol}^{-1}$. Because the stability of the ACID-p1/BASE-p1 heterodimer is independent of pH from pH 7 to pH 4.5 (Fig. 5a), the free energy of unfolding under the exchange conditions (pH 4.7, PBS) is estimated to be $-6.7 \text{ kcal mol}^{-1}$, corresponding to an equilibrium constant of 10^5 . At pH 4.7, 20°C the half-time for exchange of an unprotected amide proton is approximately 0.4 minutes [43]; thus, protons that are half-exchanged after 70 hours have protection factors of 10^4 .

$\alpha 4$ are protected from exchange by factors of only 10^3 – 10^5 , compared to the maximum of 10^{16} expected from the global stability of the molecule [27,28]. These NMR data and the results of other experiments suggest that $\alpha 4$ contains fluctuating tertiary structure [28,29].

The global stability of the ACID-p1/BASE-p1 heterodimer, under the conditions in which hydrogen exchange was studied, is $-6.7 \text{ kcal mol}^{-1}$. Thus, the maximum degree of protection from exchange is expected to be about 10^5 . At a time when protons that are protected by a factor of approximately 10^4 are expected to be half-exchanged, twelve amide proton resonances are present in the spectrum (Fig. 4). This suggests strongly that the interface between the ACID-p1 and BASE-p1 helices is well-packed [28,29].

Characterization of the disulfide-bonded heterodimer and homodimers

Our design predicts that the ACID and BASE peptides will fold as parallel, helical dimers but, at practical peptide concentrations, the ACID-p1 and BASE-p1 homodimers are too unstable to study. One way to stabilize dimeric coiled-coil peptides is to join the peptides with a disulfide-bond linker [1,2,19,30,31]. Therefore, versions of the ACID and BASE peptides containing an amino-terminal cysteine followed by two glycines, designated ACID-p1N and BASE-p1N, were made and studied. The glycines allow disulfide-bond formation without distortion of the coiled-coil structure [2]. CD studies demonstrate that the disulfide-bonded heterodimer and homodimers are greater than 80% helical at 0°C and that a cooperative thermal unfolding transition is observed for each dimer, indicating that the ACID and BASE homodimers can fold when stabilized with a disulfide-bond linker.

To determine if the orientation of the helices is parallel, the concentration dependence of stability was studied for each of the disulfide-bonded peptide dimers [1,2]. A dimer in which the helices are joined in the preferred orientation is expected to have stability and structure that is independent of peptide concentration. Alternatively, a dimer in which the helices are covalently linked in the unfavorable orientation is expected to have concentration-dependent stability and structure, arising from the intermolecular association of dimers.

When the ACID-p1N and BASE-p1N peptides are joined with a disulfide bond (that is, with a parallel orientation), the T_m of the heterodimer and each homodimer was found to be independent of peptide concentration from approximately 2.5 to $170 \mu\text{M}$ (see Fig. 5 legend). In contrast, when the heterodimer is disulfide-bonded in the antiparallel orientation (by joining the peptide ACID-p1N to a BASE peptide that has a carboxy-terminal sequence Gly-Gly-Cys), the CD signal is dependent on peptide concentration, indicating that higher-order oligomers are formed. In PBS, pH 7.0 containing 2.25 M GuHCl at 0°C , the $[\theta]_{222}$ values of the antiparallel heterodimer change from $-11\,100 \text{ deg cm}^2 \text{ dmol}^{-1}$ at $9 \mu\text{M}$ to $-25\,300 \text{ deg cm}^2 \text{ dmol}^{-1}$ at $25 \mu\text{M}$. Collectively, these results indicate that the folded conformations of the ACID and BASE heterodimer and homodimers are parallel.

Mechanism of specificity

Our design strategy sought to drive heterodimer formation by electrostatic repulsion in the homodimers. Nonetheless, because the ACID and BASE peptides contain oppositely charged residues at the **e** and **g** positions of the coiled-coil repeat, it is very plausible instead that electrostatic attraction between these peptides provides a major driving force for heterodimer formation.

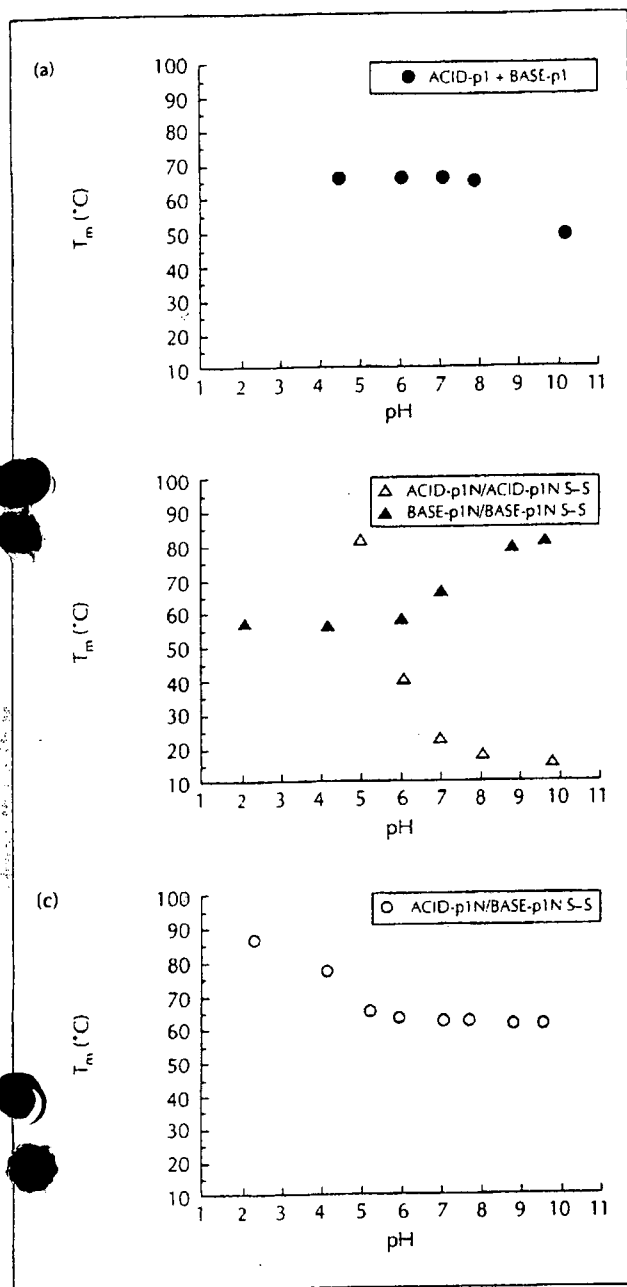


Fig. 5. The pH-dependence of stability indicates that the ACID and BASE homodimers are destabilized by unfavorable electrostatic interactions, whereas the net electrostatic contribution to the stability of the heterodimer is minimal. (a) The stability of the heterodimer, measured in the absence of a disulfide bond, is relatively independent of pH. (b) In contrast, the T_m of the disulfide-bonded ACID-p1N homodimer increases at low pH and the T_m of the disulfide-bonded BASE-p1N homodimer increases at high pH. (c) The stability of the disulfide-bonded ACID-p1N/BASE-p1N heterodimer in 3M GuHCl increases at low pH, suggesting that the heterodimer is somewhat destabilized by acidic residues at neutral pH. In PBS and at pH 7, the T_m values of the disulfide-bonded ACID-p1N/ACID-p1N, BASE-p1N/BASE-p1N, and ACID-p1/BASE-p1 dimers are $21 \pm 1^\circ\text{C}$, $66 \pm 1^\circ\text{C}$ and $> 100^\circ\text{C}$, respectively, over the concentration range 2.5–170 μM . Because the disulfide-bonded ACID-p1N/BASE-p1N heterodimer is too stable to denature thermally, the T_m of a 2.5 μM solution of heterodimer was measured in PBS containing 3M GuHCl and determined to be $62 \pm 1^\circ\text{C}$.

Assuming that one of these mechanisms predominates, some simple predictions can be made about the pH- and ionic strength-dependence of stability. If electrostatic destabilization of homodimers is dominant, then the homodimers will be more stable when the charges are removed by titration at extremes of pH or when the ionic strength is increased. In contrast, if electrostatic attraction between the ACID and BASE peptides in the heterodimer provides the dominant driving force for preferential heterodimer formation, then the heterodimer will be less stable as charges are removed by titration at pH extremes (that is, a bell-shaped curve for the pH dependence of stability would result) and the stability of the heterodimer should decrease at higher ionic strength.

The stability of both homodimers in the more stable disulfide-bonded species is highly dependent on both pH and ionic strength (Figs 5 and 6). The T_m of the ACID-p1N/ACID-p1N homodimer is more than 80°C higher at acidic pH than at pH 7, indicating clearly that it is destabilized by negatively charged residues at neutral pH (Fig. 5b). Similarly, the BASE-p1N/BASE-p1N homodimer is approximately 20°C more stable at basic pH than at pH 7, indicating that it is destabilized at neutral pH by positively charged basic residues. Both homodimers are stabilized substantially by increasing ionic strength at neutral pH (Fig. 6b), indicating that they are destabilized by repulsive electrostatic interactions.

At neutral pH, the BASE-p1N/BASE-p1N homodimer is substantially more stable than the ACID-p1N/ACID-p1N homodimer (Fig. 5b). It is likely that part of the reason for this difference in stability is the difference in the length of the side chains of residues at positions **e** and **g**. Whereas ACID-p1N has glutamate, containing two methylene groups, at positions **e** and **g**, BASE-p1N has lysine, which contains four methylene groups. The longer lysine side chain allows for more flexibility and better solvation of the terminal charged group. This is supported by studies of BASE peptides containing the non-natural amino acids ornithine (ORN-p1; three methylene groups) or diamminobutyric acid (DAB-p1; two methylene groups) at positions **e** and **g**. Unlike BASE-p1N, disulfide-bonded homodimers containing these non-natural amino acids do not show evidence for structure at 0°C , nor for a cooperative thermal unfolding transition. Additionally, the stability of the corresponding ACID/BASE heterodimer decreases with decreasing side chain length: in PBS at pH 7, the disulfide-bonded ACID-p1N/DAB-p1N and ACID-p1N/ORN-p1N heterodimers have T_m values of approximately 63°C and 80°C , respectively (the ACID-p1N/BASE-p1N heterodimer has a T_m over 100°C).

Because the disulfide-bonded ACID-p1N/BASE-p1N heterodimer is exceedingly stable, the pH and ionic

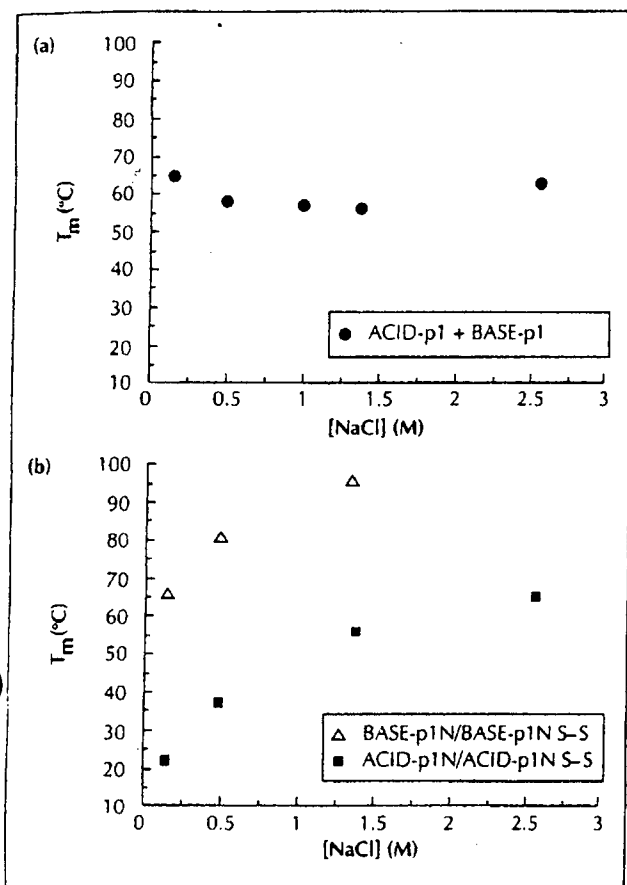


Fig. 6. The ionic strength-dependence of stability indicates that the ACID and BASE homodimers are destabilized by unfavorable electrostatic interactions, whereas the net electrostatic contribution to the stability of the heterodimer is minimal. (a) The stability of the heterodimer, measured in the absence of a disulfide bond, is relatively independent of ionic strength. (b) In contrast, the stability of the disulfide-bonded homodimers increases at high ionic strength.

strength-dependence of stability for the heterodimer was studied in the absence of a disulfide bond. At pH 7, the stability of the ACID-p1/BASE-p1 heterodimer is relatively independent of ionic strength (Fig. 6a). In addition, there is little change in the stability of the heterodimer between pH 4.5 and pH 9 (Fig. 5a). It is, however, difficult to assess the stability of the heterodimer at extremes of pH (< 3.5 and > 10.5) because at these pH extremes the thermal denaturation curves contain evidence for more than one transition, suggesting that other species, such as homodimers, are formed or that folding is not two-state.

To evaluate the stability of the heterodimer at extremes of pH, the pH-dependence was also studied in the presence of the disulfide bond but in 3 M GuHCl to destabilize the heterodimer (Fig. 5c). A comparable concentration of NaCl has little effect on the stability of the heterodimer (Fig. 6a). The striking result is that the stability of the disulfide-bonded heterodimer increases by approximately 25 °C at low pH (Fig. 5c). Sedimentation equilibrium studies show definitively that the

amino-terminal disulfide-bonded ACID-p1N/BASE-p1N heterodimer remains dimeric at pH 2: the observed molecular weight was 7200 D at pH 2 and 7700 D at pH 7 (the expected value is 7556 D). Thus, the negatively charged glutamate residues have a net destabilizing effect in the heterodimer.

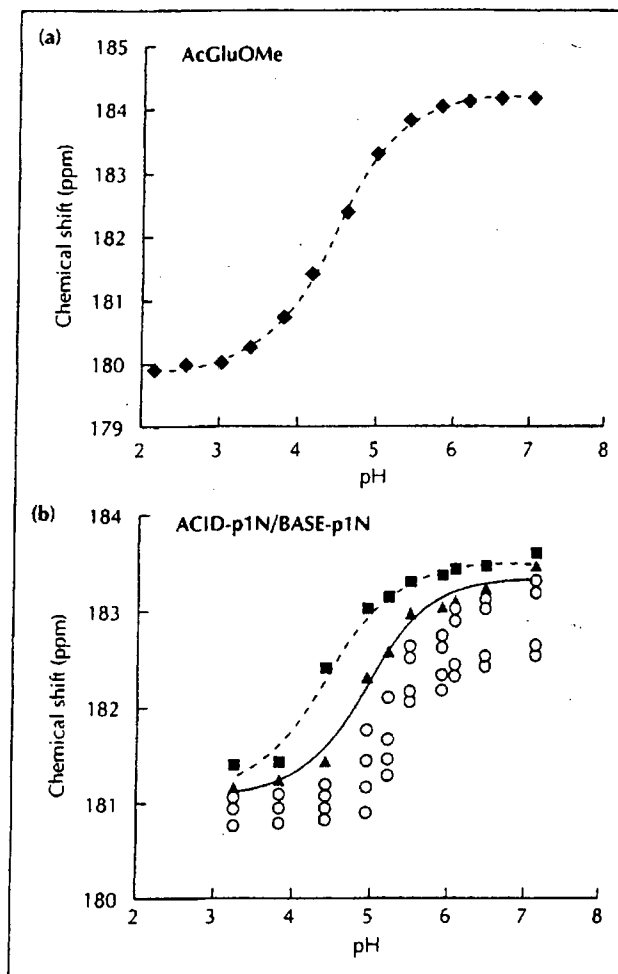


Fig. 7. ^{13}C -NMR spectroscopy indicates that the pK_a values for the glutamic acid side chains in the heterodimer are close to normal, or slightly increased, in PBS at 20 °C. (a) The changes in chemical shift with pH of the carboxylate ^{13}C resonances of AcGluOMe indicate that the pK_a of the carboxylic acid group of AcGluOMe is 4.5. (b) The changes in chemical shift with pH of the carboxylate ^{13}C resonance of disulfide-bonded ACID-p1N/BASE-p1N indicates that the pK_a values of the glutamic acid residues are either close to normal ($pK_a = 4.4$, ■, and $pK_a = 4.9$, ▲) or slightly increased ($pK_a = 5.2-5.3$, ○).

The measurements described above do not, however, rule out extremely large pK_a shifts for some ionizable groups in the heterodimer, which can result from stable salt bridge formation (for example, see [32]). The pK_a values for the glutamic acid residues in the heterodimer were therefore measured directly by natural-abundance ^{13}C NMR. The disulfide-bonded form of the heterodimer was studied to prevent formation of alternate species such as the ACID homodimer at low pH. In contrast to the decrease in pK_a expected if the glutamic

acid residues were involved in stabilizing salt bridges, the observed pK_a values for all glutamic acid residues in the heterodimer are close to or higher than the pK_a for blocked glutamic acid measured under the same conditions (Fig. 7).

The finding that some of the glutamic acid residues have increased pK_a values is consistent with the observation that the heterodimer is more stable at low pH (Fig. 5c). The most likely explanation for the increased stability at low pH is that electrostatic repulsion between the glutamic acid residues in the individual ACID helix is destabilizing, even in the heterodimer. Similarly, the Fos/Jun leucine zipper heterodimer [1,4] and previously designed coiled coils that contain acidic residues at positions **b** and **e** [18,19] are more stable at low pH than at neutral pH, suggesting that there is substantial electrostatic repulsion within the helices of these coiled coils at neutral pH [1,4]. In general, however, intrahelical destabilization can be minimized in designed coiled coils by utilizing a 14-residue repeat in which residues at a particular position in the heptad repeat are different.

It is puzzling that these studies do not indicate that interhelical salt bridges substantially stabilize the heterodimer, given that the heterodimer has charged side chains that might be expected to form salt bridges of the same type seen in the crystal structure of the GCN4 leucine zipper [3]. It is possible that salt bridges do not form in the heterodimer. Alternatively, the salt bridges may form but not be significantly stabilizing, as appears to be the case for the GCN4 leucine zipper peptide. Finally, the stabilizing effect of salt bridges could be offset by destabilizing factors. For example, the protonated form of a glutamic acid residue has a higher helix propensity than the ionized form [33, 34] and, as discussed above, electrostatic repulsion between residues of like charge within the ACID peptide helix is destabilizing even within the heterodimer.

Even if stabilizing salt bridges are present in the heterodimer, these results indicate that the net electrostatic contribution to specificity from the heterodimer *per se* is either minimal or unfavorable. In contrast, both homodimers have stabilities that are dependent strongly on pH and ionic strength, in a manner that would be expected if the homodimers have substantial net destabilizing electrostatic interactions at neutral pH. We conclude that our design strategy was successful: preferential heterodimer formation is driven primarily by destabilization of the homodimers.

Quantitation of specificity

Because the preference for heterodimer formation in a mixture of the ACID and BASE peptides is so large, the ratio of heterodimer to homodimer cannot be measured readily from an equilibrium mixture of the two peptides. However, the degree of specificity can be estimated because K_{spec} , the equilibrium constant

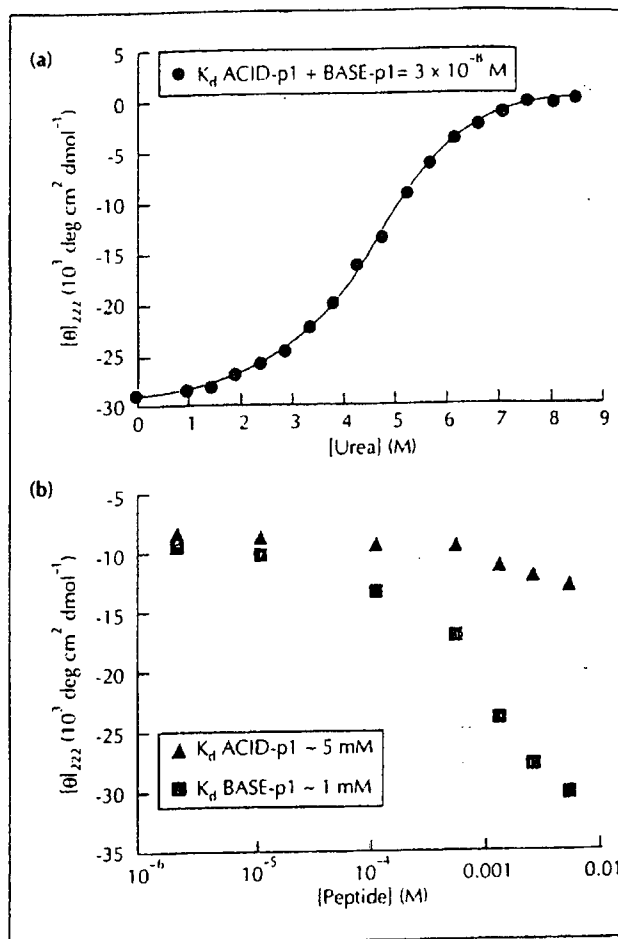


Fig. 8. Estimation of the dissociation constant for the ACID-p1/BASE-p1 heterodimer and homodimers. (a) The dissociation constant for the ACID-p1/BASE-p1 heterodimer is estimated to be 3×10^{-8} M from a curve fit of the urea dependence of the CD signal (the line from this fit is shown). (b) The dissociation constants for the homodimers of ACID-p1 and BASE-p1, estimated from plots of the CD signal at 222 nm as a function of peptide concentration, are greater than 5×10^{-3} M and approximately 1×10^{-3} M respectively.

describing the ratio of heterodimer to homodimers, can be expressed in terms of the dissociation constants for each dimer [4]. Assuming a two-state model for monomer-dimer equilibrium,

$$K_{\text{spec}} = K_d(\text{AB}) / [K_d(\text{AA})^{1/2} \times K_d(\text{BB})^{1/2}]$$

where $K_d(\text{AB})$, $K_d(\text{AA})$ and $K_d(\text{BB})$ are the dissociation constants of the ACID-p1/BASE-p1 heterodimer, the ACID-p1 homodimer and the BASE-p1 homodimer, respectively.

The dissociation constant for each dimer was determined so that the degree of specificity, ΔG_{spec} , given by $-RT \ln K_{\text{spec}}$, could be estimated. A dissociation constant of 3×10^{-8} M for the ACID-p1/BASE-p1 heterodimer was determined by fitting the CD signal as a function of urea concentration to a monomer-dimer equilibrium (Fig. 8a). An estimate of the dissociation constant for each homodimer was obtained from measurements of the helical CD signal as a function of

peptide concentration. The dissociation constant for the BASE-p1 homodimer is approximately 1×10^{-3} M and that for the ACID-p1 homodimer is greater than 5×10^{-3} M (Fig. 8b). These dissociation constants place a lower limit on the degree of preference for heterodimer, ΔG_{spec} , of $-6.5 \text{ kcal mol}^{-1}$ ($> 10^5$ -fold preference for heterodimer).

A different, albeit less reliable, estimate of specificity can be made by measuring the difference between the T_m of the heterodimer and the average of the T_m values for the homodimers [4]. This difference (ΔT_m) has been measured for other disulfide-bonded leucine-zipper peptides and has been shown to be related to ΔG_{spec} by a proportionality constant of $7.4^\circ\text{C/kcal mol}^{-1}$ [4]. ΔT_m for the amino-terminally disulfide-bonded ACID-p1N and BASE-p1N peptides is greater than 56°C (see Fig. 5 legend). If the same proportional relationship between ΔT_m and ΔG_{spec} exists with the ACID and BASE peptides, this lower limit for ΔT_m implies that ΔG_{spec} is at least $-7.5 \text{ kcal mol}^{-1}$ (greater than $10^{5.6}$ -fold preference for heterodimer).

Thus, the heterodimer is preferred over the ACID-p1 and BASE-p1 homodimers by at least 10^5 -fold. This degree of specificity is much greater than that observed for the Fos and Jun peptides, which show only a 10^2 -fold preference for heterodimer formation [4]. The increased specificity of the ACID/BASE heterodimer is likely to result from the increased number of repulsive interactions in the designed homodimers.

Conclusions

Our results indicate that the designed ACID and BASE peptides fold as parallel, helical dimers that have a very high preference for the heterodimeric state. As the ACID and BASE peptides were designed using principles learned from the study of preferential heterodimer formation in the Fos and Jun peptide system, our results support previous conclusions about the basis and mechanism of Fos/Jun specificity [1,4]. In both cases, unfavorable electrostatic interactions in the homodimers contribute more to dimerization specificity than favorable electrostatic interactions in the heterodimer.

Our results have implications for protein design, because they demonstrate that it is possible to design simple helical sequences that pair with substantial specificity. The design of simple molecules consisting of two secondary structural elements may lead to stepwise strategies for the design of larger proteins. In contrast to many other designed peptides, the ACID/BASE heterodimer does not contain fluctuating tertiary structure, as judged by amide proton exchange studies, possibly because a polar group was introduced into the otherwise hydrophobic interface [3,29,35,36].

Finally, the ACID-p1 and BASE-p1 peptides can be thought of as peptide 'Velcro': the individual peptides have little self-affinity under physiological conditions of temperature, pH and ionic strength, but the two peptides have high affinity for each other. It may be possible to use these properties for practical applications, including use in affinity reagents, in place of an epitope tag or as a way to bring together two molecules in a cell.

Materials and methods

Peptide synthesis

Peptides were synthesized using small-scale Fmoc HBTU reaction cycles and acetic anhydride capping on an Applied Biosystems Model 431A peptide synthesizer [37]. Peptides were cleaved from the resin with trifluoroacetic acid (TFA) using standard cleavage methods and were desalted on a Sephadex G-10 column in 5% acetic acid. Final purification was by reverse-phase high performance liquid chromatography (HPLC) with a Vydac preparative C18 column ($2.2 \times 25 \text{ cm}$) at 25°C using linear acetonitrile/ H_2O gradients in the presence of 0.1% TFA. Peptide purity was greater than 90%, as judged by analytical HPLC. The identity of each peptide was confirmed by laser desorption mass spectrometry on a Finnegan Lasermat and each was found to be within 2D of the expected mass. All peptides are acetylated at the amino terminus and amidated at the carboxyl terminus.

CD spectroscopy

CD studies were performed on an Aviv Model 60DS or 62DS CD spectrophotometer equipped with a thermoelectric controller. Peptide concentrations were determined by absorbance at 280 nm in 6M GuHCl [25] and samples were prepared in PBS (150 mM NaCl, 10 mM sodium phosphate). CD spectra (Fig. 3a) were measured at 37°C , pH 7.0 using a peptide concentration of $10 \mu\text{M}$ ACID-p1, $10 \mu\text{M}$ BASE-p1, or $5 \mu\text{M}$ ACID-p1 plus $5 \mu\text{M}$ BASE-p1. Urea denaturation curves shown in Figure 3b were determined at 37°C , pH 7.0 using a peptide concentration of $20 \mu\text{M}$ ACID-p1, $20 \mu\text{M}$ BASE-p1, or $10 \mu\text{M}$ ACID-p1 plus $10 \mu\text{M}$ BASE-p1. Thermal melting curves were determined by monitoring the CD signal at 222 nm as a function of temperature. For studies of the pH-dependence of stability (Fig. 5), melting curves were recorded with $5 \mu\text{M}$ homodimer or $5 \mu\text{M}$ heterodimer in PBS with the pH adjusted to the desired value. For studies of the ionic strength-dependence (Fig. 6), melting curves were recorded at pH 7.0 with $5 \mu\text{M}$ heterodimer or with $2.5 \mu\text{M}$ homodimer in 10 mM sodium phosphate in the presence of the indicated concentration of NaCl. The T_m values were estimated by taking the first derivative of the CD signal with respect to temperature $^{-1}$ and finding the minimum of this function [38]. All thermal melts performed at pH values below pH 8 are reversible; more than 90% of the CD signal at 0°C was recovered after the melt. The urea denaturation curve shown in Figure 8a was determined at 20°C , pH 7.0 with $10 \mu\text{M}$ ACID-p1 plus $10 \mu\text{M}$ BASE-p1. A two-state model for unfolding was assumed and the urea denaturation curve for the heterodimer was fit with a non-linear least-squares fitting program (Kaleidagraph, Synergy Software) to the following equation that describes the urea dependence of the CD signal for a monomer-dimer equilibrium:

$$\theta = \theta_d + (\theta_m - \theta_d) \left\{ \exp((\Delta G^\circ + m[\text{urea}])/RT)/4C_T \right\} + \left\{ \exp((\Delta G^\circ + m[\text{urea}])/RT)^2/16C_T^2 \right\} + \left\{ \exp((\Delta G^\circ + m[\text{urea}])/RT)/2C_T^{1/2} \right\}$$

where θ = CD signal at 222 nm; θ_d and θ_m are linear equations describing the urea dependence of the CD signal of the dimer and unfolded monomer respectively; ΔG° = free

energy of folding under standard conditions of 20°C and 1 M concentration of peptide chains; m = the dependence of the free energy of folding on denaturant concentration; R = gas constant; T = temperature in K; C_T = total concentration of peptide chains in M. Measurements of the CD signal at 222 nm as a function of peptide concentration for the ACID-p1 and BASE-p1 homodimers were performed at 20°C, pH 7.0 (Fig. 8b).

Analytical centrifugation

Sedimentation equilibrium was performed at 20°C using a Beckman XL-A ultracentrifuge at rotor speeds between 30 and 40 krpm. Three samples at a heterodimer concentration of 22 μ M, 44 μ M and 66 μ M in PBS were analyzed. Samples were dialysed (> 12 hours) against the reference buffer (PBS). Data were fit to an ideal model plot of radial distance squared versus log(concentration). The residuals were analyzed to reveal the presence of species other than the dimer; in no case were systematic deviations of the residuals observed. Partial molar volumes and solvent densities were calculated as described by Laue *et al.* [39].

NMR spectroscopy

One-dimensional ^1H -NMR spectra were recorded on a Bruker AMX 500 spectrometer operating at 500.1 MHz for ^1H at 20°C with a sweep width of 6024.1 Hz and a delay of 1.2 seconds. The sample was approximately 3 mM ACID-p1/BASE-p1 in PBS, pH 4.7 and internally referenced with trimethylsilylpropionic acid [40]. 8192 points were collected and water was suppressed by continuous irradiation. The pH values were uncorrected for the isotope effect [41]. Data were processed with the FTNMR software package (Hare Research Inc.) and spectra were apodized with a Gaussian window.

One-dimensional ^{13}C -NMR spectra were recorded at natural abundance on a Bruker AMX 500 spectrometer operating at 125.8 MHz for ^{13}C at 20°C with a spectral width of 26315.79 Hz and a recycle delay of 3 seconds. Samples were prepared in deuterated PBS and were either 38 mM in AcGluOMe (Bachem Feichemikalien AG, Bubendorf, Switzerland) or approximately 2 mM in disulfide-bonded ACID-p1N/BASE-p1N. ^1H were decoupled using WALTZ-16 broadband decoupling [42]. Data sets consisting of 4096 to 25000 transients defined by 4096 points were collected and zero-filled twice before Fourier transformation. The pH values were uncorrected for the isotope effect [41].

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22ND FLOOR
CHICAGO, IL 60606 (US)(57) **ABSTRACT**

A chimeric, carboxy-terminal truncated hepatitis B virus nucleocapsid protein (HBc) is disclosed that contains an immunogen for inducing the production of antibodies to the influenza M2 protein. An immunogenic influenza epitope is preferably expressed at or near the N-terminus or in the HBc immunogenic loop sequence. The chimera preferably contains an influenza-specific T cell epitope and is preferably engineered for both enhanced stability of self-assembled particles and enhanced yield of those chimeric particles. Methods of making and using the chimeras are also disclosed.

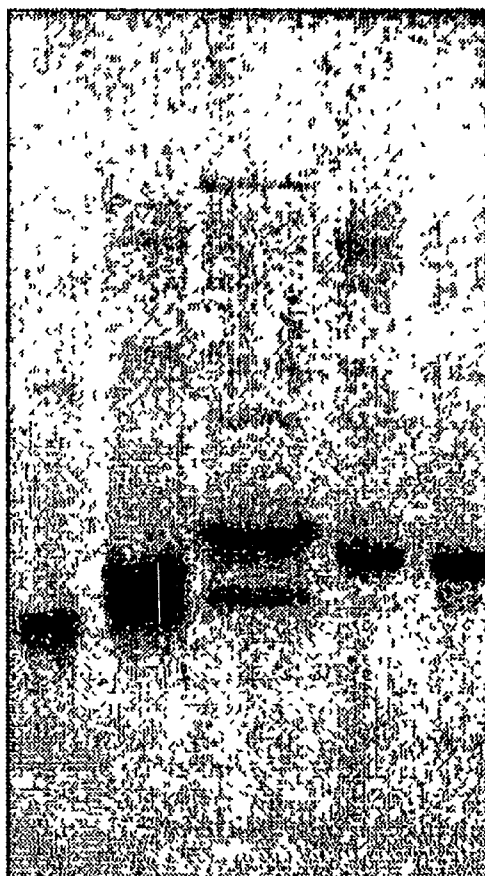
(21) **Appl. No.: 10/080,299**(22) **Filed: Feb. 21, 2002****Related U.S. Application Data**(63) **Continuation-in-part of application No. 09/930,915,**
filed on Aug. 15, 2001.**1 2 3 4 5**

FIG. 1A

Ground Squirrel	mylfhlclvf acvpcptvqa sklclglwld
	1
HBC AYW	mdidpykefg atvellsflp sdfpfsvrld ldtasalyre
HBC ADW	mdidpykefg atvellsflp sdfpfsvrld ldtasalyre
HBC ADW2	mdidpykefg atvellsflp sdfpfsvrld ldtasalyre
HBC ADYW	mdidpykefg atvellsflp sdfpfsvrld ldtaaalryd
Woodchuck	mdidpykefg ssyqlnflp ldffpdlnal vdtatalyee
Ground Squirrel	mdidpykefg ssyqlnflp ldffpdlnal vdtaaalyee
	41
HBC AYW	alespehcsp hhtalrqail cwgelmtlat wvgvnledpa
HBC ADW	alespehcsp hhtalrqail cwgelmtlat wvgnnlqdp
HBC ADW2	alespehcsp hhtalrqail cwgelmtlat wvgnnledpa
HBC ADYW	alespehcsp hhtalrqail cwgdlnltlat wvgtnledpa
Woodchuck	eltgrehcsp hhtairqalv cwdeltklia wmsnntseq
Ground Squirrel	eltgrehcsp hhtairqalv cweeltrlit wmsentteev

FIG. 1B

81
HBC AYW srdlvvsyvn tnmglkfrql lwfhiscldtf gretvleylv
HBC ADW srdlvvnynv tnmglkirql lwfhiscldtf gretvleylv
HBC ADW2 srdlvvnynv tnmglkirql lwfhiscldtf gretvleylv
HBC ADYW srdlvvsyvn tnmglkfrql lwfhiscldtf gretvleylv
Woodchuck vrtiivnhvn dtwglkvrqs lwfhiscldtf gqhtvqeflv
Ground Squirrel rriivdhvnn twglkvrqt1 wfhlscldtf gqhtvqeflv

121
HBC AYW sfgvwirtpp ayrppnapil stlpettvvr rrgrsprrrt
HBC ADW sfgvwirtpp ayrppnapil stlpettvvr rrdrgsprrr
HBC ADW2 sfgvwirtpp ayrppnapil stlpettvvr rrdrgsprrr
HBC ADYW sfgvwirtpp ayrppnapil stlpettvvr rrgrsprrrt
Woodchuck sfgvwirtpp ayrppnapil stlpettvvr rrdrgsprrr
Ground Squirrel fgvwirtpp ayrppnapil stlpettvvr rrdrgsprrr

161
HBC AYW psprrrrsqs prrrrsqsre sqc
HBC ADW rtpsprrrrs qsprrrrsqs resqc
HBC ADW2 rtpsprrrrs qsprrrrsqs resqc
HBC ADYW psprrrrsqs prrrrsqsre sqc
Woodchuck prrtpsprrr rrsqsprrrr sqc
Ground Squirrel rrtpsprrr rrsqsprrrr sqc

FIG. 2

HindIII

pKK223-3

TTCACACAGGAAACAGAATTCCCGGGGATCCGTCGACCTGCAGCCAAGCTT

SEQ ID NO:180

pKK223-3N

TTCACATAAGGAGGAAAAAccatggGATCCG-----
----AAGCTT

NcoI

SEQ ID NO:181

24

Fig. 3

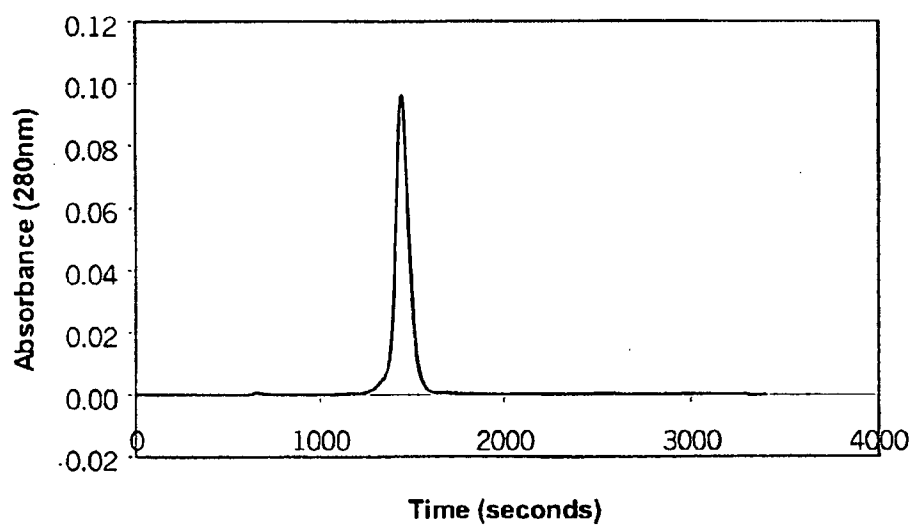


Fig. 4

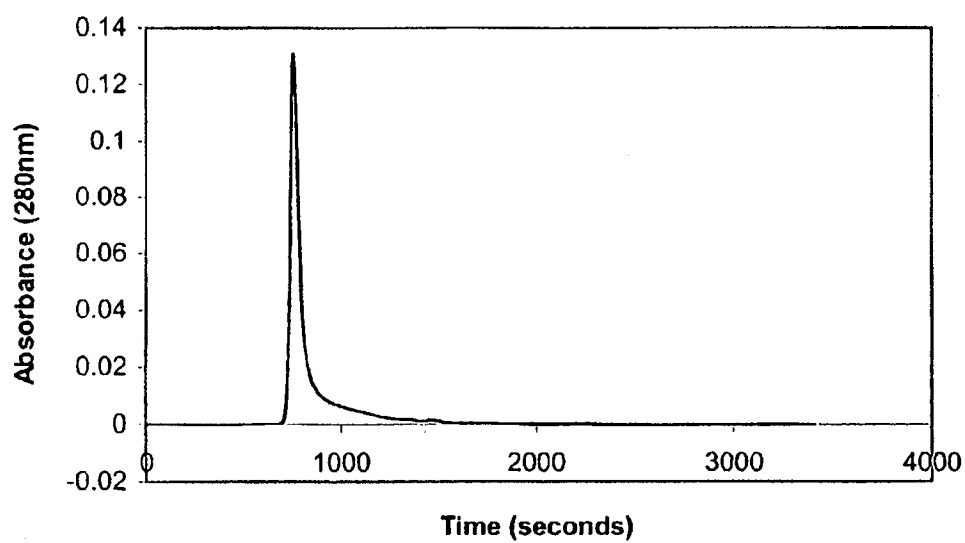


Fig. 5

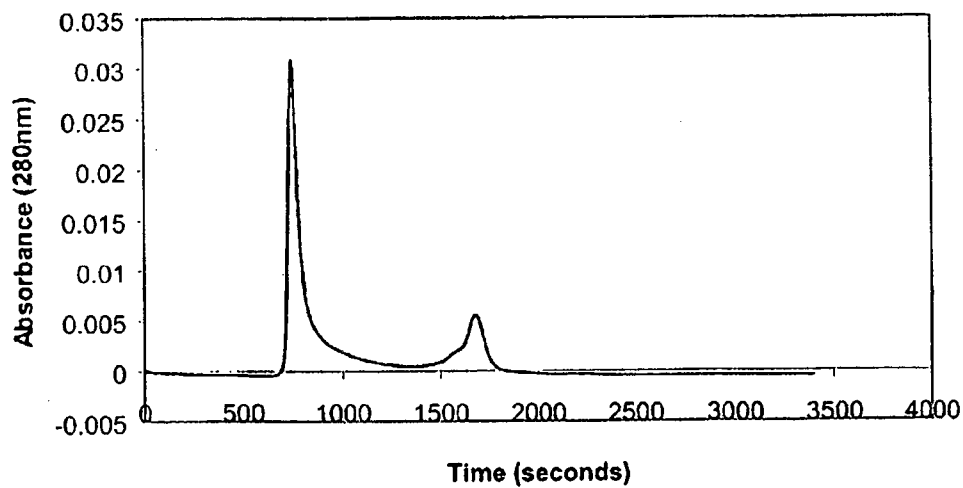
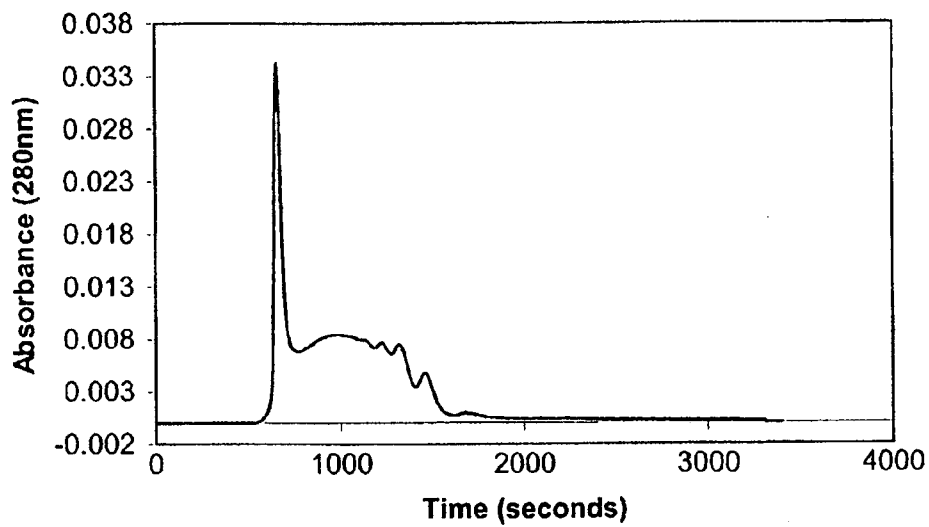


Fig. 6



216

Fig. 7

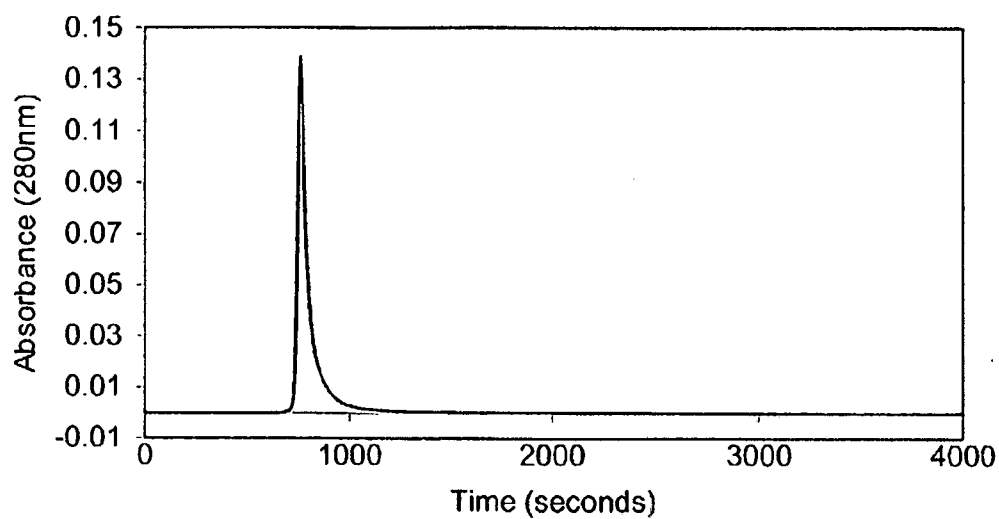


Fig. 8

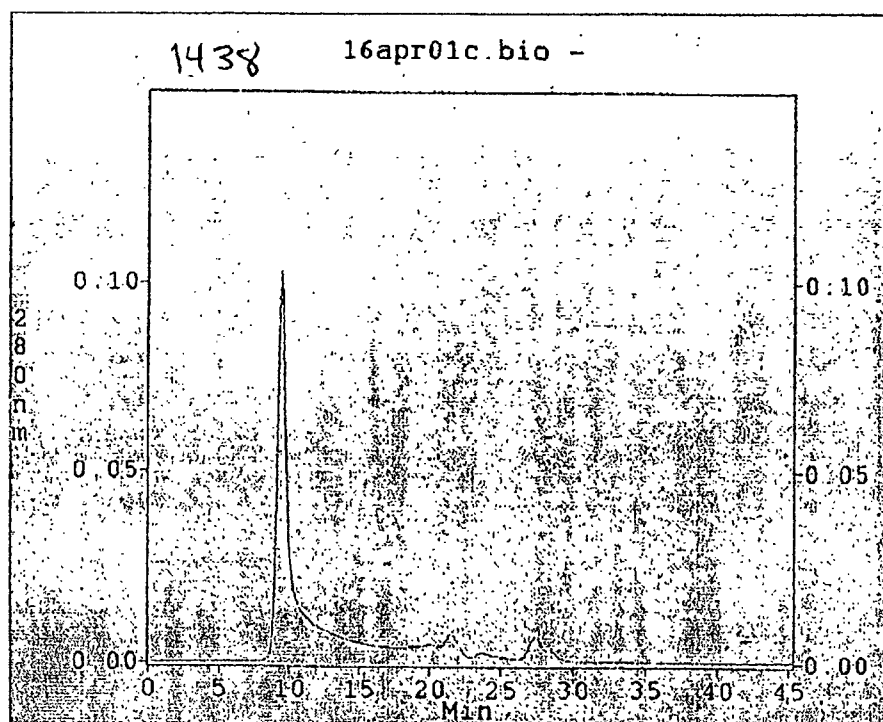


Fig. 9

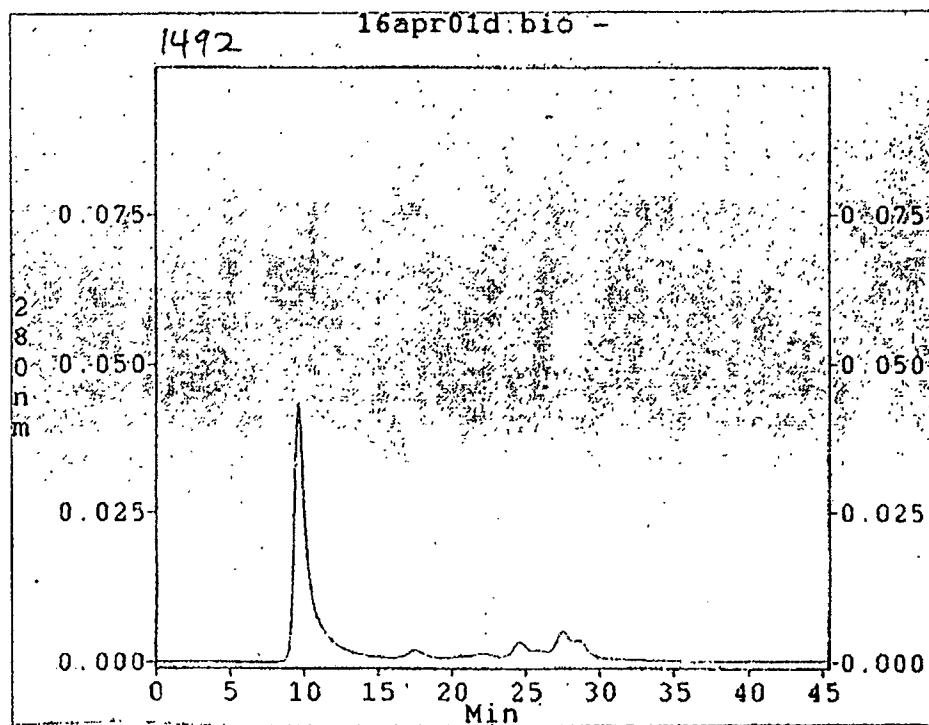
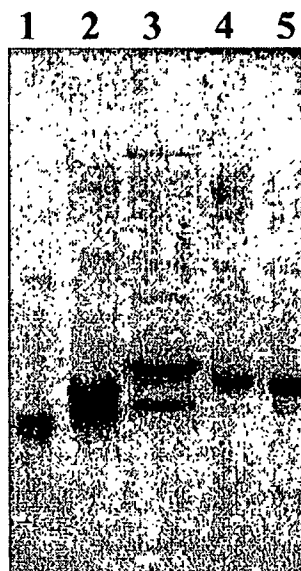


Fig. 10



INFLUENZA IMMUNOGEN AND VACCINE

DESCRIPTION

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This is a continuation-in-part of application Serial No. 09/930,915, filed Aug. 15, 2001, whose disclosures are incorporated herein by reference.

TECHNICAL FIELD

[0002] The present invention relates to the intersection of the fields of immunology and protein engineering, and particularly to an immunogen and vaccine useful in prevention of influenza infection by influenza A virus.

BACKGROUND OF THE INVENTION

[0003] The family hepadnaviridae are enveloped DNA-containing animal viruses that can cause hepatitis B in humans (HBV). The hepadnavirus family includes hepatitis B viruses of other mammals, e.g., woodchuck (WHV), and ground squirrel (GSHV), and avian viruses found in ducks (DHV) and herons (HeHV). Hepatitis B virus (HBV) used herein refers to a member of the family hepadnaviridae that infects mammals, as compared to a virus that infects an avian host, unless the discussion refers to a specific example of a non-mammalian virus.

[0004] The nucleocapsid or core of the mammalian hepatitis B virus (HBV or hepadnavirus) contains a sequence of 183 or 185 amino acid residues, depending on viral subtype, whereas the duck virus capsid contains 262 amino acid residues. Hepatitis B core protein monomers of the several hepadnaviridae self-assemble in infected cells into stable aggregates known as hepatitis B core protein particles (HBc particles). Two three-dimensional structures are reported for C-terminally truncated HBc particles. A first that comprises a minor population contains 90 copies of the HBc subunit protein as dimers or 180 individual monomeric proteins, and a second, major population that contains 120 copies of the HBc subunit protein as dimers or 240 individual monomeric proteins. These particles are referred to as T=4 or T=3 particles, respectively, wherein "T" is the triangulation number. These HBc particles of the human-infecting virus (human virus) are about 30 or 34 nm in diameter, respectively. Pumpens et al. (1995) *Intervirology*, 38:63-74; and Metzger et al. (1998) *J. Gen. Virol.*, 79:587-590.

[0005] Conway et al., (1997) *Nature*, 386:91-94, describe the structure of human HBc particles at 9 Å resolution, as determined from cryo-electron micrographs. Botcher et al. (1997), *Nature*, 386:88-91, describe the polypeptide folding for the human HBc monomers, and provide an approximate numbering scheme for the amino acid residues at which alpha-helical regions and their linking loop regions form. Zheng et al., (1992) *J. Biol. Chem.*, 267(13):9422-9429 report that core particle formation is not dependent upon the arginine-rich C-terminal domain, the binding of nucleic acids or the formation of disulfide bonds based on their study of mutant proteins lacking one or more cysteines and others' work with C-terminal-truncated proteins [Birnbaum et al., (1990) *J. Virol.* 64, 3319-3330]. The low resolution structure of HBc particles reported by Conway et al., (1997) and Botcher et al., (1997) has been confirmed by

a 3.3 Å resolution crystal structure of the T=4 particles reported by Wynne et al., (1999) *Mol. Cell*, 3(6):70-80.

[0006] The hepatitis B nucleocapsid or viral core protein (HBc) has been disclosed as an immunogenic carrier moiety that stimulates the T cell response of an immunized host animal. See, for example, U.S. Pats. No. 4,818,527, U.S. Pat. No. 4,882,145 and U.S. Pat. No. 5,143,726. A particularly useful application of this carrier is its ability to present foreign or heterologous B cell epitopes at the site of the immunodominant loop that is present at about residue positions 70-90, and more usually recited as about positions 75 through 85 from the amino-terminus (N-terminus) of the protein. Clarke et al. (1991) F. Brown et al. eds., *Vaccines* 91, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp.313-318.

[0007] During viral replication, HBV nucleocapsids associate with the viral RNA pre-genome, the viral reverse transcriptase (Pol), and the terminal protein (derived from Pol) to form replication competent cores. The association between the nucleocapsid and the viral RNA pre-genome is mediated by an arginine-rich domain at the carboxyl-terminus (C-terminus). When expressed in heterologous expression systems, such as *E. coli* where viral RNA pre-genome is absent, the protamine-like C-terminus; i.e., residues at positions 150 through 183, can bind *E. coli* RNA. Zhang et al. (1992) *JBC*, 267(13) 9422-29.

[0008] HBcAg is a particulate protein derived from the hepatitis B virus that has been proposed as a carrier for heterologous epitopes. The relative immunogenicity of HBsAg (HBs) has been compared with HBcAg (HBc), and the ability of each to evoke immune responses in different genetic backgrounds [Milich et al., *Science*, (1986) 234(4782): p. 1398-1401]. These data emphasize the higher immunogenicity of HBc relative to HBs, and the universal responsiveness to HBc, irrespective of genetic background.

[0009] For example, HBc is more than 300 times more immunogenic than HBs in BALB/c mice; and, although both B10.S and B10.M mice are non-responders to HBs, every strain tested is responsive to HBc. These results re-emphasize the suitability of HBc as a vaccine carrier and specifically, its superiority over HBs, hence the selection of HBc as opposed to HBs to carry heterologous epitopes. These facets of HBc are thought to be important in influenza vaccine development, because they address issues of genetic restriction and inadequate antibody titers.

[0010] Another advantage of the HBc carrier is the fact that it does not require complex adjuvants for efficacy. This is due to the high inherent immunogenicity of the particle. A comparison of the immunogenicity of HBc-*P. berghei* particles showed that alum, which is approved for human use, was more effective than either IFA or CFA [Schodel et al., *J. Exp. Med.*, (1994) 180(3): p. 1037-46]. The importance of this observation is highlighted by toxicity problems associated with newer, more complex adjuvants as was recently noted in clinical trials of SKB's candidate malaria vaccine [Stoute et al., *N. Engl. J. Med.*, [1997] 336(2): p. 86-91].

[0011] In an application as a vaccine carrier moiety, it may be preferable that the HBV nucleocapsids not bind nucleic acid derived from the host. Birnbaum et al. (1990) *J. Virol.*, 64:3319-3330 showed that the protamine-like C-terminal

domain of HBV nucleocapsids could be deleted without interfering with the protein's ability to assemble into virus-like particles. It is thus reported that proteins truncated to about position 144; i.e., containing the HBc sequence from position one through about 144, can self-assemble, whereas deletions beyond residue 139 abrogate capsid assembly [F. Birnbaum & M. Nassal (1990) *J. Virol.*, 64: 3319-30][Seifer et al., (1995) *Intervirology*, 38:47-62].

[0012] Zlotnick et al., (1997) *Proc. Natl. Acad. Sci., USA*, 94:9556-9561 studied the assembly of full length and truncated HBc proteins in to particles. In addition to discussing full length molecules, those authors reported the preparation of a truncated protein that contained the HBc sequence from position 1 through 149 in which the cysteines at positions 48, 61 and 107 were each replaced by alanines and in which a cysteine residue was added at the C-terminus (position 150). That C-terminal mercaptan was used for linkage to a gold atom cluster for labeling in electron microscopy.

[0013] More recently, Metzger et al. (1998) *J. Gen. Virol.*, 79:587-590 reported that the proline at position 138 (Pro-138 or P138) of the human viral sequence is required for particle formation. Those authors also reported that assembly capability of particles truncated at the carboxy-terminus to lengths of 142 and 140 residues was affected, with assembly capability being completely lost with truncations resulting in lengths of 139 and 137 residues.

[0014] Several groups have shown that truncated particles exhibit reduced stability relative to standard hepatitis B core particles [Gallina et al. (1989) *J. Virol.*, 63:4645-4652; Inada, et al. (1989) *Virus Res.*, 14:27-48], evident by variability in particle sizes and the presence of particle fragments in purified preparations [Maassen et al., (1994) *Arch. Virol.*, 135:131-142]. Thus, prior to the report of Metzger et al., above, Pumpens et al., (1995) *Intervirology*, 38:63-74 summarized the literature reports by stating that the carboxy-terminal border for HBc sequences required for self-assembly was located between amino acid residues 139 and 144, and that the first two or three amino-terminal residues could be replaced by other sequences, but elimination of four or eleven amino-terminal residues resulted in the complete disappearance of chimeric protein in transformed *E. coli* cells.

[0015] Recombinantly-produced hybrid HBc particles bearing internal insertions (referred to in the art as HBc chimeric particles or HBc chimeres) containing various inserted polypeptide sequences have been prepared by heterologous expression in a wide variety of organisms, including *E. coli*, *B. subtilis*, *Vaccinia*, *Salmonella typhimurium*, *Saccharomyces cerevisiae*. See, for example Pumpens et al. (1995) *Intervirology*, 38:63-74, and the citations therein that note the work of several research groups. Native HBc particles have also been produced in plants (Tsuda et al., 1998) *Vox Sang.*, 74(3):148-155.

[0016] Such HBc chimeres often appear to have a less ordered structure, when analyzed by electron microscopy, compared to particles that lack heterologous epitopes [Schodel et al., (1994) *J. Exp. Med.*, 180:1037-1046]. In some cases the insertion of heterologous epitopes into C-terminally truncated HBc particles has such a dramatic destabilizing affect that hybrid particles cannot be recovered following heterologous expression [Schodel et al. (1994) *Infect. Immunol.*, 62:1669-1676]. Thus, many chimeric HBc

particles are so unstable that they fall apart during purification to such an extent that they are unrecoverable or they show very poor stability characteristics, making them problematic for vaccine development.

[0017] The above Pumpens et al. (1995) *Intervirology*, 38:63-74 report lists particle-forming chimeres in which the inserted polypeptide sequence is at the N-terminus, the C-terminus and between the termini. Insert lengths reported in that article are 24 to 50 residues at the N-terminus, 7 to 43 residues internally, and 11 to 741 residues at the C-terminus.

[0018] Kratz et al., (1999) *Proc. Natl. Acad. Sci., U.S.A.*, 96:1915-1920 recently described the *E. coli* expression of chimeric HBc particles comprised of a truncated HBc sequence internally fused to the 238-residue green fluorescent protein (GFP). This chimera contained the inserted GFP sequence flanked by a pair of glycine-rich flexible linker arms replacing amino acid residues 79 and 80 of HBc. Those particles were said to effectively elicit antibodies against native GFP in rabbits as host animals.

[0019] U.S. Pat. No. 5,990,085 describes two fusion proteins formed from an antigenic bovine inhibin peptide fused into (i) the immunogenic loop between residues 78 and 79 and (ii) after residue 144 of carboxy-terminal truncated HBc. Expressed fusion proteins were said to induce the production of anti-inhibin antibodies when administered in a host animal. The titers thirty days after immunization reported in that patent are relatively low, being 1:3000-15,000 for the fusion protein with the loop insertion and 1:100-125 for the insertion after residue 144.

[0020] U.S. Pat. No. 6,231,864 teaches the preparation and use of a strategically modified hepatitis B core protein that is linked to a hapten. The modified core protein contains an insert of one to about 40 residues in length that contains a chemically-reactive amino acid residue to which the hapten is pendently linked.

[0021] Recently published WO 01/27281 teaches that the immune response to HBc can be changed from a Th1 response to a Th2 response by the presence or absence, respectively, of the C-terminal cysteine-containing sequence of the native molecule. That disclosure also opines that disulfide formation by C-terminal cysteines could help to stabilize the particles. The presence of several residues the native HBc sequence immediately upstream of the C-terminal cysteine was said to be preferred, but not required. One such alternative that might be used to replace a truncated C-terminal HBc sequence was said to include a C-terminal cysteine and an optional sequence that defines an epitope from other than HBc.

[0022] Published PCT application WO 01/98333 teaches the deletion of one or more of the four arginine repeats present at the C-terminus of native HBc, while maintaining the C-terminal cysteine residue. That application also teaches that the deleted region can be replaced by an epitope from a protein other than HBc so that the HBc portion of the molecule so formed acts as a carrier for the added epitope.

[0023] Published PCT applications corresponding to PCT/US01/25625 and PCT/US01/41759 of the present inventor teach that stabilization of C-terminally truncated HBc particles can be achieved through the use of one or more added cysteine residues in the chimera proteins from which the

particles are assembled. Those added cysteine residues are taught to be at or near the C-terminus of the chimeric protein.

[0024] A structural feature whereby the stability of full-length HBc particles could be retained, while abrogating the nucleic acid binding ability of full-length HBc particles, would be highly beneficial in vaccine development using the hepadnaviral nucleocapsid delivery system. Indeed, Ulrich et al. in their recent review of the use of HBc chimeras as carriers for foreign epitopes [*Adv. Virus Res.*, 50: 141-182 (1998) Academic Press] note three potential problems to be solved for use of those chimeras in human vaccines. A first potential problem is the inadvertent transfer of nucleic acids in a chimera vaccine to an immunized host. A second potential problem is interference from preexisting immunity to HBc. A third possible problem relates to the requirement of reproducible preparation of intact chimera particles that can also withstand long-term storage.

[0025] The above four published PCT applications appear to contain teachings that can be used to overcome the potential problems disclosed by Ulrich et al. As disclosed hereinafter, the present invention provides another HBc chimera that provides unexpectedly high titers of antibodies against influenza, and in one aspect also provides a solution to the problems of HBc chimera stability as well as the substantial absence of nucleic acid binding ability of the construct. In addition, a contemplated recombinant chimera exhibits reduced antigenicity toward preexisting anti-HBc antibodies.

[0026] The above particle instability findings related to N-terminal truncated HBc chimera molecules notwithstanding, Neirynck et al., (October 1999) *Nature Med.*, 5(10):1157-1163 reported that particle formation occurred on *E. coli* expression of a HBc chimera that contained the N-terminal 24-residue portion of the influenza M2 protein fused at residue 5 to full length HBc.

[0027] The previously discussed use of hybrid HBc proteins with truncated C-termini for vaccine applications offers several advantages over their full-length counterparts, including enhanced expression levels and lack of bound *E. coli* RNA. However, C-terminally truncated particles engineered to display heterologous epitopes are often unstable, resulting in particles that either fail to associate into stable particulate structures following expression, or that readily dissociate into non-particulate structures during and/or following purification. Such a lack of stability is exhibited by particles comprised of chimeric HBc molecules that are C-terminally truncated to HBc position 149 and also contain the above residues 1-24 of the influenza A M2 protein.

[0028] Others have reported that in wild type hepadnaviral core antigens a cysteine residue upstream of the HBcAg start codon is directly involved in the prevention of particle formation [Schodel et al. (Jan. 15, 1993) *J. Biol. Chem.*, 268(2):1332-1337; Wasenauer et al. (March 1993) *J. Virol.*, 67(3):1315-1322; and Nassal et al. (July 1993) *J. Virol.*, 67(7):4307-4315]. All three groups reported that in wild type HBcAg, the cysteine residue at position -7 of the pre-core sequence, which is present when the core gene is translated from an upstream initiator methionine at position -30, is responsible for preventing particle formation and therefore facilitating the transition from particulate HBcAg to secreted, non-particulate HBcAg.

[0029] Based upon the above three publications, one would expect the inclusion of one or more cysteine residues at a position prior to the initiator methionine of HBc; i.e., at a residue position of less than one relative to the N-terminus of the sequence of SEQ ID NO: 1, to actually destabilize hybrid particles rather than stabilize them. As will be seen from the discussion that follows, the present invention provides results that are contrary to those expectations.

BRIEF SUMMARY OF THE INVENTION

[0030] The present invention contemplates an immunogen for inducing antibodies to influenza A, and an inoculum and a vaccine comprising that immunogen dispersed in a physiologically tolerable diluent. A contemplated immunogen is a self-assembled particle comprised of recombinant hepatitis B virus core (HBc) chimera protein molecules. Each of those molecules has a length of about 150 to about 325 amino acid residues and contains four peptide-linked amino acid residue sequence domains from the N-terminus that are denominated Domains I, II, III and IV.

[0031] The first domain, Domain I, comprises about 75 to about 110 amino acid residues. The sequence of this Domain includes at least the sequence of the residues of position 4 through position 75 of HBc. One to three cysteine residues are also present at a position in the chimera molecule of about one to about -20 relative to the N-terminus of HBc of SEQ ID NO: 247 [N-terminal cysteine residue(s)]. The one or more N-terminal cysteine residues are present within a sequence other than that of the pre-core sequence of HBc. Domain I can further include a sequence of about 6 to about 24 residues of an influenza A M2 polypeptide $X_1X_2X_3X_4X_5X_6X_7X_8$ TPIRNE $X_{15}X_{16}X_{17}X_{18}X_{19}X_{20}X_{21}X_{22}X_{23}X_{24}$ of SEQ ID NO: 9 that are peptide-bonded to or within about 15 residues of the N-terminus of the HBc sequence, and whose subscripted X residues are defined hereinafter, as well as one or more HBc residues 1-4.

[0032] The second domain, Domain II, comprises about 10 to about 60 amino acid residues peptide-bonded to residue 75 of Domain I of which (i) zero to all residues in the sequence of HBc positions 76 through 85 are present peptide-bonded to (ii) an optional sequence of about 6 to about 48 residues that constitute one or more repeats of the above influenza A M2 polypeptide of SEQ ID NO: 9.

[0033] The third domain, Domain III, is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85.

[0034] The fourth domain, Domain IV, comprises (i) the residues of positions 136-140 plus up to nine residues of an HBc amino acid residue sequence from position 141 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero to three cysteine residues, (iii) fewer than three arginine or lysine residues, or mixtures thereof adjacent to each other, and (iv) up to about 100 amino acid residues in a sequence heterologous to HBc from position 164 to the C-terminus.

[0035] A contemplated chimera molecule (i) contains no more than 10 percent conservatively substituted amino acid residues in the HBc sequence, (ii) self-assembles into particles that are substantially free of binding to nucleic acids on expression in a host cell, and those particles are more stable on formation than are particles formed from an

otherwise identical HBc chimera that lacks said N-terminal cysteine residue(s) or in which an N-terminal cysteine residue present in the chimera molecule is replaced by another residue.

[0036] It is preferred that the HBc sequence of Domain I include the residues of position 4 through position 75 along plus at least an N-terminal cysteine residue. It is further preferred that a contemplated immunogen contain one cysteine residue within Domain IV alone or in an amino acid residue sequence heterologous to that of HBc from position 164 to the C-terminus. It is particularly preferred that that heterologous sequence comprise a T cell epitope of influenza A.

[0037] Another embodiment comprises an inoculum or vaccine that comprises an above HBc chimera particle that is dissolved or dispersed in a pharmaceutically acceptable diluent composition that typically also contains water. When administered in an immunogenic effective amount to an animal such as a mammal or bird, an inoculum induces antibodies that immunoreact specifically with the chimera particle. The antibodies so induced also immunoreact specifically with (bind to) the N-terminal portion of the M2 protein.

[0038] The present invention has several benefits and advantages.

[0039] A particular benefit of the invention is that its use as a vaccine provides extraordinary antibody titers against influenza A.

[0040] An advantage of the invention is that those very high antibody titers have been produced with the aid of an adjuvant approved for use in humans.

[0041] Another benefit of the invention is that the recombinant immunogen can be prepared easily and in large quantities using well-known cell culture techniques to grow transformed host cells.

[0042] Another advantage of the invention is that the immunogen is easily prepared using well-known recombinant techniques.

[0043] Yet another benefit of the invention is that a preferred immunogen exhibits greater stability at elevated temperatures than to other HBc chimeras.

[0044] Yet another advantage of the invention is that a contemplated immunogen is substantially free of nucleic acids.

[0045] Still further benefits and advantages will be apparent to the worker of ordinary skill from the disclosure that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[0046] In the drawings forming a portion of this disclosure:

[0047] FIG. 1, shown in two panels as FIG. 1A and FIG. 1B, provides an alignment of six published sequences for mammalian HBc proteins from six viruses. The first (SEQ ID NO: 1), human viral sequence is of the ayw subtype and was published in Galibert et al. (1983) *Nature*, 281:646-650; the second human viral sequence (SEQ ID NO: 2), of the adw subtype, was published by Ono et al. (1983) *Nucleic*

Acids Res., 11(6): 1747-1757; the third human viral sequence (SEQ ID NO: 3), is of the adw2 subtype and was published by Valenzuela et al., *Animal Virus Genetics*, Field et al. eds., Academic Press, New York (1980) pages 57-70; the fourth human viral sequence (SEQ ID NO: 4), is of the adyw subtype that was published by Pasek et al. (1979) *Nature*, 282:575-579; the fifth sequence (SEQ ID NO: 5), is that of the woodchuck virus that was published by Galibert et al. (1982) *J. Virol.*, 41:51-65; and the sixth mammalian sequence, (SEQ ID NO: 6), is that of the ground squirrel that was published by Seeger et al. (1984) *J. Virol.*, 51:367-375.

[0048] FIG. 2 shows the modifications made to commercial plasmid vector pKK223-3 in the preparation of plasmid vector pKK223-3N used herein for preparation of recombinant HBc chimeras. The modified sequence (SEQ ID NO: 7) is shown below the sequence of the commercially available vector (SEQ ID NO: 8). The bases of the added NcoI site are shown in lower case letters and the added bases are shown with double underlines, whereas the deleted bases are shown as dashes. The two restriction sites present in this segment of the sequence (NcoI and HindIII) are indicated.

[0049] FIG. 3 is an analytical size exclusion chromatography elution profile for ICC-1603 particles in which absorbance at 280 nm is shown on the ordinate and time in seconds is shown on the abscissa.

[0050] FIG. 4 is an analytical size exclusion chromatography elution profile for ICC-1590 particles as discussed for FIG. 3.

[0051] FIG. 5 is an analytical size exclusion chromatography elution profile for ICC-1560 particles as discussed for FIG. 3.

[0052] FIG. 6 is an analytical size exclusion chromatography elution profile for ICC-1605 particles as discussed for FIG. 3.

[0053] FIG. 7 is an analytical size exclusion chromatography elution profile for ICC-1604 particles as discussed for FIG. 3.

[0054] FIG. 8 is an analytical size exclusion chromatography elution profile for ICC-1438 particles as discussed for FIG. 3.

[0055] FIG. 9 is an analytical size exclusion chromatography elution profile for ICC-1492 particles as discussed for FIG. 3.

[0056] FIG. 10 is a photograph of an SDS-PAGE analysis under reducing conditions following particle preparation that shows the ICC-1438 monomer construct was unstable after aging (Lane 2) as compared to the ICC-1492 construct (Lane 3), with HBc-149 (Lane 1), ICC-1475 (Lane 4) and ICC-1473 (Lane 5) serving as additional molecular weight controls.

DEFINITIONS

[0057] Numerals utilized in conjunction with HBc chimeras indicate the position in the HBc ayw amino acid residue sequence of SEQ ID NO: 1 at which one or more residues has been added to or deleted from the sequence, regardless of whether additions or deletions to the amino acid residue sequence are present. Thus, HBc149 indicates that the chimera ends at residue 149, whereas HBc149+C150 indi-

cates that that same chimera contains a cysteine residue at Hbc position 150 relative to the sequence numbers of SEQ ID NO: 1.

[0058] The term "antibody" refers to a molecule that is a member of a family of glycosylated proteins called immunoglobulins, which can specifically bind to an antigen.

[0059] The word "antigen" has been used historically to designate an entity that is bound by an antibody or receptor, and also to designate the entity that induces the production of the antibody. More current usage limits the meaning of antigen to that entity bound by an antibody or receptor, whereas the word "immunogen" is used for the entity that induces antibody production or binds to the receptor. Where an entity discussed herein is both immunogenic and antigenic, reference to it as either an immunogen or antigen is typically made according to its intended utility.

[0060] "Antigenic determinant" refers to the actual structural portion of the antigen that is immunologically bound by an antibody combining site or T-cell receptor. The term is also used interchangeably with "epitope".

[0061] The word "conjugate" as used herein refers to a hapten operatively linked to a carrier protein, as through an amino acid residue side chain.

[0062] The term "conservative substitution" as used herein denotes that one amino acid residue has been replaced by another, biologically similar residue. Examples of conservative substitutions include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another such as between arginine and lysine, between glutamic and aspartic acids or between glutamine and asparagine and the like.

[0063] The term "corresponds" in its various grammatical forms as used in relation to peptide sequences means the peptide sequence described plus or minus up to three amino acid residues at either or both of the amino- and carboxy-termini and containing only conservative substitutions in particular amino acid residues along the polypeptide sequence.

[0064] The term "Domain" is used herein to mean a portion of a recombinant Hbc chimera molecule that is identified by (i) residue position numbering relative to the position numbers of HBcAg subtype ayw as reported by Galibert et al., (1979) *Nature*, 281:646-650 (SEQ ID NO: 1). The polypeptide portions of at least chimera Domains I, II and III are believed to exist in a similar tertiary form to the corresponding sequences of naturally occurring HBcAg.

[0065] As used herein, the term "fusion protein" designates a polypeptide that contains at least two amino acid residue sequences not normally found linked together in nature that are operatively linked together end-to-end (head-to-tail) by a peptide bond between their respective carboxy- and amino-terminal amino acid residues. The fusion proteins of the present invention are Hbc chimera molecules that induce the production of antibodies that immunoreact with a polypeptide that corresponds in amino acid residue sequence to the polypeptide portion of the fusion protein.

[0066] The phrase "hepatitis B" as used here refers in its broadest context to any member of the family of mammalian hepadnaviridae, as discussed before.

[0067] The words "polypeptide" and "peptide" are used interchangeably throughout the specification and designate a linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent amino acids. Polypeptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms that are salts. It is well understood in the art that amino acid residue sequences contain acidic and basic groups, and that the particular ionization state exhibited by the peptide is dependent on the pH value of the surrounding medium when the peptide is in solution, or that of the medium from which it was obtained if the peptide is in solid form. Thus, "polypeptide" or its equivalent terms is intended to include the appropriate amino acid residue sequence referenced. A peptide or polypeptide is always shown herein from left to right and in the direction from amino-terminus (N-terminus) to carboxy-terminus (C-terminus).

[0068] The term "residue" is used interchangeably with the phrase amino acid residue. All amino acid residues identified herein are in the natural or L-configuration. In keeping with standard polypeptide nomenclature, [*J. Biol. Chem.*, 243, 3557-59 (1969)], abbreviations for amino acid residues are as shown in the following Table of Correspondence.

TABLE OF CORRESPONDENCE

1-Letter	3-Letter	AMINO ACID
Y	Tyr	L-tyrosine
G	Gly	glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine
V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
Z	Glx	L-glutamic acid or L-glutamine
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
B	Asx	L-aspartic acid or L-asparagine
C	Cys	L-cysteine

DETAILED DESCRIPTION OF THE INVENTION

[0069] The present invention contemplates an immunogen and a vaccine or inoculum comprising that immunogen against the influenza A virus. A contemplated immunogen is a particle comprised of recombinant hepatitis B virus core (Hbc) protein chimera molecules with a length of about 150 to about 325 and preferably about 155 to 225 amino acid residues that contains four peptide-linked amino acid residue sequence domains from the N-terminus that are denomi-

nated Domains I, II, III and IV. At least one polypeptide containing 6 to about 24 residues of the influenza A M2 polypeptide of SEQ ID NO: 9, as defined hereinbelow, is present peptide-bonded to the chimer molecule.

[0070] (a) Domain I comprises about 71 to about 110 amino acid residues whose sequence includes at least the sequence of the residues of position 5 through position 75 of HBc. One to three cysteine residues is(are) also present at a position in the chimer molecule of about one to about -20 relative to the N-terminus of HBc of SEQ ID NO: 247 [N-terminal cysteine residue(s)]. The one or more N-terminal cysteine residues is(are) present within a sequence other than that of the pre-core sequence of HBc.

[0071] Domain I can, and preferably does, further include a (i) sequence of 6 to about 24 residues of an above-noted influenza A M2 polypeptide $X_1X_2X_3X_4X_5X_6X_7X_8$ TPIRNX₁₅X₁₆X₁₇X₁₈X₁₉X₂₀X₂₁X₂₂X₂₃X₂₄ of SEQ ID NO: 9 that are peptide-bonded to or within about 15 residues of the N-terminus of the HBc sequence, as well as (ii) one or more HBc residues 1-4. In that influenza A M2 polypeptide sequence,

[0072] residues X_1 through X_8 are absent or present, and when present are the residues naturally present in the M2 protein sequence that are methionine, serine, leucine, leucine, threonine, glutamic acid, valine, and glutamic acid, respectively, with the proviso that when one subscripted X residue is present, any remaining subscripted X with a higher subscript number up to 8 is also present,

[0073] residues X_{15} and X_{16} are present or absent, and when present are tryptophan and glycine, respectively,

[0074] residues X_{17} and X_{19} are present or absent, and when present are independently cysteine, serine, or alanine,

[0075] residue X_{18} is present or absent, and when present is arginine, and

[0076] residues X_{20} through X_{24} are present or absent, and when present are the residues naturally present in the M2 protein sequence that are asparagine, aspartic acid, serine, serine and aspartic acid respectively, with the proviso that when one subscripted X residue is present, any remaining subscripted X residue with a lower subscript number down to 15 is also present.

[0077] (b) Domain II comprises about 10 to about 60 amino acid residues peptide-bonded to residue 75. This sequence includes (i) zero to all of the residues of a sequence of HBc from HBc position 76 through 85 peptide-bonded to (ii) an optional sequence of about 6 to about 48 residues that constitute one or more repeats of the above influenza A M2 polypeptide of SEQ ID NO: 9.

[0078] (c) Domain III is an HBc sequence from position 86 through position 135 that is peptide-bonded to residue 85.

[0079] d) Domain IV comprises (i) the residues of positions 136-140 plus up to nine residues of an HBc amino acid residue sequence from position 141 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero to three cysteine residues, (iii) fewer than three arginine or

lysine residues, or mixtures thereof adjacent to each other, and (iv) up to about 100 amino acid residues in a sequence heterologous to HBc from position 164 to the C-terminus. Thus, Domain IV contains at least the 5 residues of positions 136-140.

[0080] A contemplated chimer molecule (i) contains up to about 10 percent conservatively substituted amino acid residues in the HBc sequence, (ii) self-assembles into particles that are substantially free of binding to nucleic acids on expression in a host cell, and those particles are more stable on formation than are particles formed from an otherwise identical HBc chimer that lacks said N-terminal cysteine residue(s) or in which an N-terminal cysteine residue present in the chimer molecule is replaced by another residue.

[0081] In examining the length of a contemplated HBc chimer, such a recombinant protein can have a length of about 150 to about 325 amino acid residues. Preferably, that length is about 155 to about 225 residues. More preferably, the length is about 155 to about 170 residues. These differences in length arise from changes in the length of Domains I, II and IV.

[0082] HBc chimers having a Domain I that contains more than a deletion of the first three amino-terminal (N-terminal) residues have been reported to result in the complete disappearance of HBc chimer protein in *E. coli* cells. Pumpens et al., (1995) *Intervirology*, 38:63-74. On the other hand, a recent study in which an immunogenic 23-mer polypeptide from the influenza M2 protein was fused to the HBc N-terminal sequence reported that the resultant fusion protein formed particles when residues 1-4 of the native HBc sequence were replaced. Neirynck et al. (October 1999) *Nature Med.*, 5(10):1157-1163. Thus, the art teaches that particles can form when an added amino acid sequence is present peptide-bonded the one of residues 1-5 of HBc, whereas particles do not form if no additional sequence is present and more than residues 1-3 are deleted from the N-terminus of HBc.

[0083] An N-terminal sequence peptide-bonded to one of the first five N-terminal residues of HBc can contain a sequence of up to about 40 residues that are heterologous to HBc; i.e., a portion of a pre-core sequence can be present in a contemplated chimer molecule. Exemplary sequences include an influenza A B cell or T cell epitope such as are discussed hereinafter, a sequence of another (heterologous) protein such as β -galactosidase as can occur in fusion proteins as a result of the expression system used, or another hepatitis B-related sequence such as that from the Pre-S1 or Pre-S2 regions or the major HbsAg immunogenic sequence.

[0084] Domain I preferably has the sequence of residues of positions 2-, 3- or 4- through 75 of HBc. Domain I also contains one to three, preferably one, added cysteine residue(s) and also preferably includes about 6 to about 24 residues of the sequence of the extracellular region of the influenza A M2 protein peptide-bonded at the amino-terminus as discussed herein below. Domain I therefore contains a deletion of at least the methionine residue of position 1 of HBc and can include deletions of the residues at positions 2, 3 and 4.

[0085] The one or more cysteine residues present in Domain I is(are) located at a position in the chimer molecule

of about one to about -20 relative to the N-terminus of HBc of SEQ ID NO: 1 [N-terminal cysteine residue(s)]. Thus, using the sequence of SEQ ID NO: 1 as a reference point, the N-terminal cysteine residue(s) is located in the chimer molecule at a position that corresponds to the methionine at position 1 of SEQ ID NO: 1 (FIG. 1), or at a position up to about 20 residues downstream from that position. More preferably, an N-terminal cysteine is located at a position of about one to about minus 14 relative to position 1 of SEQ ID NO: 1.

[0086] The one or more N-terminal cysteine residues are present within a sequence other than that of the pre-core sequence of HBc. As was noted previously, the HBcAg molecule contains the pre-core sequence that includes a cysteine residue. That molecule does not form particles, whereas particles are desired herein. Thus, although an N-terminal cysteine residue can be adjacent to a pre-core sequence, such a residue is not present within a pre-core sequence or a contemplated chimer molecule.

[0087] Domain I can have a length of about 110 residues. Preferably, Domain I has a length of about 95 to about 100 amino acid residues, and includes an influenza A M2 polypeptide epitope sequence of SEQ ID NO: 9, that preferably includes the C-terminal 23 residues.

[0088] Domain II, which is peptide-bonded to residue 75, contains about 10 to about 60 amino acid residues. This Domain includes zero through all of the sequence of HBc residues of positions 76 through 85. Domain II also optionally includes a sequence of about 6 to about 48 residues that constitute one or more repeats of the before-mentioned influenza A M2 polypeptide of SEQ ID NO: 9. The influenza A M2 polypeptide sequence, when present, is preferably peptide-bonded between HBc residues 78 and 79, and all of the HBc sequence from position 76 through 85 is present.

[0089] Preferred influenza A M2 polypeptide sequences for insertion into Domains I or II, or both, of a contemplated recombinant HBc chimer are enumerated in Table A, below. A sequence beginning with a methionine residue (M) is designed to be N-terminal sequence for insertion into the N-terminus of Domain I, whereas a sequence free of an N-terminal M residue is designed for insertion into Domain II.

TABLE A

Influenza A M2 Protein B Cell Epitopes	
Sequence	SEQ ID NO
SLLTEVETPIRNEWGCRNGSSD	10
SLLTEVETPIRNEWGCRNDSSD	11
SLLTEVETPIRNEWGARNDSSD	12
SLLTEVETPIRNEWGSRSDSSD	13
SLLTEVETPIRNEWGSRCDSSD	14
SLLTEVETPIRNEWGCRSDSSD	15
SLLTEVETPIRNEWGCRANDSSD	16
SLLTEVETPIRNEWGARCNDSSD	17
MSLLTEVETPIRNEWGCRCDSSD	18

TABLE A-continued

Influenza A M2 Protein B Cell Epitopes	
Sequence	SEQ ID NO
MSLLTEVETPIRNEWGSRSDSSD	19
MGISLLTEVETPIRNEWGCRCDSSDELGLWLGI	20
MSLLTEVETPIRNEWGARNDSSD	21
MSLLTEVETPIRNEWGCRANDSSD	22
MSLLTEVETPIRNEWGARCNDSSD	23
MSLLTEVETPIRNEWGCRSDSSD	24
MSLLTEVETPIRNEWGSRCDSSD	25
X ₁ X ₂ X ₃ X ₄ X ₅ X ₆ X ₇ X ₈ TPIRNX ₁₅ X ₁₆ X ₁₇ X ₁₈ X ₁₉ X ₂₀ - X ₂₁ X ₂₂ X ₂₃ X ₂₄	9
Influenza B Protein NNATFNNTNVNPISHIR	83

[0090] In the polypeptide of SEQ ID NO: 9, X₁ through X₈ are absent or present, and when present are the residues naturally present in the reported M2 protein sequence; i.e., methionine, serine, leucine, leucine, threonine, glutamic acid, valine, and glutamic acid, respectively, with the proviso that when one subscripted X is present, any remaining subscripted X residue with a higher subscript number up to 8 is also present. Thus, when X₁ is present, each of X₂ through X₈ is also present. Similarly, when X₃ is present, each of X₄ through X₈ is also present, and the like. On the other hand, X₈ can be present without any other of the remaining X residues having a lower valued subscript number being present. The residues X₁₅ and X₁₆ are present or absent, and when present are tryptophan and glycine, respectively. Residues X₁₇ and X₁₉ are present or absent, and when present are independently cysteine, serine, or alanine. It is preferred that one of X₁₇ and X₁₉ be cysteine, particularly when an M2 polypeptide epitope is present at the N-terminus of the chimer molecule. Residue X₁₈ is present or absent, and when present is arginine. Residues X₂₀ through X₂₄ are present or absent, and when present are the residues naturally present in the reported M2 protein sequence; i.e., asparagine, aspartic acid, serine, serine and aspartic acid respectively, with the proviso that when one subscripted X is present, any remaining X residue with a lower subscript number through 15 is also present. Thus, for example, when X₂₃ is present, so are each of residues X₁₅ through X₂₂.

[0091] Domain III contains the sequence of HBc position 86 through position 135 peptide-bonded at its N-terminus to residue 85.

[0092] The fourth domain, Domain IV, comprises (i) the residues of positions 136 through 140 plus up to nine residues of an HBc amino acid residue sequence from position 141 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero to three cysteine residues, and preferably one cysteine residue, (iii) fewer than three arginine or lysine residues, or mixtures thereof adjacent to each other, and (iv) up to about 100 amino acid residues, preferably up to 50 amino acid residues, and more

preferably up to about 25 residues, in a sequence heterologous to HBc from position 164 to the C-terminus.

[0093] It is preferred that Domain IV contain up to fourteen residues of an HBc sequence from position 136 through position 149 peptide-bonded to residue 135; i.e., an HBc sequence that begins with the residue of position 136 that can continue through position 149. Thus, if the residue of position 148 is present, so is the sequence of residues of positions 136 through 147, or if residue 141 is present, so is the sequence of residues of positions 136 through 140.

[0094] Domain IV can also contain zero to three cysteine residues and those Cys residues are present within about 30 residues of the carboxy-terminus (C-terminus) of the chimer molecule. Preferably, one cysteine (Cys) residue is present, and that Cys is preferably present as the carboxy-terminal (C-terminal) residue, unless an influenza T cell epitope is present as part of Domain IV. When such a T cell epitope is present, the preferred Cys is preferably within the C-terminal last five residues of the HBc chimera.

[0095] The presence of the above-discussed N-terminal cysteine residue(s) provides an unexpected enhancement of the ability of the chimera molecules to form stable immunogenic particles (discussed hereinafter). Thus, a contemplated HBc chimera immunogen tends to form particles that stay together upon collection and initial purification as measured by analytical size exclusion chromatography, whose details are discussed hereinafter.

[0096] The contemplated particles can also be more stable to decomposition at 37° C. after aging than are similar chimera particles lacking that cysteine residue. This latter type of enhanced stability can be measured using 15% SDS-PAGE gels with particles dispersed in sample buffer (reducing). Gels are stained using Coomassie Blue, and then analyzed. This type of stability is believed to be exhibited against hydrolysis, whereas the stability determined by size exclusion chromatography is that of initial particle formation.

[0097] Particles that additionally contain one or more C-terminal cysteine residues exhibit enhanced stability in formation and also toward decomposition on aging, with some particles containing both N- and C-terminal cysteines usually exhibiting greater stability in either measure than those particles having only an added cysteine at either the N- or C-terminus.

[0098] Domain IV contains fewer than three arginine or lysine residues, or mixtures thereof adjacent to each other. Arginine and lysines are present in the C-terminal region of HBc that extends from position 164 through the C-terminus of the native molecule. That region is sometimes referred to as the "protamine" or "arginine-rich" region of the molecule and binds nucleic acids. A contemplated HBc chimera molecule and particle are substantially free of bound nucleic acids.

[0099] The substantial freedom of nucleic acid binding can be readily determined by a comparison of the absorbance of the particles in aqueous solution measured at both 280 and 260 nm; i.e., a 280/260 absorbance ratio. The contemplated particles do not bind substantially to nucleic acids that are oligomeric and/or polymeric DNA and RNA species originally present in the cells of the organism used to express the protein. Such nucleic acids exhibit an absor-

bance at 260 nm and relatively less absorbance at 280 nm, whereas a protein such as a contemplated chimera absorbs relatively less at 260 nm and has a greater absorbance at 280 nm.

[0100] Thus, recombinantly expressed HBc particles or chimeric HBc particles that contain the arginine-rich sequence at residue positions 150-183 (or 150-185) exhibit a ratio of absorbance at 280 nm to absorbance at 260 nm (280:260 absorbance ratio) of about 0.8, whereas particles free of the arginine-rich nucleic acid binding region of naturally occurring HBc such as those that contain fewer than three arginine or lysine residues or mixtures thereof adjacent to each other, or those having a native or chimeric sequence that ends at about HBc residue position 140 to position 149, exhibit a 280:260 absorbance ratio of about 1.2 to about 1.6.

[0101] Chimeric HBc particles of the present invention are substantially free of nucleic acid binding and exhibit a 280:260 absorbance ratio of about 1.2 to about 1.6, and more typically, about 1.4 to about 1.6. This range is due in large part to the number of aromatic amino acid residues present in Domains II and IV of a given chimeric HBc particle. That range is also in part due to the presence of the Cys in Domain IV of a contemplated chimera, whose presence can diminish the observed ratio by about 0.1 for a reason that is presently unknown.

[0102] The contemplated chimera HBc particles are more stable in aqueous buffer at 37° C. over a time period of about two weeks to about one month than are particles formed from a HBc chimera containing the same peptide-linked Domain II, III and IV sequences and an otherwise same Domain I sequence in which the one to three cysteine residues [N-terminal cysteine residue(s)] are absent or a single N-terminal residue present is replaced by another residue such as an alanine residue.

[0103] Thus, for example, particles containing an influenza A M2 polypeptide in Domain I [e.g. ICC-1590 particles] that include two cysteine residues is more stable than otherwise identical particles [ICC-1603 particles] assembled from chimera molecules whose N-terminal M2 variant sequence contains serine residues in place of the cysteines. Similarly, particles containing the above influenza B cell epitope in Domain I and a single cysteine at the C-terminus [ICC-1605 particles] are more stable than are otherwise identical particles in which that cysteine is absent, but are less stable than are the particles containing the two N-terminal cysteines, ICC-1590 particles or those particles that contained both N-terminal and C-terminal cysteines [ICC-1604 particles].

[0104] A contemplated particle containing a N-terminal cysteine residue is also typically prepared in greater yield than is a particle assembled from a chimera molecule lacking a N-terminal cysteine. This increase in yield can be seen from the mass of particles obtained or from integration of traces from analytical gel filtration analysis using Superose® 6 HR as discussed hereinafter.

[0105] Although the T cell help afforded by HBc is highly effective in enhancing antibody responses (i.e. B cell-mediated) to 'carried' epitopes following vaccination, HBc does not activate influenza-specific T cells, except in restricted individuals for whom the B cell epitope is also a T cell

epitope. To help ensure universal priming of influenza-specific T helper cells, in addition to B cells, one or more influenza-specific T helper epitopes is preferably incorporated into a contemplated immunogen and is located in Domain IV of the immunogen.

[0106] A plurality of the above or another T cell epitopes can be present in Domain IV or another B cell epitope can be present. In preferred practice, Domain IV has up to about 50 residues in a sequence heterologous to HBc. More preferably, that sequence is up to about 25 residues and includes a T cell epitope.

[0107] A contemplated recombinant HBc chimer molecule is typically present and is used in an immunogen or vaccine as a self-assembled particle. These particles are comprised of 180 to 240 chimer molecules that separate into protein molecules in the presence of disulfide reducing agents such as 2-mercaptoethanol, and the individual molecules are therefore thought to be bound together into the particle primarily by disulfide bonds. These particles are similar to the particles observed in patients infected with HBV, but these particles are non-infectious. Upon expression in various prokaryotic and eukaryotic hosts, the individual recombinant HBc chimer molecules assemble in the host into particles that can be readily harvested from the host cells.

[0108] In addition to the before-discussed N- and C-truncations and insertion of influenza M2 polypeptide epitopes, a contemplated chimer molecule can also contain conservative substitutions in the amino acid residues that constitute HBc Domains I, II, III and IV. Conservative substitutions are as defined before.

[0109] More rarely, a "nonconservative" change, e.g., replacement of a glycine with a tryptophan is contemplated. Analogous minor variations can also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity can be found using computer programs well known in the art, for example LASERGENE software (DNASTAR Inc., Madison, Wis.)

[0110] The HBc portion of a chimer molecule of the present invention [the portion having the HBc sequence that has other than a sequence of an added epitope, or heterologous residue(s) that are a restriction enzyme artifact] most preferably has the amino acid residue sequence at positions 2 through 149 of subtype ayw that is shown in FIG. 1 (SEQ ID NO: 1), when present. Somewhat less preferred are the corresponding amino acid residue sequences of subtypes adw, adw2 and adyw that are also shown in FIG. 1 (SEQ ID NOS: 2, 3 and 4). Less preferred still are the sequences of woodchuck and ground squirrel at aligned positions 2 through 149 that are the last two sequences of FIG. 1 (SEQ ID NOS: 5 and 6). As noted elsewhere, portions of different sequences from different mammalian HBc proteins can be used together in a single chimer.

[0111] When the HBc portion of a chimer molecule of the present invention has other than a sequence of a mammalian HBc molecule at positions 2 through 149, when present, because one or more conservative substitutions has been made, it is preferred that no more than 10 percent, and more preferably no more than 5 percent, and most preferably no more than 3 percent of the amino acid residues are substituted as compared to SEQ ID NO: 1 from position 2 through

149. A contemplated chimer of 149 HBc residues can therefore contain up to about 15 residues that are different from those of SEQ ID NO: 1 at positions 2 through 149, and preferably about 7 or 8 residues. More preferably, up to about 5 residues are different from the ayw sequence (SEQ ID NO: 1) at residue positions 2-149. Where an HBc sequence is truncated further at one or both termini, the number of substituted residues is proportionally different. Deletions elsewhere in the molecule are considered conservative substitutions for purposes of calculation.

[0112] Chimer Preparation

[0113] A contemplated chimeric immunogen is prepared using the well-known techniques of recombinant DNA technology. Thus, sequences of nucleic acid that encode particular polypeptide sequences are added and deleted from the precursor sequence that encodes HBV.

[0114] A contemplated chimeric immunogen typically utilizes a cysteine residue present in the M2 sequence as the N-terminal cysteine. Primers for the preparation of such chimer molecules by in vitro mutagenesis of a polynucleotide encoding an HBc molecule are discussed hereinafter. When a cysteine-containing M2 polypeptide epitope is not present at the N-terminus, the N-terminal cysteine can be provided by in vitro mutagenesis using a primer that encodes just a cysteine-containing portion of the M2 polypeptide or a simple N-terminal start sequence such as Met-Cys- or Met-Gly-Cys-.

[0115] As was noted previously, the HBc immunodominant loop is usually recited as being located at about positions 75 through 85 from the amino-terminus (N-terminus) of the intact protein. The influenza A M2 B cell epitope-containing sequence can be placed into that immunodominant loop sequence of Domain II. That placement substantially eliminates the HBc immunogenicity and antigenicity of the HBc loop sequence, while presenting the influenza A M2 B cell epitope in an extremely immunogenic position in the assembled chimer particles.

[0116] One of two well-known strategies is particularly useful for placing the influenza A M2 B cell sequence into the loop sequence at a desired location such as between residues 78 and 79. A first, less successful strategy is referred to as replacement in which DNA that codes for a portion of the loop is excised and replaced with DNA that encodes an influenza A M2 B cell sequence. The second strategy is referred to as insertion in which an influenza A M2 B cell sequence is inserted between adjacent residues in the loop.

[0117] Site-directed mutagenesis using the polymerase chain reaction (PCR) is used in one exemplary replacement approach to provide a chimeric HBc DNA sequence that encodes a pair of different restriction sites, e.g. EcoRI and SacI, one near each end of the immunodominant loop-encoding DNA. Exemplary residues replaced are 76 through 81. The loop-encoding section is excised, an influenza A M2 B cell epitope-encoding sequence flanked on each side by appropriate HBc sequence residues is ligated into the restriction sites and the resulting DNA is used to express the HBc chimer. See, for example, Table 2 of Pumpens et al., (1995) *Intervirology*, 38:63-74 for exemplary uses of a similar technique.

[0118] Alternatively, a single restriction site or two sites can be encoded into the region, the DNA cut with a

restriction enzyme(s) to provide "sticky" or ends, and an appropriate sticky- or blunt-ended heterologous DNA segment ligated into the cut region. Examples of this type of sequence replacement into HBc can be found in the work reported in Schodel et al., (1991) F. Brown et al. eds., *Vaccines 91*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp.319-325, Schodel et al., *Behring Inst. Mitt.*, 1997(98): p. 114-119 and Schodel et al., *J. Exp. Med.*, (1994) 180(3): p. 1037-4, the latter two papers discussing the preparation of vaccines against malarial pathogens *P. yoelii* and *P. berghei*, respectively. A replacement strategy that results in a net removal of residues from the immunodominant loop is usually not used herein.

[0119] Insertion is preferred. In an illustrative example of the insertion strategy, site-directed mutagenesis is used to create two restriction sites adjacent to each other and between codons encoding adjacent amino acid residues, such as those at residue positions 78 and 79. This technique adds twelve base pairs that encode four amino acid residues (two for each restriction site) between formerly adjacent residues in the HBc loop.

[0120] Upon cleavage with the restriction enzymes, ligation of the DNA coding for the influenza A M2 sequence and expression of the DNA to form HBc chimeres, the HBc loop amino acid sequence is seen to be interrupted on its N-terminal side by the two residues encoded by the 5' restriction site, followed toward the C-terminus by the influenza A M2 B-cell epitope sequence, followed by two more heterologous, non-loop residues encoded by the 3' restriction site and then the rest of the loop sequence. This same strategy is also preferably used for insertion into Domain IV of a T cell epitope or one or more cysteine residues that are not a part of a T cell epitope. A similar strategy using an insertion between residues 82 and 83 is reported in Schoedel et al., (1990) F. Brown et al. eds., *Vaccines 90*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp.193-198.

[0121] For example, a DNA sequence that encodes a C-terminal truncated HBc sequence (HBc149) is engineered to contain adjacent EcoRI and SacI sites between residues 78 and 79. Cleavage of that DNA with both enzymes provides one fragment that encodes HBc positions 1-78 3'-terminated with an EcoRI sticky end, whereas the other fragment has a 5'-terminal SacI sticky end and encodes residues of positions 79-149. Ligation of a synthetic nucleic acid having a 5' AATT overhang followed by a sequence that encodes a desired influenza A M2 B cell epitope and a AGCT 3' overhang provides a HBc chimer sequence that encodes that B cell epitope flanked on each side by two heterologous residues (GI and EL, respectively) between residues 78 and 79, while destroying the EcoRI site and preserving the SacI site.

[0122] A similar strategy can be used for insertion of a C-terminal cysteine-containing sequence. Here, EcoRI and HindIII restriction sites are engineered in to the HBc DNA sequence after amino acid residue position 149. After digestion with EcoRI and HindIII, a synthetic DNA having the above AATT 5' overhang followed by a T cell epitope-encoding sequence, a stop codon and a 3' AGCT overhang were ligated into the digested sequence to form a sequence that encoded HBc residues 1-149 followed by two heterologous residues (GI), the stop codon and the HindIII site.

[0123] PCR amplification using a forward primer having a SacI restriction site followed by a sequence encoding HBc

beginning at residue position 79, followed by digestion with SacI and HindIII provided a sequence encoding HBc positions 79-149 plus the two added residues and the T cell epitope at the C-terminus. Digestion of that construct with SacI and ligation provides the complete gene encoding a desired recombinant HBc chimer immunogen having the sequence, from the N-terminus, of HBc positions 1-78, two added residues, the influenza A M2 B cell epitope, two added residues, HBc positions 79-149, two added residues, and the T cell epitope.

[0124] It is noted that the preferred use of two heterologous residues on either side of (flanking) a B cell or T cell epitope is a matter of convenience. As a consequence, one can also use zero to three or more added residues that are not part of the HBc sequence on either or both sides of an inserted sequence. One or both ends of the insert and HBc nucleic acid can be "chewed back" with an appropriate nuclease (e.g. S1 nuclease) to provide blunt ends that can be ligated together. Added heterologous residues that are neither part of the inserted B cell or T cell epitopes nor a part of the HBc sequence are not counted in the number of residues present in a recited Domain.

[0125] It is also noted that one can also synthesize all or a part of a desired recombinant HBc chimer nucleic acid using well-known synthetic methods as is discussed and illustrated in U.S. Pat. No.5,656,472 for the synthesis of the 177 base pair DNA that encodes the 59 residue ribulose bis-phosphate carboxylase-oxygenase signal peptide of *Nicotiana tabacum*. For example, one can synthesize Domains I and II with a blunt or "sticky" end that can be ligated to Domains III and IV to provide a construct that expresses a contemplated HBc chimer that contains zero added residues to the N-terminal side of the B cell epitope and zero to three added residues on the C-terminal side or at the Domain II/III junction or at some other desired location.

[0126] A nucleic acid sequence (segment) that encodes a previously described HBc chimer molecule or a complement of that coding sequence is also contemplated herein. Such a nucleic acid segment is present in isolated and purified form in some preferred embodiments.

[0127] In living organisms, the amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid (DNA) sequence of the gene that codes for the protein. Thus, through the well-known degeneracy of the genetic code additional DNAs and corresponding RNA sequences (nucleic acids) can be prepared as desired that encode the same chimer amino acid residue sequences, but are sufficiently different from a before-discussed gene sequence that the two sequences do not hybridize at high stringency, but do hybridize at moderate stringency.

[0128] High stringency conditions can be defined as comprising hybridization at a temperature of about 50°-55° C. in 6×SSC and a final wash at a temperature of 68° C. in 1-3×SSC. Moderate stringency conditions comprise hybridization at a temperature of about 50° C. to about 65° C. in 0.2 to 0.3 M NaCl, followed by washing at about 50° C. to about 55° C. in 0.2×SSC, 0.1% SDS (sodium dodecyl sulfate).

[0129] A nucleic sequence (DNA sequence or an RNA sequence) that (1) itself encodes, or its complement encodes,

a chimera molecule whose HBc portion from residue position 1 through 136, when present, is that of SEQ ID NOS: 1, 2, 3, 4, 5 or 6 and (2) hybridizes with a DNA sequence of SEQ ID NOS: 77, 78, 79, 80, 81 or 82 at least at moderate stringency (discussed above); and (3) whose HBc sequence shares at least 80 percent, and more preferably at least 90 percent, and even more preferably at least 95 percent, and most preferably 100 percent identity with a DNA sequence of SEQ ID NOS: 77, 78, 79, 80, 81 and 82, is defined as a DNA variant sequence. As is well-known, a nucleic acid sequence such as a contemplated nucleic acid sequence is expressed when operatively linked to an appropriate promoter in an appropriate expression system as discussed elsewhere herein.

[0130] An analog or analogous nucleic acid (DNA or RNA) sequence that encodes a contemplated chimera molecule is also contemplated as part of this invention. A chimera analog nucleic acid sequence or its complementary nucleic acid sequence encodes a HBc amino acid residue sequence that is at least 80 percent, and more preferably at least 90 percent, and most preferably is at least 95 percent identical to the HBc sequence portion from residue position 1 through residue position 136 shown in SEQ ID NOS: 1, 2, 3, 4, 5 and 6. This DNA or RNA is referred to herein as an "analog of" or "analogous to" a sequence of a nucleic acid of SEQ ID NOS: 77, 78, 79, 80, 81 and 82, and hybridizes with the nucleic acid sequence of SEQ ID NOS: 77, 78, 79, 80, 81 and 82 or their complements herein under moderate stringency hybridization conditions. A nucleic acid that encodes an analogous sequence, upon suitable transfection and expression, also produces a contemplated chimera.

[0131] Different hosts often have preferences for a particular codon to be used for encoding a particular amino acid residue. Such codon preferences are well known and a DNA sequence encoding a desired chimera sequence can be altered, using in vitro mutagenesis for example, so that host-preferred codons are utilized for a particular host in which the enzyme is to be expressed. In addition, one can also use the degeneracy of the genetic code to encode the HBc portion of a sequence of SEQ ID NOS: 1, 2, 3, 4, 5 or 6 that avoids substantial identity with a DNA of SEQ ID NOS: 77, 78, 79, 80, 81 or 82 or their complements. Thus, a useful analogous DNA sequence need not hybridize with the nucleotide sequences of SEQ ID NOS: 77, 78, 79, 80, 81 or 82 or a complement under conditions of moderate stringency, but can still provide a contemplated chimera molecule.

[0132] A recombinant nucleic acid molecule such as a DNA molecule, comprising a vector operatively linked to an exogenous nucleic acid segment (e.g., a DNA segment or sequence) that defines a gene that encodes a contemplated chimera, as discussed above, and a promoter suitable for driving the expression of the gene in a compatible host organism, is also contemplated in this invention. More particularly, also contemplated is a recombinant DNA molecule that comprises a vector comprising a promoter for driving the expression of the chimera in host organism cells operatively linked to a DNA segment that defines a gene for the HBc portion of a chimera or a DNA variant that has at least 90 percent identity to the chimera gene of SEQ ID NOS: 77, 78, 79, 80, 81 or 82 and hybridizes with that gene under moderate stringency conditions.

[0133] Further contemplated is a recombinant DNA molecule that comprises a vector containing a promoter for

driving the expression of a chimera in host organism cells operatively linked to a DNA segment that is an analog nucleic acid sequence that encodes an amino acid residue sequence of a HBc chimera portion that is at least 80 percent identical, more preferably 90 percent identical, and most preferably 95 percent identical to the HBc portion of a sequence of SEQ ID NOS: 1, 2, 3, 4, 5 or 6. That recombinant DNA molecule, upon suitable transfection and expression in a host cell, provides a contemplated chimera molecule.

[0134] It is noted that because of the 30 amino acid residue N-terminal sequence of ground squirrel HBc does not align with any of the other HBc sequences, that sequence and its encoding nucleic acid sequences and their complements are not included in the above percentages of identity, nor are the portions of nucleic acid that encode that 30-residue sequence or its complement used in hybridization determinations. Similarly, sequences that are truncated at either or both of the HBc N- and C-termini are not included in identity calculations, nor are those sequences in which residues of the immunodominant loop are removed for insertion of a heterologous epitope. Thus, only those HBc-encoding bases or HBc sequence residues that are present in a chimera molecule are included and compared to an aligned nucleic acid or amino acid residue sequence in the identity percentage calculations.

[0135] Inasmuch as the coding sequences for the gene disclosed herein is illustrated in SEQ ID NOS: 77, 78, 79, 80, 81 and 82, isolated nucleic acid segments, preferably DNA sequences, variants and analogs thereof can be prepared by in vitro mutagenesis, as is well known in the art and discussed in *Current Protocols In Molecular Biology*, Ausubel et al. eds., John Wiley & Sons (New York: 1987) p. 8.1.1-8.1.6, that begin at the initial ATG codon for a gene and end at or just downstream of the stop codon for each gene. Thus, a desired restriction site can be engineered at or upstream of the initiation codon, and at or downstream of the stop codon so that other genes can be prepared, excised and isolated.

[0136] As is well known in the art, so long as the required nucleic acid, illustratively DNA sequence, is present, (including start and stop signals), additional base pairs can usually be present at either end of the segment and that segment can still be utilized to express the protein. This, of course, presumes the absence in the segment of an operatively linked DNA sequence that represses expression, expresses a further product that consumes the enzyme desired to be expressed, expresses a product that consumes a wanted reaction product produced by that desired enzyme, or otherwise interferes with expression of the gene of the DNA segment.

[0137] Thus, so long as the DNA segment is free of such interfering DNA sequences, a DNA segment of the invention can be about 500 to about 15,000 base pairs in length. The maximum size of a recombinant DNA molecule, particularly an expression vector, is governed mostly by convenience and the vector size that can be accommodated by a host cell, once all of the minimal DNA sequences required for replication and expression, when desired, are present. Minimal vector sizes are well known. Such long DNA segments are not preferred, but can be used.

[0138] DNA segments that encode the before-described chimera can be synthesized by chemical techniques, for

example, the phosphotriester method of Matteucci et al. (1981) *J. Am. Chem. Soc.*, 103:3185. Of course, by chemically synthesizing the coding sequence, any desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence. However, DNA segments including sequences discussed previously are preferred.

[0139] A contemplated HBc chimer can be produced (expressed) in a number of transformed host systems, typically host cells although expression in acellular, in vitro, systems is also contemplated. These host cellular systems include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g. baculovirus); plant cell systems transformed with virus expression vectors (e.g. cauliflower mosaic virus; tobacco mosaic virus) or with bacterial expression vectors (e.g., Ti plasmid); or appropriately transformed animal cell systems such as CHO or COS cells. The invention is not limited by the host cell employed.

[0140] DNA segments containing a gene encoding the HBc chimer are preferably obtained from recombinant DNA molecules (plasmid vectors) containing that gene. Vectors capable of directing the expression of a chimer gene into the protein of a HBc chimer is referred to herein as an "expression vector".

[0141] An expression vector contains expression control elements including the promoter. The chimer-coding gene is operatively linked to the expression vector to permit the promoter sequence to direct RNA polymerase binding and expression of the chimer-encoding gene. Useful in expressing the polypeptide coding gene are promoters that are inducible, viral, synthetic, constitutive as described by Poszkowski et al. (1989) *EMBO J.*, 3:2719 and Odell et al. (1985) *Nature*, 313:810, as well as temporally regulated, spatially regulated, and spatiotemporally regulated as given in Chua et al. (1989) *Science*, 244:174-181.

[0142] One preferred promoter for use in prokaryotic cells such as *E. coli* is the Rec 7 promoter that is inducible by exogenously supplied nalidixic acid. A more preferred promoter is present in plasmid vector JHEX25 (available from Promega) that is inducible by exogenously supplied isopropyl- β -D-thiogalacto-pyranoside (IPTG). A still more preferred promoter, the lac promoter, is present in plasmid vector pKK223-3 and is also inducible by exogenously supplied IPTG. The pKK223-3 plasmid can be successfully expressed in a number of *E. coli* strains, such as XL-1, TB1, BL21 and BLR, using about 25 to about 100 μ M IPTG for induction. Surprisingly, concentrations of about 25 to about 50 μ M IPTG have been found to provide optimal results in 2 L shaker flasks and fermentors.

[0143] Several strains of Salmonella such as *S. typhi* and *S. typhimurium* and *S. typhimurium-E. coli* hybrids have been used to express immunogenic transgenes including prior HBc chimer particles both as sources of the particles for use as immunogens and as live, attenuated whole cell vaccines and inocula, and those expression and vaccination systems can be used herein. See, U.S. Pat. No. 6,024,961; U.S. Pat. No. 5,888,799; U.S. Pat. No. 5,387,744; U.S. Pat. No. 5,297,441; Ulrich et al., (1998) *Adv. Virus Res.*, 50:141-182; Tacket et al., (August 1997) *Infect. Immun.*,

65(8):3381-3385; Schodel et al., (February 1997) *Behring Inst. Mitt.*, 98:114-119; Nardelli-Haeffliger et al., (December 1996) *Infect. Immun.*, 64(12):5219-5224; Londono et al., (April 1996) *Vaccine*, 14(6):545-552, and the citations therein.

[0144] Expression vectors compatible with eukaryotic cells, such as those compatible with yeast cells or those compatible with cells of higher plants or mammals, are also contemplated herein. Such expression vectors can also be used to form the recombinant DNA molecules of the present invention. Vectors for use in yeasts such as *S. cerevisiae* or *Pichia pastoris* can be episomal or integrating, as is well known. Eukaryotic cell expression vectors are well known in the art and are available from several commercial sources. Normally, such vectors contain one or more convenient restriction sites for insertion of the desired DNA segment and promoter sequences. Optionally, such vectors contain a selectable marker specific for use in eukaryotic cells. Exemplary promoters for use in *S. cerevisiae* include the *S. cerevisiae* phosphoglyceric acid kinase (PGK) promoter and the divergent promoters GAL 10 and GAL 1, whereas the alcohol oxidase gene (AOX1) is a useful promoter for *Pichia pastoris*.

[0145] For example, to produce chimer in the methylophilic yeast, *P. pastoris*, a gene that encodes a desired chimer is placed under the control of regulatory sequences that direct expression of structural genes in *Pichia*. The resultant expression-competent forms of those genes are introduced into *Pichia* cells.

[0146] More specifically, the transformation and expression system described by Cregg et al. (1987) *Biotechnology*, 5:479-485; (1987) *Molecular and Cellular Biology*, 12:3376-3385 can be used. A gene for a chimer V12.Pf3.1 is placed downstream from the alcohol oxidase gene (AOX1) promoter and upstream from the transcription terminator sequence of the same AOX1 gene. The gene and its flanking regulatory regions are then introduced into a plasmid that carries both the *P. pastoris* HIS4 gene and a *P. pastoris* ARS sequence (Autonomously Replicating Sequence), which permit plasmid replication within *P. pastoris* cells [Cregg et al. (1987) *Molecular and Cellular Biology*, 12:3376-3385].

[0147] The vector also contains appropriate portions of a plasmid such as pBR322 to permit growth of the plasmid in *E. coli* cells. The resultant plasmid carrying a chimer gene, as well as the various additional elements described above, is illustratively transformed into a his4 mutant of *P. pastoris*; i.e. cells of a strain lacking a functional histidinol dehydrogenase gene.

[0148] After selecting transformant colonies on media lacking histidine, cells are grown on media lacking histidine, but containing methanol as described Cregg et al. (1987) *Molecular and Cellular Biology*, 12:3376-3385, to induce the AOX1 promoters. The induced AOX1 promoters cause expression of the chimer protein and the production of chimer particles in *P. pastoris*.

[0149] A contemplated chimer gene can also be introduced by integrative transformation, which does not require the use of an ARS sequence, as described by Cregg et al. (1987) *Molecular and Cellular Biology*, 12:3376-3385.

[0150] Production of chimer particles by recombinant DNA expression in mammalian cells is illustratively carried

out using a recombinant DNA vector capable of expressing the chimer gene in Chinese hamster ovary (CHO) cells. This is accomplished using procedures that are well known in the art and are described in more detail in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratories (1989).

[0151] In one illustrative example, the simian virus (SV40) based expression vector, pKSV-10 (Pharmacia Fine Chemicals, Piscataway, N.J.), is subjected to restriction endonuclease digestion by NcoI and HindIII. A NcoI/HindIII sequence fragment that encodes the desired HBc chimer prepared as described in Example 1 is ligated into the expression plasmid, which results in the formation of a circular recombinant expression plasmid denominated pSV-Pf.

[0152] The expression plasmid pSV-Pf contains an intact *E. coli* ampicillin resistance gene. *E. coli* RR101 (Bethesda Research Laboratories, Gaithersburg, Md.), when transformed with pSV-Pf, can thus be selected on the basis of ampicillin resistance for those bacteria containing the plasmid. Plasmid-containing bacteria are then cloned and the clones are subsequently screened for the proper orientation of the inserted coding gene into the expression vector.

[0153] The above obtained plasmid, pSV-Pf, containing the gene that encodes a desired HBc chimer is propagated by culturing *E. coli* containing the plasmid. The plasmid DNA is isolated from *E. coli* cultures as described in Sambrook et al., above.

[0154] Expression of a chimer is accomplished by the introduction of pSV-Pf into the mammalian cell line, e.g., CHO cells, using the calcium phosphate-mediated transfection method of Graham et al. (1973) *Virol.*, 52:456, or a similar technique.

[0155] To help ensure maximal efficiency in the introduction of pSV-Pf into CHO cells in culture, the transfection is carried out in the presence of a second plasmid, pSV2NEO (ATCC #37149) and the cytotoxic drug G418 (GIBCO Laboratories, Grand Island, N.Y.) as described by Southern et al. (1982) *J. Mol. Appl. Genet.*, 1:327. Those CHO cells that are resistant to G418 are cultured, have acquired both plasmids, pSV2NEO and pSV-Pf, and are designated CHO/pSV-Pf cells. By virtue of the genetic architecture of the pSV-Pf expression vector, a chimer is expressed in the resulting CHO/pSV-Pf cells and can be detected in and purified from the cytoplasm of these cells. The resulting composition containing cellular protein is separated on a column as discussed elsewhere herein.

[0156] The choice of which expression vector and ultimately to which promoter a chimer-encoding gene is operatively linked depends directly on the functional properties desired, e.g. the location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention can direct the replication, and preferably also the expression (for an expression vector) of the chimer gene included in the DNA segment to which it is operatively linked.

[0157] In one preferred embodiment, the host that expresses the chimer is the prokaryote, *E. coli*, and a preferred vector includes a prokaryotic replicon; i.e., a DNA

sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a prokaryotic host cell transformed therewith. Such replicons are well known in the art.

[0158] Those vectors that include a prokaryotic replicon can also include a prokaryotic promoter region capable of directing the expression of a contemplated HBc chimer gene in a host cell, such as *E. coli*, transformed therewith. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing one or more convenient restriction sites for insertion of a contemplated DNA segment. Typical of such vector plasmids are pUC8, pUC9, and pBR329 available from Biorad Laboratories, (Richmond, Calif.) and pPL and pKK223-3 available from Pharmacia, Piscataway, N.J.

[0159] Typical vectors useful for expression of genes in cells from higher plants and mammals are well known in the art and include plant vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers et al. (1987) *Meth. in Enzymol.*, 153:253-277 and mammalian expression vectors pKSV-10, above, and pCIneo (Promega Corp., #E1841, Madison, Wis.). However, several other expression vector systems are known to function in plants including pCaMVCN transfer control vector described by Fromm et al. (1985) *Proc. Natl. Acad. Sci. USA*, 82:58-24. Plasmid pCaMVCN (available from Pharmacia, Piscataway, N.J.) includes the cauliflower mosaic virus CaMV 35S promoter.

[0160] The above plant expression systems typically provide systemic or constitutive expression of an inserted transgene. Systemic expression can be useful where most or all of a plant is used as the source to a contemplated chimer molecule or resultant particles or where a large part of the plant is used to provide an oral vaccine. However, it can be more efficacious to express a chimer molecule or particles in a plant storage organ such as a root, seed or fruit from which the particles can be more readily isolated or ingested.

[0161] One manner of achieving storage organ expression is to use a promoter that expresses its controlled gene in one or more preselected or predetermined non-photosynthetic plant organs. Expression in one or more preselected storage organs with little or no expression in other organs such as roots, seed or fruit versus leaves or stems is referred to herein as enhanced or preferential expression. An exemplary promoter that directs expression in one or more preselected organs as compared to another organ at a ratio of at least 5:1 is defined herein as an organ-enhanced promoter. Expression in substantially only one storage organ and substantially no expression in other storage organs is referred to as organ-specific expression; i.e., a ratio of expression products in a storage organ relative to another of about 100:1 or greater indicates organ specificity. Storage organ-specific promoters are thus members of the class of storage organ-enhanced promoters.

[0162] Exemplary plant storage organs include the roots of carrots, taro or manioc, potato tubers, and the meat of fruit such as red guava, passion fruit, mango, papaya, tomato, avocado, cherry, tangerine, mandarin, palm, melons such as cantaloupe and watermelons and other fleshy fruits such as squash, cucumbers, mangos, apricots, peaches, as well as the seeds of maize (corn), soybeans, rice, oil seed rape and the like.

[0163] The CaMV 35S promoter is normally deemed to be a constitutive promoter. However, recent research has shown that a 21-bp region of the CaMV 35S promoter, when operatively linked into another, heterologous usual green tissue promoter, the *rbcS*-3A promoter, can cause the resulting chimeric promoter to become a root-enhanced promoter. That 21-bp sequence is disclosed in U.S. Pat. No. 5,023,179. The chimeric *rbcS*-3A promoter containing the 21-bp insert of U.S. Pat. No. 5,023,179 is a useful root-enhanced promoter herein.

[0164] A similar root-enhanced promoter, that includes the above 21-bp segment is the -90 to +8 region of the CAMV 35S promoter itself. U.S. Pat. No. 5,110,732 discloses that that truncated CaMV 35S promoter provides enhanced expression in roots and the radical of seed, a tissue destined to become a root. That promoter is also useful herein.

[0165] Another useful root-enhanced promoter is the -1616 to -1 promoter of the oil seed rape (*Brassica napus* L.) gene disclosed in PCT/GB92/00416 (WO 91/13922 published Sep. 19, 1991). *E. coli* DH5.alpha. harboring plasmid pRLambdaS4 and bacteriophage lambda.bet.1 that contain this promoter were deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, GB on Mar. 8, 1990 and have accession numbers NCIMB40265 and NCIMB40266. A useful portion of this promoter can be obtained as a 1.0 kb fragment by cleavage of the plasmid with HaeIII.

[0166] A preferred root-enhanced promoter is the mannopine synthase (*mas*) promoter present in plasmid pKan2 described by DiRita and Gelvin (1987) *Mol. Gen. Genet.*, 207:233-241. This promoter is removable from its plasmid pKan2 as a XbaI-XbaII fragment.

[0167] The preferred mannopine synthase root-enhanced promoter is comprised of the core mannopine synthase (*mas*) promoter region up to position -138 and the mannopine synthase activator from -318 to -213, and is collectively referred to as AmasPmas. This promoter has been found to increase production in tobacco roots about 10- to about 100-fold compared to leaf expression levels.

[0168] Another root specific promoter is the about 500 bp 5' flanking sequence accompanying the hydroxyproline-rich glycopeptide gene, HRGPnt3, expressed during lateral root initiation and reported by Keller et al. (1989) *Genes Dev.*, 3:1639-1646. Another preferred root-specific promoter is present in the about -636 to -1 5' flanking region of the tobacco root-specific gene ToRBF reported by Yamamoto et al. (1991) *Plant Cell*, 3:371-381. The cis-acting elements regulating expression are more specifically located by those authors in the region from about -636 to about -299 5' from the transcription initiation site. Yamamoto et al. reported steady state mRNA production from the ToRBF gene in roots, but not in leaves, shoot meristems or stems.

[0169] Still another useful storage organ-specific promoter are the 5' and 3' flanking regions of the fruit-ripening gene E8 of the tomato, *Lycopersicon esculentum*. These regions and their cDNA sequences are illustrated and discussed in Deikman et al. (1988) *EMBO J.*, 7(11):3315-3320 and (1992) *Plant Physiol.*, 100:2013-2017.

[0170] Three regions are located in the 2181 bp of the 5' flanking sequence of the gene and a 522 bp sequence 3' to the poly (A) addition site appeared to control expression of

the E8 gene. One region from -2181 to -1088 is required for activation of E8 gene transcription in unripe fruit by ethylene and also contributes to transcription during ripening. Two further regions, -1088 to -863 and -409 to -263, are unable to confer ethylene responsiveness in unripe fruit but are sufficient for E8 gene expression during ripening.

[0171] The maize sucrose synthase-1 (Sh) promoter that in corn expresses its controlled enzyme at high levels in endosperm, at much reduced levels in roots and not in green tissues or pollen has been reported to express a chimeric reporter gene, β -glucuronidase (GUS), specifically in tobacco phloem cells that are abundant in stems and roots. Yang et al. (1990) *Proc. Natl. Acad. Sci., U.S.A.*, 87:4144-4148. This promoter is thus useful for plant organs such as fleshy fruits like melons, e.g. cantaloupe, or seeds that contain endosperm and for roots that have high levels of phloem cells.

[0172] Another exemplary tissue-specific promoter is the lectin promoter, which is specific for seed tissue. The lectin protein in soybean seeds is encoded by a single gene (Le1) that is only expressed during seed maturation and accounts for about 2 to about 5 percent of total seed mRNA. The lectin gene and seed-specific promoter have been fully characterized and used to direct seed specific expression in transgenic tobacco plants. See, e.g., Vodkin et al. (1983) *Cell*, 34:1023 and Lindstrom et al. (1990) *Developmental Genetics*, 11:160.

[0173] A particularly preferred tuber-specific expression promoter is the 5' flanking region of the potato patatin gene. Use of this promoter is described in Twell et al. (1987) *Plant Mol. Biol.*, 9:365-375. This promoter is present in an about 406 bp fragment of bacteriophage LPOTI. The LPOTI promoter has regions of over 90 percent homology with four other patatin promoters and about 95 percent homology over all 400 bases with patatin promoter PGT5. Each of these promoters is useful herein. See, also, Wenzler et al. (1989) *Plant Mol. Biol.*, 12:41-50.

[0174] Still further organ-enhanced and organ-specific promoter are disclosed in Benfey et al. (1988) *Science*, 244:174-181.

[0175] Each of the promoter sequences utilized is substantially unaffected by the amount of chimer molecule or particles in the cell. As used herein, the term "substantially unaffected" means that the promoter is not responsive to direct feedback control (inhibition) by the chimer molecules or particles accumulated in transformed cells or transgenic plant.

[0176] Transfection of plant cells using *Agrobacterium tumefaciens* is typically best carried out on dicotyledonous plants. Monocots are usually most readily transformed by so-called direct gene transfer of protoplasts. Direct gene transfer is usually carried out by electroporation, by poly-ethyleneglycol-mediated transfer or bombardment of cells by microprojectiles carrying the needed DNA. These methods of transfection are well-known in the art and need not be further discussed herein. Methods of regenerating whole plants from transfected cells and protoplasts are also well-known, as are techniques for obtaining a desired protein from plant tissues. See, also, U.S. Pat. No. 5,618,988 and 5,679,880 and the citations therein.

[0177] A transgenic plant formed using *Agrobacterium* transformation, electroporation or other methods typically

contains a single gene on one chromosome. Such transgenic plants can be referred to as being heterozygous for the added gene. However, inasmuch as use of the word "heterozygous" usually implies the presence of a complementary gene at the same locus of the second chromosome of a pair of chromosomes, and there is no such gene in a plant containing one added gene as here, it is believed that a more accurate name for such a plant is an independent segregant, because the added, exogenous chimer molecule-encoding gene segregates independently during mitosis and meiosis. A transgenic plant containing an organ-enhanced promoter driving a single structural gene that encodes a contemplated HBc chimeric molecule; i.e., an independent segregant, is a preferred transgenic plant.

[0178] More preferred is a transgenic plant that is homozygous for the added structural gene; i.e., a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants produced for enhanced chimer particle accumulation relative to a control (native, non-transgenic) or an independent segregant transgenic plant. A homozygous transgenic plant exhibits enhanced chimer particle accumulation as compared to both a native, non-transgenic plant and an independent segregant transgenic plant.

[0179] It is to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous (heterologous) genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes that encode a chimeric HBc molecule. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

[0180] A transgenic plant of this invention thus has a heterologous structural gene that encodes a contemplated chimeric HBc molecule. A preferred transgenic plant is an independent segregant for the added heterologous chimeric HBc structural gene and can transmit that gene to its progeny. A more preferred transgenic plant is homozygous for the heterologous gene, and transmits that gene to all of its offspring on sexual mating.

[0181] Inasmuch as a gene that encodes a chimeric HBc molecule does not occur naturally in plants, a contemplated transgenic plant accumulates chimeric HBc molecule particles in a greater amount than does a non-transformed plant of the same type or strain when both plants are grown under the same conditions.

[0182] The phrase "same type" or "same strain" is used herein to mean a plant of the same cross as or a clone of the untransformed plant. Where allelic variations among siblings of a cross are small, as with extensively inbred plant, comparisons between siblings can be used or an average arrived at using several siblings. Otherwise, clones are preferred for the comparison.

[0183] Seed from a transgenic plant is grown in the field greenhouse, window sill or the like, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true

breeding lines that are evaluated for chimeric HBc molecule particle accumulation, preferably in the field, under a range of environmental conditions.

[0184] A transgenic plant homozygous for chimeric HBc molecule particle accumulation is crossed with a parent plant having other desired traits. The progeny, which are heterozygous or independently segregatable for chimeric HBc molecule particle accumulation, are backcrossed with one or the other parent to obtain transgenic plants that exhibit chimeric HBc molecule particle accumulation and the other desired traits. The backcrossing of progeny with the parent may have to be repeated more than once to obtain a transgenic plant that possesses a number of desirable traits.

[0185] An insect cell system can also be used to express a HBc chimer. For example, in one such system *Autographa californica* nuclear polyhedrosis virus (AcNPV) or baculovirus is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae.

[0186] The sequences encoding a chimer can be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of chimer sequence renders the polyhedrin gene inactive and produces recombinant virus lacking coat protein. The recombinant viruses can then be used to infect, for example, *S. Frugiperda* cells or *Trichoplusia* larvae in which the HBc chimer can be expressed. E. Engelhard et al. (1994) *Proc. Natl. Acad. Sci., USA*, 91:3224-3227; and V. Luckow, Insect Cell Expression Technology, pp. 183-218, in *Protein Engineering: Principles and Practice*, J. L. Cleland et al. eds., Wiley-Liss, Inc, 1996). Heterologous genes placed under the control of the polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) are often expressed at high levels during the late stages of infection.

[0187] Recombinant baculoviruses containing the chimeric gene are constructed using the baculovirus shuttle vector system (Luckow et al. (1993) *J. Virol.*, 67:4566-4579), sold commercially as the Bac-To-Bac™ baculovirus expression system (Life Technologies). Stocks of recombinant viruses are prepared and expression of the recombinant protein is monitored by standard protocols (O'Reilly et al., *Baculovirus Expression Vectors: A Laboratory Manual*, W. H. Freeman and Company, New York, 1992; and King et al., *The Baculovirus Expression System: A Laboratory Guide*, Chapman & Hall, London, 1992).

[0188] A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted into the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

[0189] Alternatively, synthetic linkers containing one or more restriction endonuclease sites can be used to join the DNA segment to the expression vector, as noted before. The synthetic linkers are attached to blunt-ended DNA segments by incubating the blunt-ended DNA segments with a large excess of synthetic linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase.

[0190] Thus, the products of the reaction are DNA segments carrying synthetic linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction endonuclease and ligated into an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the synthetic linker. Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including New England BioLabs, Beverly, Mass. A desired DNA segment can also be obtained using PCR technology in which the forward and reverse primers contain desired restriction sites that can be cut after amplification so that the gene can be inserted into the vector. Alternatively PCR products can be directly cloned into vectors containing T-overhangs (Promega Corp., A3600, Madison, Wis.) as is well known in the art.

[0191] The expressed chimeric protein self-assembles into particles within the host cells, whether in single cells or in cells within a multicelled host. The particle-containing cells are harvested using standard procedures, and the cells are lysed using a French pressure cell, lysozyme, sonicator, bead beater or a microfluidizer (Microfluidics International Corp., Newton Mass.). After clarification of the lysate, particles are precipitated with 45% ammonium sulfate, resuspended in 20 mM sodium phosphate, pH 6.8 and dialyzed against the same buffer. The dialyzed material is clarified by brief centrifugation and the supernatant subjected to gel filtration chromatography using Sepharose® CL-4B. Particle-containing fractions are identified, subjected to hydroxyapatite chromatography, and reprecipitated with ammonium sulfate prior to resuspension, dialysis and sterile filtration and storage at -70° C.

[0192] Inocula and Vaccines

[0193] A before-described recombinant HBc chimer immunogen preferably in particulate form is dissolved or dispersed in an immunogenic effective amount in a pharmaceutically acceptable vehicle composition that is preferably aqueous to form an inoculum or vaccine. When administered to a host animal in need of immunization or in which antibodies are desired to be induced such as a mammal (e.g., a mouse, dog, goat, sheep, horse, bovine, monkey, ape, or human) or bird (e.g., a chicken, turkey, duck or goose), an inoculum induces antibodies that immunoreact with the influenza A M2 B cell epitope present in the immunogen. In a vaccine, those induced antibodies also believed to immunoreact in vivo with (bind to) the virus or virally-infected cells and protect the host from influenza infection. A composition that is a vaccine in one animal can be an inoculum an inoculum for another host, as where the antibodies are induced in a second host that is not infected by influenza A.

[0194] The amount of recombinant HBc chimer immunogen utilized in each immunization is referred to as an immunogenic effective amount and can vary widely, depending inter alia, upon the recombinant HBc chimer immunogen, animal host immunized, and the presence of an adjuvant in the vaccine, as discussed below. Immunogenic effective amounts for a vaccine and an inoculum provide the protection or antibody activity, respectively, discussed hereinbefore.

[0195] Vaccines or inocula typically contain a recombinant HBc chimer immunogen concentration of about 1 microgram to about 1 milligram per inoculation (unit dose),

and preferably about 10 micrograms to about 50 micrograms per unit dose. Immunizations in mice typically contain 10 or 20 µg of chimer particles.

[0196] The term "unit dose" as it pertains to a vaccine or inoculum of the present invention refers to a physically discrete unit suitable as an unitary dosage for animals, each unit containing a predetermined quantity of active material calculated to individually or collectively produce the desired immunogenic effect in association with the required diluent; i.e., carrier, or vehicle. A single unit dose or a plurality of unit doses can be used to provide an immunogenic effective amount of recombinant HBc chimer immunogen particles.

[0197] Vaccines or inocula are typically prepared from a recovered recombinant HBc chimer immunogen particles by dispersing the particles in a physiologically tolerable (acceptable) diluent vehicle such as water, saline phosphate-buffered saline (PBS), acetate-buffered saline (ABS), Ringier's solution or the like to form an aqueous composition. The diluent vehicle can also include oleaginous materials such as peanut oil, squalane or squalene as is discussed hereinafter.

[0198] The immunogenic active ingredient is often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, an inoculum or vaccine can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents that enhance the immunogenic effectiveness of the composition.

[0199] A contemplated vaccine or inoculum advantageously also includes an adjuvant. Suitable adjuvants for vaccines and inocula of the present invention comprise those adjuvants that are capable of enhancing the antibody responses against B cell epitopes of the chimer, as well as adjuvants capable of enhancing cell mediated responses towards T cell epitopes contained in the chimer, if present. Adjuvants are well known in the art (see, for example, *Vaccine Design—The Subunit and Adjuvant Approach*, 1995, Pharmaceutical Biotechnology, Volume 6, Eds. Powell, M. F., and Newman, M. J., Plenum Press, New York and London, ISBN 0-306-44867-X).

[0200] Exemplary adjuvants include complete Freund's adjuvant (CFA) that is not used in humans, incomplete Freund's adjuvant (IFA), squalene, squalane and alum [e.g., Alhydrogel™ (Superfos, Denmark)], which are materials well known in the art, and are available commercially from several sources.

[0201] Preferred adjuvants for use with immunogens of the present invention include aluminum or calcium salts (for example hydroxide or phosphate salts). A particularly preferred adjuvant for use herein is an aluminum hydroxide gel such as Alhydrogel™. For aluminum hydroxide gels (alum), the chimer protein is admixed with the adjuvant so that about 50 to about 800 micrograms of aluminum are present per dose, and preferably about 400 to about 600 micrograms are present.

[0202] Another particularly preferred adjuvant for use with an immunogen of the present invention is an emulsion. A contemplated emulsion can be an oil-in-water emulsion or a water-in-oil emulsion. In addition to the immunogenic chimer protein particles, such emulsions comprise an oil

phase of squalene, squalane, peanut oil or the like as are well-known, and a dispersing agent. Non-ionic dispersing agents are preferred and such materials include mono- and di- C_{12} - C_{24} -fatty acid esters of sorbitan and mannide such as sorbitan mono-stearate, sorbitan mono-oleate and mannide mono-oleate. An immunogen-containing emulsion is administered as an emulsion.

[0203] Preferably, such emulsions are water-in-oil emulsions that comprise squalene and mannide mono-oleate (Arlacel™ A), optionally with squalane, emulsified with the chimer protein particles in an aqueous phase. Well-known examples of such emulsions include Montanide™ ISA-720, and Montanide™ ISA 703 (Seppic, Castres, France), each of which is understood to contain both squalene and squalane, with squalene predominating in each, but to a lesser extent in Montanide™ ISA 703. Most preferably, Montanide™ ISA-720 is used, and a ratio of oil-to-water of 7:3 (w/w) is used. Other preferred oil-in-water emulsion adjuvants include those disclosed in WO 95/17210 and EP 0 399 843.

[0204] The use of small molecule adjuvants is also contemplated herein. One type of small molecule adjuvant useful herein is a 7-substituted-8-oxo- or 8-sulfo-guanosine derivative described in U.S. Pats. No. 4,539,205, U.S. Pat. No. 4,643,992, U.S. Pat. No. 5,011,828 and U.S. Pat. No. 5,093,318, whose disclosures are incorporated by reference. Of these materials, 7-allyl-8-oxoguanosine (loxoribine) is particularly preferred. That molecule has been shown to be particularly effective in inducing an antigen- (immunogen-) specific response.

[0205] Still further useful adjuvants include monophosphoryl lipid A (MPL) available from Corixa Corp. (see, U.S. Pat. No. 4,987,237), CPG available from Coley Pharmaceutical Group, QS21 available from Aquila Biopharmaceuticals, Inc., SBAS2 available from SKB, the so-called muramyl dipeptide analogues described in U.S. Pat. No. 4,767,842, and MF59 available from Chiron Corp. (see, U.S. Pats. No. 5,709,879 and U.S. Pat. No. 6,086,901).

[0206] More particularly, immunologically active saponin fractions having adjuvant activity derived from the bark of the South American tree *Quillaja Saponaria* Molina (e.g. Quil™ A) are also useful. Derivatives of Quil™ A, for example QS21 (an HPLC purified fraction derivative of Quil™ A), and the method of its production is disclosed in U.S. Pat. No. 5,057,540. In addition to QS21 (known as QA21), other fractions such as QA17 are also disclosed.

[0207] 3-De-O-acylated monophosphoryl lipid A is a well-known adjuvant manufactured by Ribi Immunochem, Hamilton, Mont. The adjuvant contains three components extracted from bacteria: monophosphoryl lipid (MPL) A, trehalose dimycolate (TDM) and cell wall skeleton (CWS) (MPL+TDM+CWS) in a 2% squalene/Tween® 80 emulsion. This adjuvant can be prepared by the methods taught in GB 2122204B. A preferred form of 3-de-O-acylated monophosphoryl lipid A is in the form of an emulsion having a small particle size less than 0.2 μ m in diameter (EP 0 689 454 B1).

[0208] The muramyl dipeptide adjuvants include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thur-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine [CGP 11637, referred to as nor-MDP], and N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-

glycero-3-hydroxyphosphoryloxy)-ethylamin [CGP 1983A, referred to as MTP-PE].

[0209] Preferred adjuvant mixtures include combinations of 3D-MPL and QS21 (EP 0 671 948 B1), oil-in-water emulsions comprising 3D-MPL and QS21 (WO 95/17210, PCT/EP98/05714), 3D-MPL formulated with other carriers (EP 0 689 454 B1), QS21 formulated in cholesterol-containing liposomes (WO 96/33739), or immunostimulatory oligonucleotides (WO 96/02555). Alternative adjuvants include those described in WO 99/52549 and non-particulate suspensions of polyoxyethylene ether (UK Patent Application No. 9807805.8).

[0210] Adjuvants are utilized in an adjuvant amount, which can vary with the adjuvant, host animal and recombinant HBc chimer immunogen. Typical amounts can vary from about 1 μ g to about 1 mg per immunization. Those skilled in the art know that appropriate concentrations or amounts can be readily determined.

[0211] Inocula and vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations that are suitable for other modes of administration include suppositories and, in some cases, oral formulation or by nasal spray. For suppositories, traditional binders and carriers can include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like.

[0212] An inoculum or vaccine composition takes the form of a solution, suspension, tablet, pill, capsule, sustained release formulation or powder, and contains an immunogenic effective amount of HBc chimer, preferably as particles, as active ingredient. In a typical composition, an immunogenic effective amount of preferred HBc chimer particles is about 1 μ g to about 1 mg of active ingredient per dose, and more preferably about 5 μ g to about 50 μ g per dose, as noted before.

[0213] A vaccine or inoculum is typically formulated for parenteral administration. Exemplary immunizations are carried out sub-cutaneously (SC) intra-muscularly (IM), intravenously (IV), intraperitoneally (IP) or intra-dermally (ID).

[0214] The HBc chimer particles and HBc chimer particle conjugates can be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein or hapten) and are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[0215] The inocula or vaccines are administered in a manner compatible with the dosage formulation, and in such amount as are therapeutically effective and immunogenic (an antibody-inducing amount or protective amount, as is

desired). The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per individual. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed in intervals (weeks or months) by a subsequent injection or other administration.

[0216] Once immunized, the host animal is maintained for a period of time sufficient for the recombinant HBc chimer immunogen to induce the production of a sufficient titer of antibodies that bind to the M2 protein. The maintenance time for the production of anti-M2 antibodies typically lasts for a period of about three to about twelve weeks, and can include a booster, second immunizing administration of the vaccine. A third immunization is also contemplated, if desired, at a time 24 weeks to five years after the first immunization. It is particularly contemplated that once a protective level titer of antibodies is attained, the vaccinated host animal is preferably maintained at or near that antibody titer by periodic booster immunizations administered at intervals of about 1 to about 5 years.

pKK223-3/433-452-F GGTGCATGCAAGGAGATG

SEQ ID NO:26

pKK223-NcoI-mod-R GCGAAGCTTCGGATCccatggTTTTTCCTCCTTATGTGAAATTCCTATCCG-
CTC

SEQ ID NO:27

[0217] The production of antibodies is readily ascertained by obtaining a plasma or serum sample from the immunized host and assaying the antibodies therein for their ability to bind to a synthetic M2 polypeptide antigen in an ELISA assay as described hereinafter or by another immunoassay such as a Western blot as is well known in the art.

[0218] It is noted that the before-described antibodies so induced can be isolated from the blood of the host using well-known techniques, and then reconstituted into a second vaccine for passive immunization as is also well known. Similar techniques are used for gamma-globulin immunizations of humans. For example, antiserum from one or a number of immunized hosts can be precipitated in aqueous ammonium sulfate (typically at 40-50 percent of saturation), and the precipitated antibodies purified chromatographically as by use of affinity chromatography in which an M2 polypeptide is utilized as the antigen immobilized on the chromatographic column.

[0219] Inocula are preparations that are substantially identical to vaccines, but are used in a host animal in which antibodies to influenza are desired to be induced, but in which protection from influenza is not desired.

[0220] Without further elaboration, it is believed that one skilled in the art can, using the preceding description and the detailed examples below, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limiting of the remainder of the disclosure in any way whatsoever.

EXAMPLE 1

B Cell Epitope-Containing Chimera Preparation

[0221] A. Preparation of plasmid vector pKK223-3N, a Modified Form of pKK223-3

[0222] Plasmid vector pKK223-3 (Pharmacia) was modified by the establishment of a unique NcoI restriction site to enable insertion of HBc genes as NcoI-HindIII restriction fragments and subsequent expression in *E. coli* host cells. To modify the pKK223-3 plasmid vector, a new SphI-HindIII fragment was prepared using the PCR primers pKK223-3/433-452-F and pKK223-NcoI-mod-R, and pKK223-3 as the template.

[0223] This PCR fragment was cut with the restriction enzymes SphI and HindIII to provide a 467 bp fragment that was then ligated with a 4106 bp fragment of the pKK223-3 vector, replacing the original 480 bp SphI-HindIII fragment. The resultant plasmid (pKK223-3N; 4573 bp) is therefore 13 bp shorter than the parent plasmid and contains modified nucleotide sequence upstream of the introduced NcoI site (see FIG. 2, in which the dashes indicate the absent bases). Restriction sites in plasmid pKK223-3N are indicated in FIG. 2 and nucleotide changes made to the pKK223-3 parent plasmid are indicated by an underline as shown below.

[0224] B. Preparation of V1, V2 and V8 Cloning Vectors

[0225] Modified HBc149 (V1 and V2) or HBc183 (V8) genes, able to accept the directional insertion of synthetic dsDNA fragments into the immunodominant loop region, were constructed using PCR. (The plasmid accepting inserts between amino acids E77 and D78 and truncated to V149 was named V1, the plasmid accepting inserts between D78 and P79 and truncated to V149 was named V2, and the plasmid accepting inserts between D78 and P79 and terminating at C183, was called V8). The HBc149 and HBc183 genes were amplified in two halves using two PCR primer pairs, one of which amplifies the amino terminus, the other amplifies the carboxyl terminus. For V1, the products of the PCR reactions (N- and C-terminus) are both 246 bp fragments; for V2, the products are a 249 bp (N-terminus) and a 243 bp fragment (C-terminus); for V8, the products are a 249 bp (N-terminus) and a bp fragment (C-terminus).

[0226] The N-terminal fragments prepared were digested with NcoI and EcoRI, and the C-terminal fragments were digested with EcoRI and HindIII. The V1, V2 and V8 fragment pairs were then ligated together at the common EcoRI overhangs. The resultant NcoI-HindIII fragments were then ligated into the pKK223-3N vector, which had been prepared by digestion with NcoI and HindIII.

[0227] To insert B cell epitopes into the V1, V2 and V8 plasmids, the appropriate plasmid was digested with EcoRI and SacI restriction enzymes. Synthetic dsDNA fragments containing 5' EcoRI and 3' SacI overhangs were then inserted. In all cases, V1, V2, and V8, glycine-isoleucine (EcoRI) and glutamic acid-leucine (SacI) amino acid pairs, flank the inserted B cell epitopes. The inserted restriction sites are underlined in the primers below.

[0228] V1

V1
HBc149/NcoI-F
5'-GGGCCATGGACATCGACCCTTA SEQ ID NO:28

HBc-E77/EcoRI-R
5'-GCGGAATTCCTTCCAAATTAACACCCACC SEQ ID NO:29

HBc-D78/EcoRI-SacI-F
5'-CGCGAATTCAAAAAGAGCTCGATCCAGCGTCTAGAGAC SEQ ID NO:30
HBc149/HindIII-R
5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG SEQ ID NO:31

V2
HBc149/NcoI -F
5'-TTGGGCCATGGACATCGACCCTTA SEQ ID NO:32

HBc-D78/EcoRI -R
5'-GCGGAATTCCATCTTCCAAATTAACACCCAC SEQ ID NO:33

HBc-P79/EcoRI -SacI-F
5'-CGCGAATTCAAAAAGAGCTCCCAGCGTCTAGAGACCTAG SEQ ID NO:34

HBc149/HindIII-R
5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG SEQ ID NO:31

V8
HBc149/NcoI-F
5'-GGGCCATGGACATCGACCCTTA SEQ ID NO:28

HBc-D78/EcoRI-R
5'-GCGGAATTCCATCTTCCAAATTAACACCCAC SEQ ID NO:29

HBc-P79/EcoRI-SacI-F
5'-CGCGAATTCAAAAAGAGCTCCCAGCGTCTAGAGACCTAG SEQ ID NO:35

HBc183/HindIII-R
5'-GGAAGCTTACTAATGAGATTCCCG SEQ ID NO:36

V34/V55
pKK-BamHI-F
5'-GCGGGATCCGGAGCTTATCGA SEQ ID NO: 37

HBc-NcoI/EcoRI/SacI-R
5'-GCGGAGCTCTTTTGAATTCCTCCATG- SEQ ID NO:38
GTTTTTCTCCTTAT

PreC-SacI-HBc-F
5'-GCGGAGCTCTTGGGTG- SEQ ID NO:39
GCTTTGGGGCATTGACATCGACCCTTATAAAG

V34
HBc149/HindIII-R
5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG SEQ ID NO:31

V55
HBc149+C/HindIII-R
5'-CGCAAGCTTACTAGCAACAACAGTAGTCTCCGGAAG SEQ ID NO:40

[0229] C. Preparation of V34 and V55 Cloning Vectors

[0230] Modified HBc149 genes, able to accept the directional insertion of synthetic dsDNA fragments into the N-terminal region, 5' to the pre-core sequence LGWLWG, were constructed using PCR. (The plasmid that encoded an HBc sequence terminating at V149 was named V34, whereas the plasmid that encoded an HBc sequence harboring an additional cysteine, C-terminal to V149, was named V55.) The HBc149 gene was amplified in two halves using two PCR primer pairs, one of which amplifies the amino terminus (for which V1 was used as a template), the other amplifies the carboxyl terminus. For V34, the products of the PCR reactions were a 293 bp (N-terminus) fragment and a

484 bp (C-terminus) fragment; for V55, the same N-terminal fragment was used and a 490 bp C-terminal fragment was prepared.

[0231] The N-terminal fragment prepared by PCR was digested with NcoI and SacI, and the C-terminal fragments were digested with SacI and HindIII. The V34 and V55 fragment pairs were then ligated together at the common SacI overhangs. The resultant NcoI-HindIII fragments were then ligated into the pKK223-3N vector, which had been prepared by digestion with NcoI and HindIII.

[0232] B cell epitope insertion was accomplished by a procedure identical to that outlined above for the V1 cloning vector. Restriction sites are underlined in the oligonucleotides primers below.

V34/V55

pKK-BamHI-F

5'-GCGGGATCCGGAGCTTATCGA

SEQ ID NO:37

HBc-NcoI/EcoRI/SacI-R

5'-GCGGAGCTCCTTTTGAATTCCTTAT

SEQ ID NO:38

PreC-SacI-HBc-F

5'-GCGGAGCTCCTTGGGTGGCTTTGGGGCATTGACATCGACCTTATAAAG

SEQ ID NO:39

V34

HBc149/HindIII-R

5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG

SEQ ID NO:31

V55

HBc149 + C/HindIII-R

5'-CGCAAGCTTACTAGCAAACAACAGTAGTCTCCGGAAG

SEQ ID NO:40

[0233] D. Preparation of V47, V48 and V54 Cloning Vectors

[0234] Modified HBc149 and HBc183 genes, able to accept the directional insertion of synthetic dsDNA fragments into the N-terminal region between amino acid residues I3 and D4 were constructed using PCR. (The plasmid encoding an HBc chimera terminating at V149 was named V47, the plasmid encoding an HBc chimera harboring an additional cysteine, C-terminal to V149, was named V54, and the plasmid encoding an HBc chimera terminating at C183 was named V48). For V47, V48 and V54, a PCR primer pairs was used to amplify the amino terminus, from the template V1, including sequence preceeding the HBc gene. The resultant PCR fragment of 190 bp for the C-terminus of V47, the HBc gene was amplified using a PCR primer pair resulting in a 469 bp fragment; for V54, the C-terminal fragment is 475 bp. For the C-terminus of V48,

the HBc183 gene was amplified using a PCR primer pair, resulting in a 574 bp fragment.

[0235] The cloning procedure used from this point was identical to that outlined for the cloning vector V1.

[0236] To insert epitopes into the V47, V48 and V54 plasmids, the plasmids were first digested with NcoI and SacI restriction enzymes. Synthetic dsDNA fragments containing 5' AflIII and 3' SacI overhangs were then inserted (note, restriction enzymes AflIII and NcoI leave compatible overhangs). In all cases, V47, V48, and V54, HBc residues D2 and I3 were deleted so that the sequence of the epitope directly follows residue M1; the glutamic acid-leucine (EL) amino acid pairs, coded for by the SacI restriction site, follows the inserted epitope. The inserted restriction sites are underlined in the oligonucleotide primers below.

V47/V48/V54

pKK(167-150)-F

5'-GCATAATTCGTGTCGCTC

SEQ ID NO:41

HBc-I3/EcoRI-R

5'-GCGGAATTCGATGTCCATGGTTTTTTCCT

SEQ ID NO:42

HBc-EcoRI/SacI/D4-F

5'- GCGGAATTCAAAAA
GAGCTCGACCCCTTATAAGAATTGGA

SEQ ID NO:43

V47

HBc149/HindIII-R

5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG

SEQ ID NO:31

V54

HBc149+C/HindIII-R

5'-CGCAAGCTTACTAGCAAACAACAGTAGTCTCCGGAAG

SEQ ID NO:40

V48

HBc183/HindIII-R

5'-GGAAAGCTTACTAACATTGAGATTCCCG

SEQ ID NO:36

EXAMPLE 2

Preparation of Chimers Containing Influenza A M2
Polypeptide Sequences

[0237] A. Insertion of Influenza A M2 N-terminal Domain
into V34, V47, V48, V54, and V55 Cloning Vectors

V47

M2 (1-24/C17S)

M S L L T E V E T P I R N E W G S R SEQ ID NO:44
CATGTCTCTGCTGACCGAAGTTGAAACCCCTATCAGAAACGAATGGGGGTCTAGA SEQ ID NO:45
AGAGACGACTGGCTTCAACTTTGGGGATAGTCTTTGCTTACCCCGAGTCT SEQ ID NO:46
C N D S S D E L
TGTAACGATTCAAGTGATGAGCT
ACATTGCTAAGTTCACTAC

M2 (1-24/C19S)

M S L L T E V E T P I R N E W G C R SEQ ID NO:47
CATGTCTCTGCTGACCGAAGTTGAAACCCCTATCAGAAACGAATGGGGGTGCGAGA SEQ ID NO:48
AGAGACGACTGGCTTCAACTTTGGGGATAGTCTTTGCTTACCCCGAGTCT SEQ ID NO:49

S N D S S D E L

TCGAACGATTCAAGTGATGAGCT
AGCTTGCTAAGTTCACTAC

[0238] B. Insertion of a mutated Influenza A M2 (M2(2-
24/C17A,C19A))into V2, V8 and V16 Expression Vectors

V2, V8, V16

M2 (2-24/C17A,C19A)

I S L L T E V E T P I R N E W G A R SEQ ID NO:50
ATTTCTCTGTTAACCAGAGTGGAGACGCCGATTGTAACGAATGGGGTGCGCGC SEQ ID NO:51
AGAGACAATTGGCTTCACTCTGCGGCTAAGCATTGCTTACCCCGAGCGG SEQ ID NO:52
A N D S S D E L
GCCAATGATAGCTCTGACGAGCT
CGGTTACTATCGAGACTGC

[0239] C. Insertion of Influenza A M2 N-terminal Domain
into Vectors V34, V47, V54, and V55

[0240] For V34 and V55 constructs, synthetic dsDNA
fragments coding for the M2 epitope (residues 1-24 of the
influenza A M2 protein; SEQ ID NO: 9) were inserted into
EcoRI/SacI restriction sites, whereas for V47 and V54
constructs, the same were inserted into NcoI/SacI restriction

sites. Synthetic dsDNA fragments were prepared by mixing
complementary single stranded DNA oligonucleotides at
equimolar concentrations, heating to 95° C. for 5 minutes,
and then cooling to room temperature at a rate of -1° C. per
minute. This annealing reaction was performed in TE buffer.
The double-stranded DNAs are shown below with the
encoded epitope sequence shown above.

V34/V55

M2 (2-24)

I S L L T E V E T P I R N E W G C R SEQ ID NO:53
AATTAGCCTGTTAACCGAAGTGGAGACGCCGATCCGTAACGAATGGGCTGCCG SEQ ID NO:54
TCGGACAATTGGCTTCACTCTGCGGCTAGGCATTGCTTACCCGACGGC SEQ ID NO:55
C N D S S D E L
CTGTAATGATTCTTCCGACGAGCT
GACATTACTAAGAAGGCTGC

V47/V54

M2 (1-24)

M S L L T E V E T P I R N E W G C R SEQ ID NO:56
CATGTCTCTGCTGACCGAAGTTGAAACCCCTATCAGAAACGAATGGGGTGCGAGA SEQ ID NO:57
AGAGACGACTGGCTTCAACTTTGGGGATAGTCTTTGCTTACCCCGAGTCT SEQ ID NO:58
C N D S S D E L
TGTAACGATTCAAGTGATGAGCT
ACATTGCTAAGTTCACTAC

-continued

M2 (1-24/C17S,C19S)
 M S L L T E V E T P I R N E W G S R SEQ ID NO:59
 CATGTCTCTGCTGACCGAAGTTGAAACCCCTATCAGAAACGAATGGGGGTCTAGA SEQ ID NO:60
 AGAGACGACTGGCTTCAACTTTGGGGATAGTCTTTGCTTACCCCCAGATCT SEQ ID NO:61
 S N D S S D E L
 TCGAACGATTCAAGTGATGAGCT
 AGCTTGCTAAGTTCCTAC

[0241] D. Construction of Truncated Version of Native M2-HBc

[0242] The original M2-HBc construct [Neirynck et al., (October 1999) *Nature Med.*, 5(10):1157-1163: WO 99/07839] that contained the 183-residue, full length HBc sequence was truncated to V149, and the entire gene was moved into the pKK223-3 expression vector. To achieve this, the plasmid 3453, which was provided by the University of Gent, was used as a template for a PCR reaction that yielded a product of 523 bp. This product was digested with restriction enzymes AflIII and HindIII, and then ligated into the pKK223-3N vector, which had been prepared by digestion with NcoI and HindIII.

AflIII-M2-F
 5'-CGCGACATGTCTCTGCTGACCG SEQ ID NO:62

HBc-HindIII-R
 5'-CGCAAGCTTAAACAACAGTAGTCTCCGGAAG SEQ ID NO:31

EXAMPLE 3

Vectors to Express Chimers Displaying Both
 N-terminal- and Loop-Inserted Influenza M2
 Sequences, Stabilized at the N-Terminus, or both
 N- and C-Termini

[0243] A. Vector to Express Hybrid Particles with both an N-terminal Fusion and a Loop Insertion, Stabilized at the N-terminus

[0244] To construct a vector containing two copies of the influenza M2 sequence, a copy of native M2 (1-24) at the N-terminus and a mutated version of M2 [M2(2-24/C17A, C19A)] in the immunodominant loop, the vector V47.M2(1-24)/V2.M2(2-24/C17A,C19A) was constructed. This vector was constructed by digesting vector V47.M2(1-24) with the restriction enzymes BglII and HindIII, isolating the digested vector, and inserting a 449 bp BglII-HindIII fragment from V2.M2(2-24/C17A,C19A).

[0245] B. Vector to Express Hybrid Particles with both an N-terminal Fusion and a Loop Insertion, stabilized at both the N- and C-termini

[0246] To construct a vector containing two copies of the influenza M2 sequence, a copy of native M2 (1-24) at the N-terminus, a mutated version of M2 [M2(2-24/C17A, C19A)] in the immunodominant loop, and a C-terminal cysteine, the vector V47.M2(1-24)/V16.M2(2-24/C17A, C19A) was constructed. This vector was constructed by digesting vector V47.M2(1-24) with the restriction enzymes BglII and HindIII, isolating the digested vector, and inserting a 452 bp BglII-HindIII fragment from V16.M2(2-24/C17A,C19A).

EXAMPLE 4

Assay Procedures

[0247] A. Antigenicity

[0248] 1. Particle ELISA

[0249] Purified particles were diluted to a concentration of 10 $\mu\text{g/mL}$ in coating buffer (50 mM sodium bicarbonate, pH 9.6) and coated onto the wells of ELISA strips (50 $\mu\text{L/well}$). The ELISA strips were incubated at room temperature overnight (about 18 hours). Next morning, the wells were washed with ELISA wash buffer [phosphate buffered saline (PBS), pH 7.4, 0.05% Tween®-20] and blocked with 3% BSA in PBS for 1 hour (75 $\mu\text{L/well}$). ELISA strips were stored, dry, at -20°C . until needed.

[0250] To determine the antigenicity of particles, antisera were diluted using 1% BSA in PBS and 50 $\mu\text{L/well}$ added to antigen-coated ELISA wells. Sera were incubated for 1 hour, washed with ELISA wash buffer (above) and probed using an anti-mouse (IgG)-HRP (The Binding Site, San Diego, Calif.; HRP=horseradish peroxidase) conjugate (50 $\mu\text{L/well}$) or other appropriate antibody for 30 minutes. After washing with ELISA wash buffer the reaction was visualized by the addition of TM blue substrate (50 $\mu\text{L/well}$). After 10 minutes, the reaction was stopped by the addition of 1N H_2SO_4 (100 $\mu\text{L/well}$) and read on an ELISA plate reader set at 450 nm.

[0251] 2. Synthetic Peptide ELISA

[0252] A 24 amino acid residue synthetic peptide M2 is diluted to a concentration of 2 $\mu\text{g/mL}$ in coating buffer (50 mM sodium bicarbonate, pH 9.6) and coated onto the wells of ELISA strips (50 $\mu\text{L/well}$). Peptides are dried onto the wells by incubating overnight (about 18 hours), in a hood with the exhaust on. Next morning, the wells are washed with ELISA wash buffer (phosphate buffered saline, pH 7.4, 0.05% Tween®-20) and blocked with 3% BSA in PBS (75 $\mu\text{L/well}$) for 1 hour. ELISA strips are stored, dry, at -20°C . until needed.

[0253] To determine antibody antigenicity of particles, antisera (monoclonal or polyclonal) are diluted using 1% BSA in PBS, and 50 $\mu\text{L/well}$ added to antigen-coated ELISA wells. Sera are incubated for 1 hour, washed with ELISA wash buffer, and probed using an anti-mouse(IgG)-HRP conjugate or other antibody (as above at 50 $\mu\text{L/well}$) for 30 minutes, washed again with ELISA wash buffer, and then visualized by the addition of TM blue substrate (50 $\mu\text{L/well}$). After 10 minutes, the reaction is stopped by the addition of 1N H_2SO_4 (100 $\mu\text{L/well}$) and read on an ELISA plate reader set at 450 nm.

[0254] B. Immunogenicity of Particles

[0255] To assay the immunogenicity of particles, mice are immunized, IP, with 20 μ g of particles in Freund's complete adjuvant, and then boosted at 4 weeks with 10 μ g in Freund's incomplete adjuvant. Mice were bled at 2, 4, 6, and 8 weeks.

EXAMPLE 5**Determination of 280:260 Absorbance Ratios**

[0256] To determine the 280:260 absorbance ratio of purified particles, the particles were diluted to a concentration of approximately 0.2 mg/mL in 20 mM sodium phosphate buffer, pH 6.8, and absorbance values determined at wavelengths of 260 and 280 nm. The absorbance measured at 280 nm was divided by the value at 260 nm to determine the 280:260 ratio. The ratios were obtained for several samples, including native particles (HBc183), HBc particles truncated after residue position 149 (HBc149), and several HBc chimeras that are identified elsewhere herein, are shown below in Table 1. Full length particles ICC-1559 are a preparation of the particles first reported in Neirynck et al., (October 1999) *Nature Med.*, 5(10):1157-1163, whereas full length particles ICC-1607 are similar particles in which the M2 polypeptide cysteines at polypeptide positions 17 and 19, (X₁₇ and X₁₉ of SEQ ID NO: 9) were mutated to serine residues.

TABLE 1

Particle Number	Full Length, (F) or C-Terminal Truncated, (T)	280/260 Absorbance Ratio
HBc183	F	0.84
HBc149	T	1.59
1438	T	1.57
1473	T	1.64
1475	T	1.04
1492	T	1.33
1559	F	0.68
1560	T	1.36
1590	T	1.51
1603	T	1.68
1604	T	1.40
1605	T	1.26
1607	F	0.73

EXAMPLE 6**Thermal Stability Protocol**

[0257] Purified particles were diluted to a concentration of 1 mg/mL using 50 mM NaPO₄, pH 6.8 and sodium azide was added to a final concentration of 0.02% to prevent bacterial growth. Samples were mixed with SDS-PAGE sample buffer (reducing) and run on 15% SDS-PAGE gels. Gels were stained using Coomassie Blue, and then analyzed.

EXAMPLE 7**Analytical Gel Filtration Analysis of Hybrid Particles**

[0258] Analytical gel filtration analysis of purified hybrid HBc particles was performed using a 25 mL Superose® 6 HR 10/30 chromatographic column (Amersham Pharmacia # 17-0537-01) and a BioCAD™ SPRINT Perfusion Chro-

matography System. The UV detector was set to monitor a wavelength of 280 nm. The column was equilibrated with 3 column volumes (CV; about 75 mL) of buffer (50 mM NaPO₄, pH 6.8) at a flow rate of 0.75 mL/minute.

[0259] The particles to be analyzed were diluted to a concentration of 1 mg/mL using 50 mM NaPO₄, pH 6.8. 200 Microliters (μ L) of the sample were then loaded onto a 200 μ L loop and injected onto the column. The sample was eluted from the column with 50 mM NaPO₄, pH 6.8 at a flow rate of 0.75 mL/minute.

[0260] Particles containing N-terminal cysteine residues or similar particles free of such cysteines were analyzed using the above procedure. Integration of the 280 nm trace was carried out using BioCAD™ software (PerSeptive™) to provide the results in

EXAMPLE 8**Influenza M2 Constructs**

[0261] Recently, Neirynck et al., (October 1999) *Nature Med.*, 5(10):1157-1163 and WO 99/07839 reported the fusion of the 24 amino acid extracellular domain of M2 to the N-terminus of full-length HBc particles (HBc183), lacking amino acid residues 1-4. A schematic representation of that construct referred to herein as IM2HBc is shown below in which the 24-mer is linked to the N-terminus of HBc.

IM2HBc
MSLLTEVETPIRNEWGCRCNDSSD-HBc (5-183) SEQ ID NO: 63

[0262] In one illustrative preparation, the M2 epitope was inserted into the immunodominant loop of hepatitis B core and particles referred to as ICC-1475 were successfully expressed and purified using techniques discussed previously for such insertions and purifications. A mutated version of the M2 epitope, in which two cysteine residues at M2 native positions 17 and 19 were substituted by alanine residues, was also expressed in the immunodominant loop (ICC-1473 particles) and the resulting particles purified. These two particles are illustrated schematically below.

ICC-1475
HBc (1-78)-GI-SLLTEVETPIRNEWGCRCNDSSD- SEQ ID NO: 64
EL-HBc (79-149)

ICC-1473
HBc (1-78)-GI-SLLTEVETPIRNEWGARANDSSD- SEQ ID NO: 65
EL-HBc (79-149)-C

[0263] The ICC-1473 particle construct yielded approximately 7-fold more purified particles when compared with the native sequence (ICC-1475). It remains to be determined if the mutation of the cysteine residues alters protective potential of the particles. However, epitopes delivered on the immunodominant loops of HBc are usually significantly more immunogenic as compared to when they are fused to other regions (including the N-terminus), and resulting particles exhibit reduced anti-HBc immunogenicity.

[0264] Particles have also been prepared in which the M2 N-terminal 24-mer epitope was fused to the N-terminus of C-terminal truncated hepatitis B core particles. That construct (ICC-1438) also contained the N-terminal pre-core

sequence (SEQ ID NO: 66). A similar construct was prepared that contained a single cysteine residue at the end of the hybrid protein (ICC-1492), in this case immediately after Val-149 of the HBC gene. These constructs are shown schematically below.

ICC-1438 SEQ ID NO:66
MGISLLTEVETPIRNEWGCRCNDSSDELLGWLWGI-HBc (2-149)

ICC-1492 SEQ ID NO:67
MGISLLTEVETPIRNEWGCRCNDSSDELLGWLWGI-HBc (2-149)-C

otides M2(17-24/C17S)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene from vector V2.Pf1. The resultant 546 bp fragment is cleaved with NcoI and HindIII and inserted into pKK-233-N, which has been cleaved with the same two enzymes.

[0270] To make V2.Pf1(N-M2(17-24/C19S)), the oligonucleotides M2(17-24/C19S)-NcoI-F and HBc149/HindIII-R are used to amplify the hybrid HBc gene in vector V2.Pf1. The resultant 540 bp fragment is cleaved with NcoI and HindIII and inserted into pKK223-3N, which had been cleaved with the same two enzymes.

M2 (17-24/C17S)-NcoI-F
M G S R C N D S S D I D P Y K E F G SEQ ID NO:68
GGCGCCATGGGGTCTAGATGTAACGATTCAAGTGACATCGACCCCTTATAAA GAATTCG SEQ ID NO:69

M2 (17-24/C19S)-NcoI-F
M G C N D S S D I D P Y K E F G SEQ ID NO:70
GCGCCATGGGGTGTAACGATTCAAGTGACATCGACCCCTTATAAAGAATTGG SEQ ID NO:71

[0265] It should be noted that to guard against translation initiation from the natural HBC initiator methionine, the codon for that residue was mutated to code for an isoleucine residue. Residues contributed by EcoRI (GI) and SacI (EL) restriction sites are underlined. The pre-core sequence is recited between the underlined EL residues and “-HBC(2-149)”

[0266] Analysis by SDS-PAGE as discussed elsewhere herein, showed that upon preparation, the ICC-1438 monomer construct was unstable (Lane 2) as compared to the ICC-1492 (Lane 3), with HBc-149 (Lane 1), ICC-1475 (Lane 4) and ICC-1473 (Lane 5) serving as additional molecular weight controls on the SDS-PAGE gel in **FIG. 10**. The instability of the ICC-1438 monomers was not evident using analytical gel filtration of particles.

[0267] Both ICC-1475 (FIG. 10, lane 4) and ICC-1473 (FIG. 10, lane 5) were expected to have slightly lower molecular weights than ICC-1438 and ICC-1492, because the former two contain the M2 epitope inserted directly into the immunodominant loop and therefore lack the pre-core sequence (SEQ ID NO: 66) present in ICC-1438 and ICC-1498. As expected, ICC-1492 was larger than ICC-1475 and ICC-1473; however, ICC-1438, which is identical to ICC-1492 save the C-terminal cysteine residue, is clearly not larger than ICC-1475 and ICC-1473 due to an apparent cleavage.

[0268] A construct containing a M2 N-terminal extracellular sequence as discussed above linked to the HBc N-terminus (Domain I) or loop (Domain II) and also containing a M2 protein C-terminal sequence such as that of SEQ ID NO: 11 (see Table A) linked the loop (Domain II) or at the C-terminus (Domain IV) of HBc is also contemplated. Such a contemplated construct also contains at least one stabilizing C-terminal cysteine residue as discussed elsewhere herein.

[0269] To modify the amino-terminus of hybrid HBc particles containing immunodominant loop fusions to incorporate a cysteine residue, and minimal M2-derived sequence, a series of synthetic oligonucleotides are synthesized. To make V2.P1f(N-M2(17-24/C17S)), the oligonucle-

EXAMPLE 9

HBc Chimera Molecules With and Without Both N- and C-Terminal Cysteine Residues

[0271] A series of HBc chimeric molecule-containing particles was prepared that contained residues 1-24 of the influenza A, M2 protein peptide-bonded at or near the N-terminus of HBc whose C-terminus was truncated at residue 149. The component chimeric protein molecules contained different N-terminal sequences that included an M2 sequence or variant, and some contained a C-terminal cysteine residue.

[0272] All purified particles listed in Table 2, below, were analyzed by analytical size exclusion chromatography to assess the retention of particulate structure following purification. Particles designated ICC-1603, which contain no N-terminal cysteine residues, displayed evidence of disassembly back to sub-particulate structures (**FIG. 3**) because the protein eluted in the 1500 second range (particles elute at approximately 1000 seconds).

[0273] Similar analysis of particles ICC-1590, which are similar to ICC-1603 ICC-particles except for the mutation of two serine residues to cysteine residues in the N-terminal M2 sequence, revealed that the construct remained particulate following purification, with elution occurring at around 1000 seconds, which is typical for a hybrid particle (**FIG. 4**). There was no evidence of disassembly for ICC-1590 particles.

[0274] Analysis of ICC-1560 particles, whose chimera protein also has two N-terminal cysteine residues, revealed that it too was particulate following purification, although it did exhibit some degree of disassembly (FIG. 5), suggesting that the stabilization was not quite as robust as it was for ICC-1590 particles. Comparison of the N-terminal configurations of ICC-1590 and ICC-1560 particles (Table 2, hereinafter), shows that the relative position of the two cysteine residues in ICC-1560 particles is shifted by 3 amino acid residues relative to ICC-1590 particles via the deletion of three amino acid residues (DEL), indicating that the cysteine residues may be required to be a minimal distance from the start of the core gene to enable optimal cross-linking.

EXAMPLE 10

Particles With an M2 or M2 Variant Sequence and
A C-Terminal Cysteine Residue

[0275] ICC-1603 particles were shown in FIG. 3 to rapidly disassemble following purification. The HBc chimer molecules that comprise ICC-1605 particles are similar to those of ICC-1603 particles, except that the ICC-1605 component chimer molecules have a single C-terminal stabilizing cysteine. A plasmid was made to direct the expression of ICC-1605 particles to investigate if the addition of a C-terminal cysteine residue to ICC-1603 particles could impart greater stability on the particle. Following purification, ICC-1605 particles were analyzed using analytical size exclusion chromatography (FIG. 6).

[0276] The results of this study demonstrated that particle stabilization was more complete than for the ICC-1603 particles, but incomplete compared to ICC-1590 particles, which contains two amino-terminal cysteine residues and no C-terminal stabilizing cysteine. Although a significant amount of ICC-1605 remained particulate, there was evidence of a heterogeneous mixture of sub-particulate structures that eluted over a broad range. These observations suggest that for this hybrid particle (ICC-1603), C-terminal stabilization as found in ICC-1605 particles was less complete than for the N-terminal stabilization found in ICC-1590 particles.

[0277] To investigate the compatibility of combined amino and carboxyl-terminal cysteine stabilization of hybrid

cation (FIG. 7), indicating that the two stabilizing methods are complementary and can be used in concert with each other.

[0278] Alternative linker sequences between the N-terminus of HBc and the N-terminal cysteine residues were investigated using particles ICC-1438 and ICC-1492. Both of these particles contain the amino acid sequence ELLGWLWGIDI (SEQ ID NO: 72) between the M2 fusion and amino acid D4 of HBc. Amino acid residues LGWLWGIDI are derived from amino acids -6 of pre-core to amino acid 13 of HBc, with the initiator codon of HBc mutated to an isoleucine to prevent translation initiation from this position, which would compromise the study. The HB pre-core sequence includes a cysteine at position -7.

[0279] These particles differed only in the fact that the ICC-1438 component chimer molecule terminated at position 149 of HBc, whereas the ICC-1492 component chimer molecule terminated at 149 of HBc and contained a terminal cysteine at position 150 relative to the HBc of SEQ ID NO: 1. When analyzed by analytical gel filtration, using an alternative but similar method to that discussed before, whereby particles elute at approximately 10 minutes, both constructs were shown to be particulate following purification (ICC-1438 in FIG. 8 and ICC-1492 in FIG. 10). This study demonstrated the compatibility of amino- and carboxyl-terminal cysteine stabilization of truncated particles, and the tolerance of substantial variability in the amino acid sequence and distance between the N-terminal cysteine residues and start of the HBc gene.

TABLE 2

Construct Name	N-terminal Fusion	HBc N-term Start	Residues Between M2 and HBc	C-term End	Bound Nucleic Acid	C-term Cysteine Stab.
ICC-1560	M2 (1-24)	D4	None	149	No	No
ICC-1603	M2 (1-24) (2C > 2S)	D4	EL	149	No	No
ICC-1590	M2 (1-24)	D4	EL	149	No	No
ICC-1604	M2 (1-24)	D4	EL	149	No	Yes (C150)
ICC-1605	M2 (1-24) (2C > 2S)	D4	EL	149	No	Yes (C150)
ICC-1438	M2 (1-24)	D2	ELLGWLWGI	149	No	No
ICC-1492	M2 (1-24)	D2	ELLGWLWGI	149	No	Yes (C150)

particles, an expression plasmid was constructed to direct the expression of ICC-1604 particles. The component chimer molecules of ICC-1604 particles contain both the two amino-terminal stabilizing cysteine residues present in a native M2 polypeptide sequence (as in ICC-1590) as well as a C-terminal stabilizing cysteine (as in ICC-1605 particles). Analysis of ICC-1604 particles showed that they retained a homogeneous particulate state following purifi-

[0280] Table 3, below, shows an alignment that illustrates the configuration of the N-termini of HBcAg, and particles designated ICC-1590, ICC-1560, ICC-1603, ICC-1604 and ICC-1605. Sequences are aligned according to amino acid residue position 4 from the N-terminus of HBc of SEQ ID NO:1 that is shared by all constructs. N-terminal cysteine residues, when present, are underlined.

TABLE 3

Construct Name	Sequence	SEQ ID NO
HBeAg	SKLCLGWLWGMDD	73
ICC-1590/ICC-1604	MSLLTEVETPIRNEWGCRQNDSSDELD	74
ICC-1560	MSLLTEVETPIRNEWGCRQNDSSD	18
ICC-1603/ICC-1605	MSLLTEVETPIRNEWGSRNDSSDELD	75
ICC-1438/ICC-1492	MGISLLTEVETPIRNEWGCRQNDSSDELLGWLWGIDID	76

[0281] Table 4, below, provides a tabulation of the results in which stability was assessed for particles containing an N-terminal influenza A M2 sequence or variant contemplated herein. As is seen, stable particles have been prepared from HBc chimera molecules that contain an N-terminal cysteine residue at a position of minus 14 (-14) relative to the N-terminus of the HBc sequence of SEQ ID NO: 1 to about the N-terminus itself.

TABLE 4

Construct	Amino Acids Between HBc D4 and N-terminal Cysteine Residues		C-terminal Cysteine	Stable Particle	
	Name	Cys 1	Cys 2	Stabilization	Formed
HBeAg	—	9	No	No	No
ICC-1603	—	—	No	No	No
ICC-1605	—	—	Yes	Yes/No	Yes/No
ICC-1590	9	7	No	Yes	Yes
ICC-1604	9	7	Yes	Yes	Yes
ICC-1560	6	4	No	Yes	Yes

TABLE 4-continued

Construct	Amino Acids Between HBc D4 and N-terminal Cysteine Residues		C-terminal Cysteine	Stable Particle
	Name	Cys 1	Cys 2	Stabilization
ICC-1438	18	16	No	Yes
ICC-1492	18	16	Yes	Yes

[0282] Each of the patents and articles cited herein is incorporated by reference. The use of the article "a" or "an" is intended to include one or more.

[0283] The foregoing description and the examples are intended as illustrative and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are possible and will readily present themselves to those skilled in the art.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 83

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<212> TYPE: PRT

<213> ORGANISM: Hepatitis B virus

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1 5 10 15

Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp
20 25 30

Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys
35 40 45

Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu
50 55 60

Leu Met Thr Leu Ala Thr Trp Val Gly Val Asn Leu Glu Asp Pro Ala
65 70 75 80

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys

-continued

	85		90		95
Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg					
	100		105		110
Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr					
	115		120		125
Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro					
	130		135		140
Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr					
	145		150		155
Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser					
	165		170		175
Gln Ser Arg Glu Ser Gln Cys					
	180				

<210> SEQ ID NO 2
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 <213> ORGANISM: Hepatitis B virus

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Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu					
1	5		10		15
Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp					
	20		25		30
Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys					
	35		40		45
Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu					
	50		55		60
Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Gln Asp Pro Ala					
	65		70		75
Ser Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Met Gly Leu Lys					
	85		90		95
Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg					
	100		105		110
Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr					
	115		120		125
Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro					
	130		135		140
Glu Thr Thr Val Val Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg					
	145		150		155
Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg					
	165		170		175
Arg Ser Gln Ser Arg Glu Ser Gln Cys					
	180		185		

<210> SEQ ID NO 3
 <211> LENGTH: 185
 <212> TYPE: PRT
 <213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 3

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1	5		10		15
Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp					

-continued

20	25	30
Thr Ala Ser Ala Leu Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys		
35	40	45
Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu		
50	55	60
Leu Met Thr Leu Ala Thr Trp Val Gly Asn Asn Leu Glu Asp Pro Ala		
65	70	75
Ser Arg Asp Leu Val Val Asn Tyr Val Asn Thr Asn Val Gly Leu Lys		
85	90	95
Ile Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg		
100	105	110
Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr		
115	120	125
Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro		
130	135	140
Glu Thr Thr Val Val Arg Arg Arg Asp Arg Gly Arg Ser Pro Arg Arg		
145	150	155
Arg Thr Pro Ser Pro Arg Arg Arg Pro Ser Gln Ser Pro Arg Arg Arg		
165	170	175
Arg Ser Gln Ser Arg Glu Ser Gln Cys		
180	185	

<210> SEQ ID NO 4

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<212> TYPE: PRT

<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 4

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1	5	10
Ser Phe Leu Pro Ser Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp		
20	25	30
Thr Ala Ala Ala Leu Tyr Arg Asp Ala Leu Glu Ser Pro Glu His Cys		
35	40	45
Ser Pro His His Thr Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Asp		
50	55	60
Leu Met Thr Leu Ala Thr Trp Val Gly Thr Asn Leu Glu Asp Pro Ala		
65	70	75
Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Val Gly Leu Lys		
85	90	95
Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg		
100	105	110
Glu Thr Val Leu Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr		
115	120	125
Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro		
130	135	140
Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr		
145	150	155
Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser		
165	170	175
Gln Ser Arg Glu Ser Gln Cys		
180		

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<210> SEQ ID NO 5
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<212> TYPE: PRT
<213> ORGANISM: Marmota monax

<400> SEQUENCE: 5

Met Asp Ile Asp Pro Tyr Lys Glu Phe Gly Ser Ser Tyr Gln Leu Leu
1 5 10 15
Asn Phe Leu Pro Leu Asp Phe Phe Pro Asp Leu Asn Ala Leu Val Asp
20 25 30
Thr Ala Thr Ala Leu Tyr Glu Glu Glu Leu Thr Gly Arg Glu His Cys
35 40 45
Ser Pro His His Thr Ala Ile Arg Gln Ala Leu Val Cys Trp Asp Glu
50 55 60
Leu Thr Lys Leu Ile Ala Trp Met Ser Ser Asn Ile Thr Ser Glu Gln
65 70 75 80
Val Arg Thr Ile Ile Val Asn His Val Asn Asp Thr Trp Gly Leu Lys
85 90 95
Val Arg Gln Ser Leu Trp Phe His Leu Ser Cys Leu Thr Phe Gly Gln
100 105 110
His Thr Val Gln Glu Phe Leu Val Ser Phe Gly Val Trp Ile Arg Thr
115 120 125
Pro Ala Pro Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro
130 135 140
Glu His Thr Val Ile Arg Arg Arg Gly Gly Ala Arg Ala Ser Arg Ser
145 150 155 160
Pro Arg Arg Arg Thr Pro Ser Pro Arg Arg Arg Ser Gln Ser Pro
165 170 175
Arg Arg Arg Arg Ser Gln Cys
180

<210> SEQ ID NO 6
<211> LENGTH: 217
<212> TYPE: PRT
<213> ORGANISM: *Spermophilus variegatus*

<400> SEQUENCE: 6

Met Tyr Leu Phe His Leu Cys Leu Val Phe Ala Cys Val Pro Cys Pro
1 5 10 15
Thr Val Gln Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Asp Met Asp
20 25 30
Ile Asp Pro Tyr Lys Glu Phe Gly Ser Ser Tyr Gln Leu Asn Phe
35 40 45
Leu Pro Leu Asp Phe Phe Pro Asp Leu Asn Ala Leu Val Asp Thr Ala
50 55 60
Ala Ala Leu Tyr Glu Glu Glu Leu Thr Gly Arg Glu His Cys Ser Pro
65 70 75 80
His His Thr Ala Ile Arg Gln Ala Leu Val Cys Trp Glu Glu Leu Thr
85 90 95
Arg Leu Ile Thr Trp Met Ser Glu Asn Thr Thr Glu Glu Val Arg Arg
100 105 110
Ile Ile Val Asp His Val Asn Asn Thr Trp Gly Leu Lys Val Arg Gln
115 120 125

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Thr Leu Trp Phe His Leu Ser Cys Leu Thr Phe Gly Gly His Thr Val
 130 135 140
 Gln Glu Phe Leu Val Ser Phe Gly Val Trp Ile Arg Thr Pro Ala Pro
 145 150 155 160
 Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro Glu His Thr
 165 170 175
 Val Ile Arg Arg Arg Gly Gly Ser Arg Ala Ala Arg Ser Pro Arg Arg
 180 185 190
 Arg Thr Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg
 195 200 205
 Arg Ser Gln Ser Pro Ala Ser Asn Cys
 210 215

<210> SEQ ID NO 7
 <211> LENGTH: 51
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: plasmid pkk223

<400> SEQUENCE: 7

ttcacacagg aaacagaatt cccggggatc cgtcgacctg cagccaagct t 51

<210> SEQ ID NO 8
 <211> LENGTH: 38
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: plasmid pkk223

<400> SEQUENCE: 8

ttcacataag gaggaaaaaa ccatgggatc cgaagctt 38

<210> SEQ ID NO 9
 <211> LENGTH: 24
 <212> TYPE: PRT
 <213> ORGANISM: Hepatitis B virus
 <220> FEATURE:
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (1)..(1)
 <223> OTHER INFORMATION: Xaa at position 1 is methionine or absent. If methionine then Xaa in positions 2 through 8 are not absent
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (2)..(2)
 <223> OTHER INFORMATION: Xaa at position 2 is serine or absent. If serine then Xaa in positions 3 through 8 are not absent.
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (3)..(3)
 <223> OTHER INFORMATION: Xaa at position 3 is leucine or absent. If leucine then Xaa in positions 4 through 8 are not absent.
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (4)..(4)
 <223> OTHER INFORMATION: Xaa at position 4 is leucine or absent. If leucine then Xaa in positions 5 through 8 are not absent.
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (5)..(5)
 <223> OTHER INFORMATION: Xaa at position 5 is threonine or absent. If threonine then Xaa in positions 6 through 8 are not absent.
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (6)..(6)
 <223> OTHER INFORMATION: Xaa at position 6 is glutamic acid or absent. If glutamic acid then Xaa in positions 7 through 8 are not absent.
 <221> NAME/KEY: MISC_FEATURE
 <222> LOCATION: (7)..(7)
 <223> OTHER INFORMATION: Xaa at position 7 is valine or absent. If valine then Xaa in position 8 is not absent.

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<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa at position 8 is glutamic acid or absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (15)..(15)
<223> OTHER INFORMATION: Xaa at position 15 is tryptophan or absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (16)..(16)
<223> OTHER INFORMATION: Xaa at position 16 is glycine or absent.
If glycine then Xaa in position 15 is not absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (17)..(17)
<223> OTHER INFORMATION: Xaa at position 17 is absent or present, if present Xaa in position 17 is cysteine, serine or alanine.
If Xaa in position 17 is present then positions 15 through 16 are not absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (18)..(18)
<223> OTHER INFORMATION: Xaa at position 18 is arginine or absent.
If arginine then Xaa in positions 15 through 17 are not absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (19)..(19)
<223> OTHER INFORMATION: Xaa at position 19 is absent or present, if present Xaa in position 19 is cysteine, serine or alanine.
If Xaa in position 19 is present then positions 15 through 18 are not absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (20)..(20)
<223> OTHER INFORMATION: Xaa at position 20 is asparagine or absent.
If asparagine then Xaa in positions 15 through 19 are not absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (21)..(21)
<223> OTHER INFORMATION: Xaa at position 21 is aspartic acid or absent.
If aspartic acid then Xaa in positions 15 through 20 are not absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (22)..(22)
<223> OTHER INFORMATION: Xaa at position 22 is serine or absent.
If serine then Xaa in positions 15 through 21 are not absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (23)..(23)
<223> OTHER INFORMATION: Xaa at position 23 is serine or absent.
If serine then Xaa in positions 15 through 22 are not absent.
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (24)..(24)
<223> OTHER INFORMATION: Xaa at position 24 is aspartic acid or absent.
If aspartic acid then Xaa in positions 15 through 23 are not absent.

<400> SEQUENCE: 9

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Thr Pro Ile Arg Asn Glu Xaa Xaa
1 5 10 15
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
20

<210> SEQ ID NO 10
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 10

Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly Cys
1 5 10 15

Arg Cys Asn Gly Ser Ser Asp
20

<210> SEQ ID NO 11
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

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<400> SEQUENCE: 11

Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly Cys
1 5 10 15

Arg Cys Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 12

<211> LENGTH: 23

<212> TYPE: PRT

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 12

Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly Ala
1 5 10 15

Arg Ala Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 13

<211> LENGTH: 23

<212> TYPE: PRT

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 13

Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly Ser
1 5 10 15

Arg Ser Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 14

<211> LENGTH: 23

<212> TYPE: PRT

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 14

Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly Ser
1 5 10 15

Arg Cys Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 15

<211> LENGTH: 23

<212> TYPE: PRT

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 15

Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly Cys
1 5 10 15

Arg Ser Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 16

<211> LENGTH: 23

<212> TYPE: PRT

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 16

Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly Cys
1 5 10 15

Arg Ala Asn Asp Ser Ser Asp

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20

<210> SEQ ID NO 17
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 17

Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly Ala
1 5 10 15
Arg Cys Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 18
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 18

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15
Cys Arg Cys Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 19
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 19

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15
Ser Arg Ser Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 20
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 20

Met Gly Ile Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu
1 5 10 15
Trp Gly Cys Arg Cys Asn Asp Ser Ser Asp Glu Leu Leu Gly Trp Leu
20 25 30
Trp Gly Ile
35

<210> SEQ ID NO 21
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 21

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15
Ala Arg Ala Asn Asp Ser Ser Asp
20

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<210> SEQ ID NO 22
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 22

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Cys Arg Ala Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 23
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 23

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Ala Arg Cys Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 24
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 24

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Cys Arg Ser Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 25
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 25

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Ser Arg Cys Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 26
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: plasmid pkk223

<400> SEQUENCE: 26

ggtgcatgca aggagatg

18

<210> SEQ ID NO 27
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: plasmid pkk223

<400> SEQUENCE: 27

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gcgaagcttc ggatcccatg gttttttcct ccttatgtga aattgttatc cgctc 55

<210> SEQ ID NO 28
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 28

gggccatgga catcgaccct ta 22

<210> SEQ ID NO 29
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 29

gcggaattcc ttccaaatta acaccacc 29

<210> SEQ ID NO 30
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 30

cgcggaattca aaaagagctc gatccagcgt ctagagac 38

<210> SEQ ID NO 31
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 31

cgcaagctta aacaacagta gtctccggaa g 31

<210> SEQ ID NO 32
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 32

ttgggccatg gacatcgacc cttta 24

<210> SEQ ID NO 33
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 33

gcggaattcc atcttccaaa ttaacacca c 31

<210> SEQ ID NO 34
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 34

cgcggaattca aaaagagctc ccagcgtcta gagacctag 39

<210> SEQ ID NO 35
<211> LENGTH: 39

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<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus
<400> SEQUENCE: 35
cgcggaattca aaaagagctc ccagcgtcta gagacctag 39

<210> SEQ ID NO 36
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus
<400> SEQUENCE: 36
ggaaagctta ctaacattga gattcccg 28

<210> SEQ ID NO 37
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: plasimd pkk223
SEQUENCE: 37
gcgggatccg gagcttatcg a 21

<210> SEQ ID NO 38
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus
<400> SEQUENCE: 38
gcggagctct tttgaattc ccattggttt ttcttcctta t 41

<210> SEQ ID NO 39
<211> LENGTH: 49
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus
<400> SEQUENCE: 39
gcggagctcc ttgggtggct ttggggcatt gacatcgacc cttataaag 49

<210> SEQ ID NO 40
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus
<400> SEQUENCE: 40
cgcaagctta ctacaaaca acagtagtct ccggaag 37

<210> SEQ ID NO 41
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: plasmid pkk223
<400> SEQUENCE: 41
gcataattcg tgctgctc 18

<210> SEQ ID NO 42
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

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<400> SEQUENCE: 42

gcggaattcc gatgtccatg gttttttctt 30

<210> SEQ ID NO 43

<211> LENGTH: 41

<212> TYPE: DNA

<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 43

gcggaattca aaaagagctc gacccttata aagaatttgg a 41

<210> SEQ ID NO 44

<211> LENGTH: 26

<212> TYPE: PRT

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 44

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Ser Arg Cys Asn Asp Ser Ser Asp Glu Leu
20 25

<210> SEQ ID NO 45

<211> LENGTH: 78

<212> TYPE: DNA

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 45

catgtctctg ctgaccgaag ttgaaacccc tatcagaaac gaatgggggt ctagatgtaa 60

cgattcaagt gatgagct 78

<210> SEQ ID NO 46

<211> LENGTH: 70

<212> TYPE: DNA

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 46

catcacttga atcgttacat ctagaccccc attcgtttct gatagggggt tcaacttcgg 60

tcagcagaga 70

<210> SEQ ID NO 47

<211> LENGTH: 26

<212> TYPE: PRT

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 47

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Cys Arg Ser Asn Asp Ser Ser Asp Glu Leu
20 25

<210> SEQ ID NO 48

<211> LENGTH: 78

<212> TYPE: DNA

<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 48

catgtctctg ctgaccgaag ttgaaacccc tatcagaaac gaatgggggt gcagatcgaa 60

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cgattcaagt gatgagct 78

<210> SEQ ID NO 49
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 49

catcacttga atcggtcgat ctgcaccccc attcggttct gataggggtt tcaacttcgg 60

tcagcagaga 70

<210> SEQ ID NO 50
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 50

Ile Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Ala Arg Ala Asn Asp Ser Ser Asp Glu Leu
20 25

<210> SEQ ID NO 51
<211> LENGTH: 78
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 51

aatttctctg ttaaccgaag tggagacgcc gattcgtaac gaatgggggtg cgcgcgccaa 60

tgatagctct gacgagct 78

<210> SEQ ID NO 52
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 52

cgtcagagct atcattggcg cgcgcacccc attcggtacg aatcggcgtc tccacttcgg 60

ttaacagaga 70

<210> SEQ ID NO 53
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 53

Ile Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Cys Arg Cys Asn Asp Ser Ser Asp Glu Leu
20 25

<210> SEQ ID NO 54
<211> LENGTH: 78
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 54

aattagcctg ttaaccgaag tggagacgcc gatccgtaac gaatgggggt ggcgctgtaa 60

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tgattttcc gacgagct 78

<210> SEQ ID NO 55
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 55

cgtcggaaga atcattacag cggcagcccc attcgttacg gatcggcgtc tccattcgg 60

ttaacaggct 70

<210> SEQ ID NO 56
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 56

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15Cys Arg Cys Asn Asp Ser Ser Asp Glu Leu
20 25

<210> SEQ ID NO 57
<211> LENGTH: 78
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 57

catgtctctg ctgaccgaag ttgaaacccc tatcagaaac gaatgggggt gcagatgtaa 60

cgattcaagt gatgagct 78

<210> SEQ ID NO 58
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 58

catcacttga atcgttacat ctgcaccccc attcgtttct gataggggtt tcaactcgg 60

tcagcagaga 70

<210> SEQ ID NO 59
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 59

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15Ser Arg Ser Asn Asp Ser Ser Asp Glu Leu
20 25

<210> SEQ ID NO 60
<211> LENGTH: 78
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 60

catgtctctg ctgaccgaag ttgaaacccc tatcagaaac gaatgggggt ctgatcgaa 60

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cgattcaagt gatgagct 78

<210> SEQ ID NO 61
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 61

catcacttga atcggtcgat ctagaccccc attgctttct gataggggtt tcaacttcgg 60

tcagcagaga 70

<210> SEQ ID NO 62
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 62

cgcgacatgt ctctgctgac cg 22

<210> SEQ ID NO 63
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 63

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15Cys Arg Cys Asn Asp Ser Ser Asp
20

<210> SEQ ID NO 64
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 64

Gly Ile Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp
1 5 10 15Gly Cys Arg Cys Asn Asp Ser Ser Asp Glu Leu
20 25

<210> SEQ ID NO 65
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 65

Gly Ile Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp
1 5 10 15Gly Ala Arg Ala Asn Asp Ser Ser Asp Glu Leu
20 25

<210> SEQ ID NO 66
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 66

Met Gly Ile Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu

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1 5 10 15

Trp Gly Cys Arg Cys Asn Asp Ser Ser Asp Glu Leu Leu Gly Trp Leu
 20 25 30

Trp Gly Ile
 35

<210> SEQ ID NO 67
<211> LENGTH: 35
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 67

Met Gly Ile Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu
1 5 10 15

Trp Gly Cys Arg Cys Asn Asp Ser Ser Asp Glu Leu Leu Gly Trp Leu
 20 25 30

Trp Gly Ile
 35

<210> SEQ ID NO 68
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 68

Met Gly Ser Arg Cys Asn Asp Ser Ser Asp Ile Asp Pro Tyr Lys Glu
1 5 10 15

Phe Gly

<210> SEQ ID NO 69
<211> LENGTH: 59
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 69

ggcgccatgg ggtctagatg taacgattca agtgacatcg acccttataa agaatttcg 59

<210> SEQ ID NO 70
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 70

Met Gly Cys Asn Asp Ser Ser Asp Ile Asp Pro Tyr Lys Glu Phe Gly
1 5 10 15

<210> SEQ ID NO 71
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Influenza A virus

<400> SEQUENCE: 71

gcgccatggg gtgtaacgat tcaagtgaca tcgaccctta taaagaattt gg 52

<210> SEQ ID NO 72
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 72

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Glu Leu Leu Gly Trp Leu Trp Gly Ile Asp Ile
1 5 10

<210> SEQ ID NO 73
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 73

Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile Asp
1 5 10

<210> SEQ ID NO 74
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 74

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Cys Arg Cys Asn Asp Ser Ser Asp Glu Leu Asp
20 25

<210> SEQ ID NO 75
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 75

Met Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu Trp Gly
1 5 10 15

Ser Arg Ser Asn Asp Ser Ser Asp Glu Leu Asp
20 25

<210> SEQ ID NO 76
<211> LENGTH: 38
<212> TYPE: PRT
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 76

Met Gly Ile Ser Leu Leu Thr Glu Val Glu Thr Pro Ile Arg Asn Glu
1 5 10 15

Trp Gly Cys Arg Cys Asn Asp Ser Ser Asp Glu Leu Leu Gly Trp Leu
20 25 30

Trp Gly Ile Asp Ile Asp
35

<210> SEQ ID NO 77
<211> LENGTH: 549
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 77

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gccttagagt ctctgagca ttgttcacct caccatactg cactcaggca agcaattctt 180
tgctgggggg aactaatgac tctagctacc tgggtgggtg ttaatttga agatccagcg 240

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tctagagacc tagtagtcag ttatgtcaac actaatatgg gcctaaagtt caggcaactc 300
ttgtggtttc acattttctg tctcactttt ggaagagaaa cagttataga gtatttggtg 360
tctttcggag tgtggattcg cactcctcca gcttatagac caccaaatgc ccctatccta 420
tcaacacttc cggagactac tgttgtaga cgacgaggca ggtcccctag aagaagaact 480
ccctcgctc gcagacgaag gtctcaatcg ccgcgtcgca gaagatctca atctcgggaa 540
tctcaatgt 549

<210> SEQ ID NO 78
<211> LENGTH: 555
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 78

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tctgacttct ttccttcctg acgagatctc ctgacaccg cctcagctct gtatcgagaa 120
gccttagagt ctctgagca ttgctcacct caccatactg cactcaggca agccattctc 180
tgctgggggg aattgatgac tctagctacc tgggtgggta ataatttgca agatccagca 240
tccagagatc tagtagtcaa ttatgttaat actaacatgg gtttaaagat caggcaacta 300
ttgtggtttc atatatcttg ccttactttt ggaagagaga ctgtacttga atatttggtc 360
tctttcggag tgtggattcg cactcctcca gcctatagac caccaaatgc ccctatctta 420
tcaacacttc cggaaactac tgttgtaga cgacgggacc gaggcaggtc ccctagaaga 480
agaactccct cgcctcgag acgcagatct caatcgccgc gtcgcagaag atctcaatct 540
cgggaatctc aatgt 555

<210> SEQ ID NO 79
<211> LENGTH: 555
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 79

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tctgacttct ttccttcctg cagagatctc ctgacaccg cctcagctct gtatcgagaa 120
gccttagagt ctctgagca ttgctcacct caccatactg cactcaggca agccattctc 180
tgctgggggg aattgatgac tctagctacc tgggtgggta ataatttgga agatccagca 240
tctaggatc ttgtagtaa ttatgttaat actaacgtgg gtttaaagat caggcaacta 300
ttgtggtttc atatatcttg ccttactttt ggaagagaga ctgtacttga atatttggtc 360
tctttcggag tgtggattcg cactcctcca gcctatagac caccaaatgc ccctatctta 420
tcaacacttc cggaaactac tgttgtaga cgacgggacc gaggcaggtc ccctagaaga 480
agaactccct cgcctcgag acgcagatct caatcgccgc gtcgcagaag atctcaatct 540
cgggaatctc aatgt 555

<210> SEQ ID NO 80
<211> LENGTH: 549
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B virus

<400> SEQUENCE: 80

-continued

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gccttagagt ctcctgagca ttgttcacct caccatactg cactcaggca agcaattctt	180
tgctggggag acttaatgac tctagctacc tgggtgggta ctaatttaga agatccagca	240
tctagggacc tagtagtcag ttatgtcaac actaatgtgg gcctaaagtt cagacaatta	300
ttgtggttcc acatttcttg tctcactttt ggaagagaaa cggttctaga gtatttggtg	360
tcttttgagg tgtggattcg cactcctcca gcttatagac caccaaatgc ccctatccta	420
tcaacgcttc cggagactac tgttgtaga cgacgaggca ggtcccctag aagaagaact	480
ccctcgcttc gcagacgaag atctcaatcg ccgcgtcgca gaagatctca atctcgggaa	540
tctcaatgt	549

<210> SEQ ID NO 81
 <211> LENGTH: 549
 <212> TYPE: DNA
 <213> ORGANISM: Marmota monax

<400> SEQUENCE: 81

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gaattttctt cctttggact tctttcctga tcttaatgct ttggtggaca ctgctactgc	120
cttgatgaa gaagaactaa caggtaggga acattgctct ccgcaccata cagctattag	180
acaagcttta gtatgctggg atgaattaac taaattgata gcttggtaga gctctaact	240
aacttctgaa caagtaagaa caatcattgt aaatcatgtc aatgatacct ggggacttaa	300
ggtgagacaa agtttatggt ttcatttgtc atgtctcact ttcggacaac atacagtcca	360
agaattttta gtaagttttg gagtatggat caggactcca gctccatata gacctcctaa	420
tgacccatt ctctcgactc ttcgggaaca tacagtcatt aggagaagag gaggtgcaag	480
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gcgtcgag	549

<210> SEQ ID NO 82
 <211> LENGTH: 651
 <212> TYPE: DNA
 <213> ORGANISM: Spermophilus variegatus

<400> SEQUENCE: 82

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tccaagctgt gccttgatg gctttgggac atggacatag atccctataa agaatttggt	120
tcttcttctc agttgttgaa ttttcttctt ttggactttt ttcctgatct caatgcattg	180
gtggacactg ctgctgctct ttatgaagaa gaattaacag gtagggagca ttgttctcct	240
catcatactg ctattagaca ggccttagtg tgttggaag aattaactag attaattaca	300
tggtgagtg aaaatacaac agaagaagtt agaagaatta ttgttgatca tgtcaataat	360
acttgaggac ttaaagtaag acagacttta tggtttcatt tatcatgtct tacttttgga	420
caacacacag ttcaagaatt tttggtagt tttggagtat ggattagaac tccagctcct	480
tatagaccac ctaatgcacc cattttatca actcttcccg aacatacagt cattaggaga	540
agaggagggt caagagctgc taggtcccc cgaagacgca ctccctctcc tcgcaggaga	600

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aggtctcaat caccgcgtcg cagacgctct caatctccag ctccaactg c

651

<210> SEQ ID NO 83
 <211> LENGTH: 17
 <212> TYPE: PRT
 <213> ORGANISM: Influenza B virus
 <400> SEQUENCE: 83

Asn Asn Ala Thr Phe Asn Tyr Thr Asn Val Asn Pro Ile Ser His Ile
 1 5 10 15

Arg

What is claimed:

1. A recombinant hepatitis B virus core (HBc) protein chimer molecule with a length of about 150 to about 325 amino acid residues that contains four peptide-linked amino acid residue sequence domains from the N-terminus that are denominated Domains I, II, III and IV, and includes at least one peptide-bonded polypeptide of about 6 to about 24 residues of the influenza A M2 polypeptide of SEQ ID NO: 9 wherein

(a) Domain I comprises (i) about 75 to about 110 amino acid residues whose sequence includes at least the sequence of the residues of position 4 through position 75 of HBc, (ii) one to three cysteine residues present at a position in the chimer molecule of about one to about -20 relative to the N-terminus of HBc of SEQ ID NO: 1 [N-terminal cysteine residue(s)], said one or more N-terminal cysteine residues being present within a sequence other than that of the pre-core sequence of HBc, and optionally includes said sequence of about 6 to about 24 residues of the influenza A M2 polypeptide of SEQ ID NO: 9 that, when present, is peptide-bonded to or within about 15 residues of the N-terminus of the HBc sequence,

(b) Domain II comprises about 10 to about 60 amino acid residues peptide-bonded to residue 75 of which (i) zero to all of the sequence of HBc is present from position 76 through 85 and (ii) an optional sequence of 6 to about 48 residues that constitute one or more repeats of the influenza A M2 polypeptide of SEQ ID NO: 9;

(c) Domain III is an HBc sequence from position 86 through position 135 peptide-bonded to residue 85; and

d) Domain IV comprises (i) the residues of positions 136-140 plus up to nine residues of an HBc amino acid residue sequence from position 141 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero to three cysteine residues, and (iii) up to about 100 amino acid residues in a sequence heterologous to HBc from position 164 to the HBc C-terminus;

said chimer molecule (i) containing no more than 10 percent conservatively substituted amino acid residues in the HBc sequence, (ii) self-assembling into particles that are substantially free of binding to nucleic acids on expression in a host cell, and said particles being more stable on formation than are particles formed from an otherwise identical HBc chimer that lacks said N-ter-

минаl cysteine residue(s) or in which an N-terminal cysteine residue present in the chimer molecule is replaced by another residue.

2. The recombinant HBc chimer protein molecule according to claim 1 wherein one of said residues X₁₇ and X₁₉ of said M2 polypeptide of SEQ ID NO: 9 is cysteine.

3. The recombinant HBc chimer protein molecule according to claim 1 wherein said M2 polypeptide of SEQ ID NO: 9 includes residues X₂ through X₂₄.

4. The recombinant HBc chimer protein molecule according to claim 1 wherein M2 polypeptide of SEQ ID NO: 9 is present in Domain I.

5. The recombinant HBc chimer protein molecule according to claim 1 wherein M2 polypeptide of SEQ ID NO: 9 is present in Domain II.

6. The recombinant HBc chimer protein molecule according to claim 1 wherein Domain I consists essentially of the HBc sequence from position 2 through position 75.

7. The recombinant HBc chimer protein molecule according to claim 1 wherein Domain IV contains zero cysteine residues.

8. The recombinant HBc chimer protein molecule according to claim 1 wherein Domain IV is free of said sequence heterologous to HBc at position 164 to the C-terminus.

9. A recombinant hepatitis B virus core (HBc) protein chimer molecule with a sequence of about 155 to about 225 amino acid residues that contains four peptide-linked domains from the N-terminus that are denominated Domains I, II, III and IV, and includes at least one peptide-bonded polypeptide of about 6 to about 24 residues of the influenza A M2 polypeptide of SEQ ID NO: 9 wherein

(a) Domain I comprises (i) about 95 to about 100 amino acid residues whose sequence includes at least the sequence of the residues of position 4 through position 75 of HBc, (ii) one to three cysteine residues present at a position in the chimer molecule of about one to about -14 relative to the N-terminus of HBc of SEQ ID NO: 1 [N-terminal cysteine residue(s)], said one or more N-terminal cysteine residues being present within a sequence other than that of the pre-core sequence of HBc, and optionally includes said sequence of about 6 to about 24 residues of the influenza A M2 polypeptide of SEQ ID NO: 9 that, when present, is peptide-bonded to or within about 15 residues of the N-terminus of the HBc sequence,

(b) Domain II comprises about 10 to about 60 amino acid residues peptide-bonded to residue 75 of which (i) zero

- to all of the sequence of HBc is present from position 76 through 85 and (ii) an optional sequence of 6 to about 48 residues that constitute one or more repeats of the influenza A M2 polypeptide of SEQ ID NO: 9;
- (c) Domain III consists essentially of the HBc sequence from position 86 through position 135; and
- d) Domain IV comprises (i) the residues of positions 136-140 plus up to nine residues of an HBc amino acid residue sequence from position 141 through 149 peptide-bonded to the residue of position 135 of Domain III, (ii) zero or one cysteine residue, and (iii) up to about 50 amino acid residues in a sequence heterologous to HBc from position 164 to the HBc C-terminus;
- said chimer molecule (i) containing no more than 5 percent conservatively substituted amino acid residues in the HBc sequence, (ii) self-assembling into particles that are substantially free of binding to nucleic acids on expression in a host cell, and said particles being more stable on formation than are particles formed from an otherwise identical HBc chimer that lacks said N-terminal cysteine residue(s) or in which an N-terminal cysteine residue present in the chimer molecule is replaced by another residue.
10. The recombinant HBc chimer protein molecule according to claim 9 wherein Domain IV includes a sequence of about nine amino acid residues of the HBc sequence from residue position 141 through about position 149 peptide-bonded to residue 140.
11. The recombinant HBc chimer protein molecule according to claim 9 wherein Domain I includes one N-terminal cysteine residue.
12. The recombinant HBc chimer protein molecule according to claim 9 wherein Domain IV includes one C-terminal cysteine residue.
13. The recombinant HBc chimer protein molecule according to claim 9 wherein Domain I includes one N-terminal cysteine residue and Domain IV includes one C-terminal cysteine residue.
14. The recombinant HBc chimer protein molecule according to claim 9 wherein one of said residues X₁₇ and X₁₉ of said M2 polypeptide of SEQ ID NO: 9 is cysteine.
15. The recombinant HBc chimer protein molecule according to claim 9 wherein said M2 polypeptide of SEQ ID NO: 9 includes residues X₂ through X₂₄.
16. The recombinant HBc chimer protein molecule according to claim 9 wherein Domain II comprises the amino acid residues of the sequence of HBc from position 76 through 85.
17. The recombinant HBc chimer protein molecule according to claim 9 wherein Domain II comprises the amino acid residues of the sequence of HBc from position 76 through 85 that further includes 6 to about 23 residues of the influenza A M2 polypeptide of SEQ ID NO: 9.
18. The recombinant HBc chimer protein molecule according to claim 9 wherein Domain I includes 6 to about 23 residues of the influenza A M2 polypeptide of SEQ ID NO: 9.
19. The recombinant HBc chimer protein molecule according to claim 9 that has a length of about 155 to about 170 residues.
20. Particles comprised of recombinant hepatitis B virus core (HBc) protein chimer molecules according to claim 1.
21. Particles comprised of recombinant hepatitis B virus core (HBc) protein chimer molecules according to claim 20 that include the C-terminal 23 residues of the influenza A M2 polypeptide of SEQ ID NO: 9.
22. Particles comprised of recombinant hepatitis B virus core (HBc) protein chimer molecules according to claim 9.
23. Particles comprised of recombinant hepatitis B virus core (HBc) protein chimer molecules according to claim 22 that include the C-terminal 23 residues of the influenza A M2 polypeptide of SEQ ID NO: 9.
24. A vaccine or inoculum comprising an immunogenic effective amount immunogenic particles according to claim 1 dissolved or dispersed in a pharmaceutically acceptable diluent.
25. A vaccine or inoculum comprising an immunogenic effective amount immunogenic particles according to claim 9 dissolved or dispersed in a pharmaceutically acceptable diluent.
26. A nucleic acid that encodes a recombinant HBc protein molecule according to claim 1, or a variant, analog or complement thereof.
27. A nucleic acid that encodes a recombinant HBc protein molecule according to claim 9, or a variant, analog or complement thereof.
28. A recombinant nucleic acid molecule that comprises a vector operatively linked to a nucleic acid segment defining a gene that encodes a recombinant HBc protein molecule according to claim 1, or a variant, analog or complement thereof, and a promoter suitable for driving the expression of the gene in a compatible host organism.
29. A recombinant nucleic acid molecule that comprises a vector operatively linked to a nucleic acid segment defining a gene that encodes a recombinant HBc protein molecule according to claim 9, or a variant, analog or complement thereof, and a promoter suitable for driving the expression of the gene in a compatible host organism.
30. A host cell transformed with a recombinant nucleic acid molecule according to claim 28.
31. The transformed host cell according to claim 30 wherein said host cell is selected from the group consisting of *E. coli*, *S. typhi*, *S. typhimurium*, and a *S. typhimurium*-*E. coli* hybrid.
32. A host cell transformed with a recombinant nucleic acid molecule according to claim 29.
33. The transformed host cell according to claim 32 wherein said host cell is selected from the group consisting of *E. coli*, *S. typhi*, *S. typhimurium*, and a *S. typhimurium*-*E. coli* hybrid.

* * * * *



Industria y Comercio
SUPERINTENDENCIA

**REQUISITOS MÍNIMOS PARA ADMISIÓN A TRAMITE
PCT**

PATENTE DE INVENCION ☒

MODELO DE UTILIDAD ☐

- ◇ Indicación de que se solicita la concesión de una patente ☒
- ◇ Datos de identificación del solicitante o de la persona que presenta la solicitud ☒
- ◇ Descripción de la invención ☒
- ◇ Dibujos de ser estos pertinentes ☒
- ◇ Comprobante de pago de las tasas establecidas ☒

COMPLETA ☒

INCOMPLETA ☐

DISEÑO INDUSTRIAL ☐

- ◇ Indicación de que se solicita el registro de Diseño Industrial ☐
- ◇ Datos de identificación del solicitante o de la persona que presenta la solicitud ☐
- ◇ Representación gráfica y fotográfica del Diseño Industrial o muestra del material que incorpora el diseño ☐
- ◇ Comprobante de pago de las tasas establecidas ☐

COMPLETA ☐

INCOMPLETA ☐

ESQUEMA DE TRAZADO ☐

- ◇ Indicación de que se solicita el registro de un Esquema de Trazado ☐
- ◇ Datos de identificación del solicitante o de la persona que presenta la solicitud ☐
- ◇ Representación gráfica del esquema trazado ☐
- ◇ Comprobante de pago de las tasas establecidas ☐

COMPLETA ☐

INCOMPLETA ☐


Firma Responsable

Fecha _____

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Fax: 3505220
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Bogotá D.C. - Colombia

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Bogotá,
2020

Doctor(a)
RAISBECK J. IAN

REFERENCIA	Radicación No.	9 35922
	Solicitud internacional	PCT/EP2007/006190
	Fecha inicio fase nacional	08/04/2009
	Trámite	11
	Evento	379
	Actuación	430
	Oficio No.	- 5134

Como resultado del examen de forma, realizado con fundamento en el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT), regla 51 bis del Reglamento y Circular Única de la Superintendencia de Industria y Comercio, se le comunica que:

1. La solicitud no contiene el poder debidamente otorgado, con el lleno de los requisitos exigidos por los artículos 63 y subsiguientes del Código de Procedimiento Civil. Adicionalmente, deberá presentar el documento que acredita la existencia y representación legal de la sociedad solicitante, cuando ésta fuere persona jurídica.

2. El solicitante debe aclarar si la solicitud corresponde al capítulo I o II del PCT por que la indicación en el petitorio no corresponde a los documentos del expediente, en caso necesario adjuntar los documentos que corresponden.

3. La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante, conforme a lo establecido por el artículo 26 literal k) de la Decisión 486 de la Comisión de la Comunidad Andina. Cuando se trate de documentos otorgados en el extranjero, estos deberán legalizarse de conformidad con lo dispuesto por los artículos 259 y 260 del Código de Procedimiento Civil. Cuando se trate de las declaraciones a que hace referencia la Regla 4.17 ii) del Tratado de Cooperación en Materia de Patentes PCT., el alcance de las tales declaraciones se determinará según indiquen claramente una cesión de derechos del inventor al solicitante o su causante, de conformidad con lo dispuesto en el numeral 5.2.15 del Capítulo Quinto, Título X de la Circular Única de Superintendencia de Industria y Comercio.

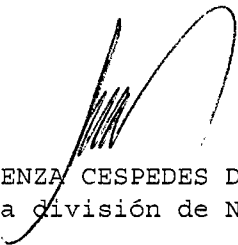
En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486, se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Unica No.10 de 2001.

Continúa en la página siguiente...

REPUBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Radicación No.: 9 35922



ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la división de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista de
fecha 08 MAYO 2009
jfernand