SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 14-98853- -13 **FECHA**: 2016-03-17 09:00:00
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 7
ACT: 519 TRASLADOINFORME

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
ANDREA TOBOS MATEUS

Asunto: Radicación: 14-98853- -13
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 2924
Folios: 7

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2012/059708				
Fecha de Presentación Internacional	11/10/2012				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado N° 14-98853-00000-0000	08/Mayo/2014
Reivindicaciones:	Radicado N° 14-98853-00000-0000	08/Mayo/2014
	Radicado N° 14-98853-00001-0000	13/Mayo/2014
	Radicado N° 14-98853-00005-0000	28/Julio/2014
	Radicado N° 14-98853-00007-0000	23/Enero/2015
	Radicado N° 14-98853-00012-0000	26/Enero/2016
Dibujos:	Radicado N° 14-98853-00000-0000	08/Mayo/2014
Secuencias:	Radicado N° 14-98853-00000-0000	08/Mayo/2014
Prioridad:	US 61/546,248	12/Octubre/2011

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones aceptadas (Art 34 D 486)

Se aceptan las modificaciones a las reivindicaciones en los folios 13 a 15 del Radicado N° 14-98853-00012-0000, ya que no amplían la materia inicialmente presentada, y sobre dicho capítulo se realiza el análisis de patentabilidad.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 17 del capítulo reivindicatorio del Radicado N° 14-98853-00012-0000.

Título de la solicitud: “PROTEÍNA NAGLU HUMANA RECOMBINANTE” (Modificado en el Radicado N° 14-98853-00012-0000)

El problema planteado en esta solicitud consiste en la necesidad de proporcionar una proteína NaGlu estable que sea enzimáticamente activa y que tenga propiedades físicas que le permitan atravesar la barrera hematoencefálica (BHE) e introducirse de manera eficaz en los lisosomas de las células diana, para su uso terapéutico, por ejemplo, en el tratamiento del síndrome de Sanfilippo de tipo B.

Para resolver este problema técnico la solicitud en estudio proporciona composiciones que comprenden la proteína NaGlu humana recombinante (rhNaGlu).

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K38/47; C12N5/07; C12N9/26.

5. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 12 a 14 y 16 no es patentable de acuerdo con el art citado, debido a que la composición farmacéutica que comprende la proteína NaGlu recombinante humana, se encuentra caracterizada porque se administra eficazmente en el cerebro de un mamífero de manera intravenosa, por lo cual el alcance de estas reivindicaciones es una excepción a la patentabilidad de conformidad con lo dispuesto en el artículo 20 lit. d) de la referida Decisión 486.

6. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

1– Las reivindicaciones 1, 6, y 17 no son claras porque definen una composición que comprende una mezcla aislada de N – acetil – α – D – glucosaminidasa recombinante (rhNaGlu), en términos de su resultado alcanzar que da cuenta de su mecanismo de acción, y del mecanismo con el cual se establece la proporción molar de M6P a proteína rhNaGlu, al indicar que dicha M6P que contiene rhNaGlu puede ser internalizada en una célula de mamífero que tiene deficiencia de NaGlu por endocitosis mediada por el

receptor de M6P y restablece 50% de la actividad de la NaGlu observada en una célula del tipo natural del mismo tipo que expresa la NaGlu endógena; que dicha rhNaGlu restablece 60, 70, 80, 90, 95 o 100% de la actividad enzimática normal de NaGlu; y que la proporción de M6P a proteína rhNaGlu es determinada por medio de cromatografía de intercambio aniónico de alto rendimiento con detección amperométrica pulsada (HPAEC-PAD). En este caso las reivindicaciones incluirían no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se le recuerda a la solicitante que una composición farmacéutica se debe definir a partir de sus características técnicas esenciales, especificando sus ingredientes, excipientes en las cantidades o proporciones específicas características de dicha composición.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 12 de octubre de 2011, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO2009/131698	"Phosphorylated Recombinant N-Acetyl-alpha-D- Glucosaminidase (NaGlu) and uses thereof"	29/Octubre/2009
D3	Jung – Ja P. K. *et al., Structural Biology,* 2009. Vol. 19, Pág. 534 – 542.	"Carbohydrate recognition by the mannose-6-phosphate receptors"	Octubre/2009

El documento D1[1] divulga proteínas de fusión recombinantes NaGl fosforiladas, métodos de producción y usos de las mismas (Abstract).

El documento D3[2] avances en los análisis estructurales de los receptores de manosa – 6 – fosfato (M6P) catión – dependiente (CD – MPR) y de manosa – 6 – fosfato (M6P) catión – independiente (CI – MPR), que evidencian las bases estructurales para el reconocimiento de los residuos fosfomanosílicos, proporcionando una mayor comprensión acerca de cómo cargan y descargan su carga esta clase de receptores (Abstract).

8. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en *Una composición que comprende una mezcla aislada de N – acetil – alfa – glucosaminidasa (rhNaGlu) humana recombinante cuya secuencia de aminoácidos se expone en 24 a 743 de SEQ ID NO: 1, en la que dicha mezcla comprende rhNaGlu que contiene estructuras de glucano que comprenden manosa – 6 – fosfato (M6P) ….."*. (Ver página 13 del radicado Nº Radicado Nº 14-0098853-00012-0000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características Esenciales	D1	D3
Composición que comprende una mezcla aislada de N – acetil – alfa – glucosaminidasa (rhNaGlu) humana recombinante	Composición que comprende una proteína de fusión aislada recombinante que contiene N – acetil – alfa – glucosaminidasa (rhNaGlu) (Pág. 21, Rv. 19).	N – acetil – alfa – glucosaminidasa (Pág. 535, Fig. 1)
Comprende la secuencia de aminoácidos que se expone en 24 a 743 de SEQ ID NO: 1	Proteína NaGlu comprende la SEQ ID NO: 1 o una secuencia de aminoácidos que presenta al menos 80% de identidad con la secuencia SEQ ID NO: 1 (Pág.	No registra

1 http://worldwide.espacenet.com/publicationDetails/biblio?CC=WO&NR=2009131698A2&KC=A2&FT=D
2 http://www.sciencedirect.com/science/article/pii/S0959440X09001328

	20, Rv. 12 – Fig. 3B).	
Comprende manosa – 6 – fosfato (M6P)	La proteína de fusión comprende manosa – 6 – fosfato (Pág. 21, Rv. 19).	Enzima lisosomal que adquiere una etiqueta Man – 6 – P (Pág. 535, Fig. 1)
Proporción de M6P a proteína rhNaGlu es 1 a 6 moles de M6P por mol de proteína	Proteína recombinante NaGlu unida a M6P (Pág. 8, líneas 1 – 11; Pág. 30 Fig. 14) (relación molar 1: 1)	Cinco potenciales sitios de fosforilación en residuos de manosa (Pág. 535, Fig. 1)

Como se dijo anteriormente, el documento D1 es considerado el estado de la técnica cercano a la composición definida en la reivindicación 1, porque divulga composiciones que comprenden una proteína de fusión recombinante aislada fosforilada con manosa – 6 – fosfato y N – acetil – alfa – glucosaminidasa (NaGlu) y un portador farmacéuticamente aceptable y/o un medio de cultivo (Pág. 4, líneas 12 - 14).

La diferencia entre la composición, definida en la reivindicación 1 y la composición que comprende una proteína de fusión aislada recombinante que contiene N – acetil – alfa – glucosaminidasa (rhNaGlu) divulgada en el documento D1, consiste en que la proporción de M6P a proteína rhNaGlu es 1 a 6 moles de M6P por mol de proteína.

No hay evidencia de avances o ventajas asociadas a esta diferencia, puesto que la composición divulgada en el documento D1 al igual que la alternativa propuesta por la solicitante, permite la internalización de una proteína de fusión recombinante humana, que comprende una proteína N – acetil – alfa – glucosaminidasa (NaGlu) fosforilada con manosa – 6 – fosfato, a través de mecanismos de endocitosis en una célula humana, la cual presenta deficiencia en la actividad enzimática de NaGlu (Pág. 26, Fig. 10A), con el objetivo de tratar una enfermedad autosómica recesiva de almacenamiento lisosomal (LSD), como el síndrome de Sanfilippo de tipo B (Pág. 17, líneas 8 - 14). Por lo tanto no es posible establecer cuál es el aporte al estado de la técnica con el diseño de la alternativa propuesta.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: Como modificar la composición divulgada en el documento D1 para que comprenda una proteína N - acetil- alfa - glucosaminidasa humana recombinante (rhNaGlu) estable, enzimáticamente activa y con propiedades físicas que le permitan atravesar la barrera hematoencefálica (BHE), siendo internalizada por endocitosis en una célula de mamífero que tiene deficiencia de NaGlu, para tratar el síndrome de Sanfilippo de tipo B.

Sin embargo, el documento D3, divulga los cinco potenciales sitios de fosforilación de residuos de manosa sobre la proteína N – acetil – alfa – glucosaminidasa NaGlu (Pág. 535, Fig. 1), indicando que la fosforilación de N –glucanos en proteínas lisosomales permite la internalización celular de dichas proteínas y su posterior transporte hacia los lisosomas mediado por la interacción del receptor de M6P; además afirma, que las enfermedades autosómicas recesivas como el síndrome Sanfilippo tipo B se originan por la deficiencia en la actividad de la GlcNAc – fosfotransferasa, la cual es la enzima encargada de generar la etiqueta de M6P (Pág. 534, Col. 2).

Por lo tanto, la persona del oficio normalmente versada en la materia, realizaría la fosforilación de uno o mas residuos de manosa, en los cinco sitios potenciales de fosforilación de la proteína N – acetil – alfa – glucosaminidasa NaGlu (Pág. 535, Fig. 1), con el objetivo de incrementar la concentración de esta enzima lisosomal divulgados en el documento D3, en la proteína NaGlu recombinante humana fosforilada (rhNaGlu) divulgada en el documento D1, en donde se indica la importancia de fosforilar la enzima recombinante con M6P, para atravesar la barrera hematoencefálica (BHE) (Pág. 16, líneas 21- 34 - Pág. 17, líneas 1- 14) para llegar así al objeto de la reivindicación 1 de la solicitud, por lo cual se considera obvia.

En consecuencia, la reivindicación 1 de acuerdo a lo divulgado en los documentos D1 y D3, carece de nivel inventivo (Art. 18).

Las reivindicaciones dependientes 2 a 11, 15 y 17 no mencionan características inventivas adicionales para la composición de la reivindicación 1, por lo cual se considera que tampoco tienen nivel inventivo.

9. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO2009/131698	1 – 11, 15, 17	Nivel inventivo
D3	Jung – Ja P. K. *et al., Structural Biology,* 2009. Vol. 19, Pág. 534 – 542.	1 – 11, 15, 17	Nivel inventivo

10. Respuesta a los argumentos del señor solicitante

En referencia al argumento de la solicitante según el cual el documento D1 no divulga una proteína NaGlu humana recombinante que sea absorbida en las células siendo adecuada para su uso terapéutico, este Despacho se permite aclarar que el documento D1 anticipa las características técnicas esenciales de la composición reclamada en la solicitud, y aunque divulga una proteína de fusión recombinante aislada en donde la proteína N – acetil – alfa – glucosaminidasa (NaGlu), es fosforilada con manosa – 6 – fosfato, esta demostró la internalización celular a través de endocitosis mediada por clatrina, empleando el mismo mecanismo de la proteína NaGlu nativa, uniéndose al receptor de la M6P, indicando que dicha proteína de fusión recombinante podría ser empleada para uso terapéutico (Pág. 17, líneas 1- 14). Esto se demostró durante la evaluación *in vivo* en ratones MPS IIIB, en donde la expresión de NaGlu en el hígado de estos organismos dio lugar a la producción de una enzima activa capaz de atravesar la barrera hematoencefálica (BHE) (Pág. 18, líneas 1- 9). También se empleó un modelo canino de MPS IIIB, en donde la expresión de la enzima quimérica en el hígado de los perros afectados MPS IIIB podría prevenir signos neurológicos de la enfermedad (Pág. 18, ejemplo 3). Adicionalmente se evidenció la reducción en el almacenamiento lisosomal relacionado con bajas concentraciones de glucosaminoglucanos (GAG) (Pág. 29, Fig. 13). Finalmente cabe destacar que el documento D1 divulga la estructura aminoácida de la proteína NaGlu humana recombinante según la SEQ ID NO: 1 (Pág. 19, R- 2), correspondiente al componente activo de la composición reclamada en la presente solicitud. En consecuencia el efecto técnico asociado a dicha composición, no es inesperado, ni sorprendente a la luz de las enseñanzas de los documentos D1.

En cuanto a la relación molar de manosa-6-fosfato (M6P) a la proteína rhNaGlu, que logró el efecto técnico deseado y permitió que rhNaGlu fuera absorbida por las células y cruzara eficientemente la barrera hematoencefálica, este Despacho se permite aclarar que el documento D1 resalta la importancia de fosforilar la enzima recombinante NaGlu con M6P, para atravesar la barrera hematoencefálica (BHE) (Pág. 16, líneas 21- 34 - Pág. 17, líneas 1- 14); y el documento D3 divulga los cinco sitios potenciales de fosforilación de residuos de manosa sobre la proteína N – acetil – alfa – glucosaminidasa NaGlu (Pág. 535, Fig. 1), indicando que la fosforilación de N –glucanos en proteínas lisosomales permite la internalización celular de dichas proteínas y su posterior transporte hacia los lisosomas mediado por la interacción del receptor de M6P. Además afirma, que las enfermedades autosómicas recesivas como el síndrome Sanfilippo tipo B se originan por la deficiencia en la actividad de la GlcNAc – fosfotransferasa, la cual es la enzima encargada de generar la etiqueta de M6P (Pág. 534, Col. 2); por lo cual una persona normalmente versada en la materia llegaría de manera obvia a la relación molar de M6P respecto a la proteína rhNaGlu, a partir de la fosforilación de los cinco residuos de manosa de la proteína NaGlu sugeridos en el documento D3, incrementando así la concentración de esta enzima lisosomal, permitiendo el desarrollo de potenciales terapias de reemplazo enzimático; y por lo tanto el

objeto reclamado en la presente solicitud no presenta altura inventiva a la luz de la combinación de las enseñanzas de los documentos D1 y D3.

En relación a las publicaciones que demuestran que la proteína NaGlu producida en diferentes líneas celulares carecía de niveles suficientes de manosa 6 – fosfato (M6P) para la absorción celular y uso terapéutico, este Despacho se permite reiterar que el documento D1 resalta la importancia de fosforilar la enzima recombinante NaGlu (Pág. 16, líneas 21- 34- Pág. 17, líneas 1- 14), y sugiere un método que permite incrementar los niveles de M6P en una proteína recombinante NaGlu (Pág. 5, líneas 1 – 8); demostrando a partir de la evaluación *in vivo* en ratones MPS IIIB, que la expresión de NaGlu en el hígado de estos organismos daba lugar a la producción de una enzima activa capaz de atravesar la barrera hematoencefálica (BHE) (Pág. 18, líneas 1- 9), lo cual resuelve el problema técnico planteado en la solicitud.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Si como respuesta a este requerimiento el solicitante llegara a modificar: i) el capítulo reivindicatorio, ii) la descripción de la invención a proteger, iii) o presente un nuevo capítulo reivindicatorio, no deberá pagar la tasa de modificación voluntaria pero sí la correspondiente a la tasa de examen de patentabilidad para que se estudie la materia modificada. En todo caso, el número de requerimientos por no patentabilidad estará supeditado a que el término del estudio de patentabilidad no sea superior a 18 meses, contados desde la publicación de la solicitud de patente de invención. Adicionalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones deberá pagar la tasa por reivindicación adicional.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2016-03-22

Elaboró: Mario Andres Forero
Revisó: Dayron Alexander Ramirez Reyes

INFORME DE BÙSQUEDA DE PATENTES PARA EL Radicado N° 14-98853-00012-0000	
CRITERIOS PARA LA BÙSQUEDA (anexar pantallazo o reporte en PDF, junto con los boleanos utilizados)	
Palabras clave utilizadas	Enzimas lisosomales, N-acetil-alfa-D-glucosaminidasa (NaGlu), Composición+ and NaGlu, enfermedad autosómica recesiva, síndrome de Sanfilippo de tipo B, receptor manosa-6-fosfato (M6P)
CLASIFICACIONES INTERNACIONALES UTILIZADAS PARA LA BÚSQUEDA	A61K38/47; C12N5/07; C12N9/26

BASES DE DATOS CONSULTADAS.			
Patentscope	Google/patents		
NCBI	PatBase		
Espacenet	Epoquenet		
Lens	Sciencedirect		

INFORMES DE EXÁMENES REALIZADOS EN OTRAS OFICINAS (documentos patentes, literatura no patente etc…)

Oficina	Fecha del examen o de reporte de búsqueda de documentos	Anterioridades encontradas para el examen o reportados en el examen de búsqueda(relacionarlos todos)	Reiv. afectadas	RELEVANCIA (requisito que afecta)	FUERON TENIDOS EN CUENTA PARA EL EXAMEN EN LA OFICINA COLOMBIANA	
					SI	NO
EUROPEAN SEARCH REPORT EPO	27/Mayo/2015	Di Natale P et al., 2005. Treatment of the mouse model of mucopolysaccharidosis type IIIB with lentiviral-NAGLU vector.	1 - 27	Nivel inventivo	X	
		Zhao H. G. *et al.,* 1996. The molecular basis of Sanfilippo síndrome type B.	1 - 27	Nivel inventivo		X
		Weber B. *et al.,* 2001. Expression and characterization of human recombinant and alpha - N – actylglucosaminidase.	1 - 27	Nivel inventivo		X
		WO2011/163652	1 - 27	Documento Y, P		X
		US2012/021436	1 - 27	Documento Y, P		X

DOCUMENTOS ADICIONALES ENCONTRADOS POR EL EXAMINADOR DE ACUERDO A LOS CRITERIOS DE BÙSQUEDA.
WO2009/131698
WO2011163652
US2012003202
P Kim *et al.,* 2009. Carbohydrate recognition by the mannose-6-phosphate receptors
Schmidtchen A. *et al.,* 1998. NAGLU Mutations Underlying Sanfilippo Syndrome Type B
Champion K. *el al.,* 2010. Identification and characterization of a novel homozygous deletion in the a-N-acetylglucosaminidase gene in a patient with Sanfilippo type B síndrome (mucopolysaccharidosis IIIB)
US2006247177
US2006247177
US2011223147
CN104131094

BÚSQUEDA DE SECUENCIAS
Número de depósito / patente y N° de secuencia
% de identidad

SEQ ID NO (de la solicitud)	NCBI	The Lens	EMBL-EBI	DDJB	Genome Quest
SEQ ID NO: 1	SEQ ID: refINP 000254.2 presenta un 100% de identidad				

Búsqueda de Secuencias

alpha-N-acetylglucosaminidase precursor [Homo sapiens]

Sequence ID: ref|NP_000254.2| **Length:** 743 **Number of Matches:** 1

▸ See 1 more title(s)

Range 1: 1 to 743 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Positives	Gaps
2489 bits(5651)	0.0	743/743(100%)	743/743(100%)	0/743(0%)

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Sbjct  1    MEAVAVAAAVGVLLLAGAGGAAGDEAREAAAVRALVARLLGPGPAADFSVSVERALAAKP  60

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Sbjct  121  LTEATPNRYRYYQNVCTQSYSFVWWDWARWEREIDWMALNGINLALAWSGQEAIWQRVYL  180

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Query  301  KEFGTDHIYGADTFNEMQPPSSEPSYLAAATTAVYEAMTAVDTEAVWLLQGWLFQHQPQF  360
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Sbjct  301  KEFGTDHIYGADTFNEMQPPSSEPSYLAAATTAVYEAMTAVDTEAVWLLQGWLFQHQPQF  360

Query  361  WGPAQIRAVLGAVPRGRLLVLDLFAESQPVYTRTASFQGQPFIWCMLHNFGGNHGLFGAL  420
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Sbjct  541  RLLLTSAPSLATSPAFRYDLLDLTRQAVQELVSLYYEEARSAYLSKELASLLRAGGVLAY  600

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Búsqueda en PatBase

7) Family number: 43925953 (WO09131698A2)

Title: PHOSPHORYLATED RECOMBINANT N-ACETYL-ALPHA-D- GLUCOSAMINIDASE (**NAGLU**) AND USES THEREOF

Abstract: The present invention relates generally to phosphorylated recombinant NaGIu fusion proteins, the production of phosphorylated recombinant NaGIu fusion proteins and uses thereof.
Classifications:
International (IPC 8-9): A61K38/47 C07K14/435 (Advanced/Invention); A61K38/43 C07K14/435 (Core/Invention)
CPC: C12N9/16 C12Y301/04045 C07K14/775 A61K38/00 C07K2319/00 C07K2319/02 C12Y302/0105
European: C07K14/775 C12N9/24 C12Y302/01050 K61K38/00 M07K319/00 M07K319/02

8) Family number: 43959995 (US2011223147A)

Title: LYSOSOMAL TARGETING PEPTIDES AND USES THEREOF

Abstract: Source: US2011223147A The present invention provides further improved **compositions** and methods for efficient lysosomal targeting based on the GILT technology. Among other things, the present invention provides methods and **compositions** for targeting lysosomal enzymes to lysosomes using furin-resistant lysosomal targeting peptides. The present invention also provides methods and **compositions** for targeting lysosomal enzymes to lysosomes using a lysosomal targeting peptide that has reduced or diminished binding affinity for the insulin receptor.
Classifications:
International (IPC 8-9): A61K A61K38/00 A61K38/27 A61K38/43 A61K38/46 A61K38/47 A61P3/00 A61P43/00 C07H21/00 C07K1/00 C07K14/47 C07K14/475 C07K14/62 C07K14/65 C07K19/00 C12N1/00 C12N1/15 C12N1/19 C12N1/21 C12N15/09 C12N15/17 C12N15/56 C12N15/62 C12N5/10 C12N9/24 C12N9/26 C12N9/96 C12P21/00 (Advanced/Invention);
C07K14/435 C07K19/00 (Core/Invention);
C07K (Subclass/Invention)
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European: C07K14/65 M07K319/06
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17) Family number: 57719982 (CN104131094A)

Title: PRIMER **COMPOSITION** FOR LYSOSOMAL DISEASE GENE SCREENING AND KIT USING THE SAME

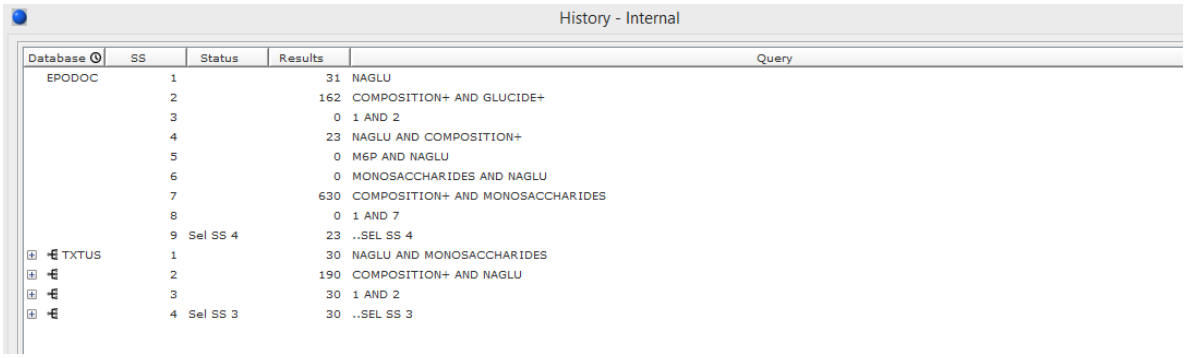
Abstract: Source: CN104131094A The invention provides a primer **composition** for lysosomal disease gene screening and a kit using the same. The primer **composition** comprises multiple primer sequences respectively aiming at non-repetitive regions of IDUA, ARSB, MAN2B1, ST3GAL5, SMPD1, CTNS, MFSD8, IDS, GUSB, GBA, ARSA, GNPTAB, SLC17A5, CLN8, SGSH, HYAL1, PSAP, GNPTAG, PPT1, CTSD, **NAGLU**, FUCA1, GLB1, SUMF1, MCOLN1, TPP1, HGSNAT, NAGA, GM2A, GALC, LIPA, CLN3, GALNS, NEU1, HEXA, GLA, NPC1, CLN5, MANBA, HEXB, ASAH1, NPC2 and CLN6 genes.
Classifications:
International (IPC 8-9): C12N15/11 C12Q1/68 (Advanced/Invention)
CPC: C12Q1/6883

Family:

Publication number	Publication date	Application number	Application date
CN104131094 A	20141105	CN201410359940	20140725

Priority:
CN201410359940 20140725
Probable Assignee: FENG ZHICHUN
Assignee(s): (std): FENG ZHICHUN
Assignee(s): PURE SEALS
Inventor(s): (std): FENG ZHICHUN ; WANG YAN ; YANG YAO

Búsqueda en EPOQUENET



History - Internal

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	6		0	MONOSACCHARIDES AND NAGLU
	7		630	COMPOSITION+ AND MONOSACCHARIDES
	8		0	1 AND 7
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TXTUS	1		30	NAGLU AND MONOSACCHARIDES
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	4	Sel SS 3	30	..SEL SS 3

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Carbohydrate recognition by the mannose-6-phosphate receptors

Jung-Ja P Kim, Linda J Olson and Nancy M Dahms

The two P-type lectins, the 46 kDa cation-dependent mannose-6-phosphate (Man-6-P) receptor (CD-MPR), and the 300 kDa cation-independent Man-6-P receptor (CI-MPR), are the founding members of the growing family of mannose-6-phosphate receptor homology (MRH) proteins. A major cellular function of the MPRs is to transport Man-6-P-containing acid hydrolases from the Golgi to endosomal/lysosomal compartments. Recent advances in the structural analyses of both CD-MPR and CI-MPR have revealed the structural basis for phosphomannosyl recognition by these receptors and provided insights into how the receptors load and unload their cargo. A surprising finding is that the CD-MPR is dynamic, with at least two stable quaternary states, the open (ligand-bound) and closed (ligand-free) conformations, similar to those of hemoglobin. Ligand binding stabilizes the open conformation; changes in the pH of the environment at the cell surface and in endosomal compartments weaken the ligand–receptor interaction and/or weaken the electrostatic interactions at the subunit interface, resulting in the closed conformation.

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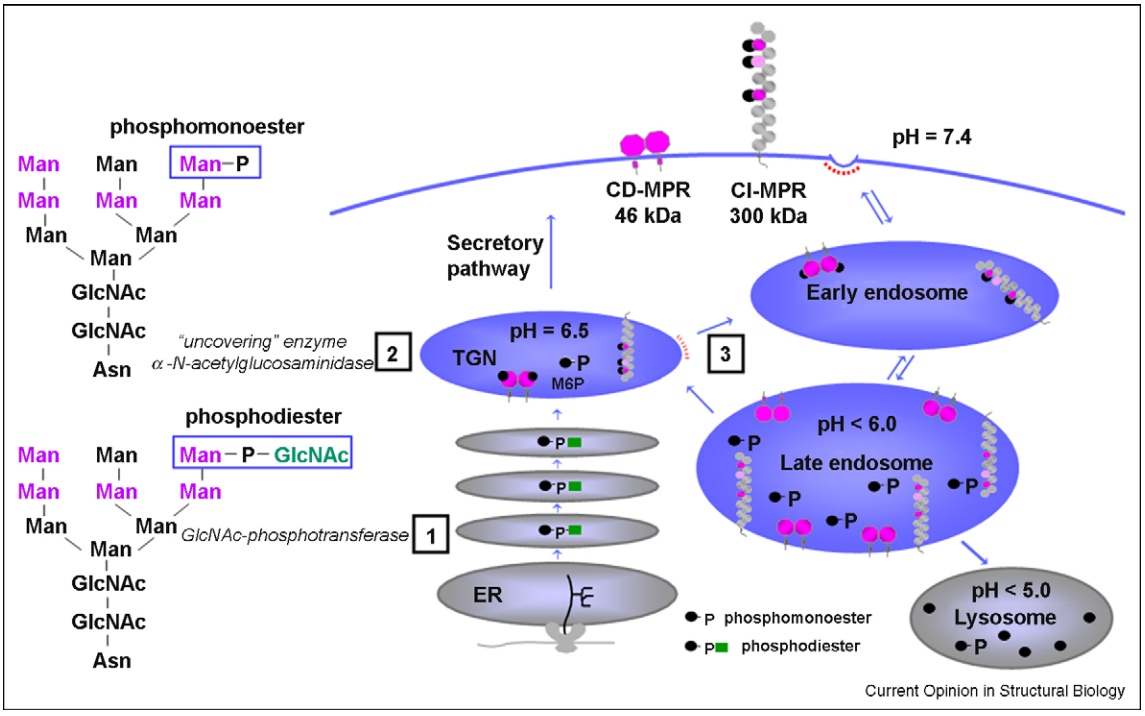
Introduction

In eukaryotic cells, mannose-6-phosphate receptors (MPRs) mediate the delivery of ~60 different newly synthesized soluble acid hydrolases to the lysosome by binding to mannose-6-phosphate (Man-6-P) residues found on their *N*-linked oligosaccharides. Lysosomal enzymes become differentiated from other proteins in the secretory pathway by acquiring Man-6-P residues in a two-step process: firstly, the GlcNAc-phosphotransferase transfers *N*-acetylglucosamine-1-phosphate to one or two mannose residues on an *N*-glycan to yield a phosphodiester intermediate [1•,2•]. Secondly, α-*N*-acetylglucosaminidase removes the *N*-acetylglucosamine residue in the *trans* Golgi network (TGN) to generate the phosphomonoester, Man-6-P [3] (Figure 1). The resulting MPR/lysosomal enzyme complex is transported from the TGN to the late endosome where the low pH of the compartment induces the complex to dissociate. The released enzymes are packaged into lysosomes [4,5] and the receptors either return to the Golgi to repeat the process or move to the plasma membrane where they function to internalize exogenous ligands (Figure 1). The importance of this phosphomannosyl recognition system in the biogenesis of lysosomes is illustrated by the existence of more than 40 different human lysosomal storage diseases that are estimated to affect 1 in 5000 live births [6]. The discovery of the MPRs originated from studies centered on determining the molecular basis of a lysosomal storage disorder, mucolipidosis II (MLII; also referred to as 'I-cell disease'). The pioneering work of Hickman and Neufeld [7] led to the finding that fibroblasts from MLII patients were capable of internalizing lysosomal enzymes secreted by normal cells, while in contrast, normal fibroblasts were unable to endocytose lysosomal enzymes secreted by MLII fibroblasts. Their hypothesis that lysosomal enzymes contained a recognition tag required for receptor-mediated uptake and transport to lysosomes was later confirmed upon the identification of the tag as Man-6-P [8–10]. I-cell disease is an autosomal recessive disorder caused by a deficiency of GlcNAc-phosphotransferase activity (IUBMB accession number EC 2.7.8.17), which is the enzyme that generates the Man-6-P tag [11] (see Figure 1, step 1).

Two distinct MPRs, the 46 kDa cation-dependent mannose-6-phosphate receptor (CD-MPR) and the ~300 kDa cation-independent mannose-6-phosphate receptor (CI-MPR) are the sole members of the P-type lectin family [12]. The CI-MPR is multifunctional in that it binds proteins bearing the Man-6-P recognition marker as well as the peptide hormone IGF-II (hence it is also called IGF-IIR/CI-MPR) [13–15]. In addition to its intracellular role in lysosome biogenesis and regulation of circulating IGF-II levels, the CI-MPR has been implicated in many other cellular functions because it binds to Man-6-P-containing proteins that are not lysosomal hydrolases. These include transforming growth factor-β (TGF-β) precursor [16], the placental angiogenic hormone proliferin [17], the cytokine leukemia inhibitory factor [18], and the T-cell activation antigen CD26 [19], to name just a few. Functional roles of the cell surface CI-MPR in interactions with these nonlysosomal proteins include activation of TGF-β precursor and renin precursor [20]

Figure 1



Lysosomal enzyme trafficking. Movements of lysosomal enzymes and MPRs between the various intracellular compartments and the cell surface are shown. Phosphorylation of mannose residues on *N*-linked oligosaccharides occurs in two steps (see text). The five potential sites of phosphorylation are indicated in pink letters. Lysosomal enzymes that acquire the Man-6-P tag in early Golgi compartments bind specifically to MPRs in the Golgi. The resulting receptor–lysosomal enzyme complex is transported from the *trans* Golgi network (TGN) to an early endosomal compartment (step 3) and to an acidified late endosomal compartment where the low pH of the compartment causes dissociation of the complex. Lysosomal enzymes that are not phosphorylated (●), contain a phosphomonoester (●-P), or contain a diester with *N*-acetylglucosamine are shown (●-P-■). The dimeric CD-MPR is depicted as two pink balls and the CI-MPR is shown as 15 repeating balls. The three Man-6-P binding domains of the CI-MPR are depicted as pink balls.

and clearance from the plasma in the case of leukemia inhibitory factor [21]. In addition to IGF-II, the CI-MPR has been shown to interact with a number of proteins that do not have phosphomannosyl residues. These include urokinase-type plasminogen activator receptor (uPAR) [22], plasminogen [23], retinoic acid [24], and heparanase [25•]. Residues essential for uPAR and plasminogen binding have been mapped to domain 1 [26] (Figure 2). However, the nature of their interactions is not clear.

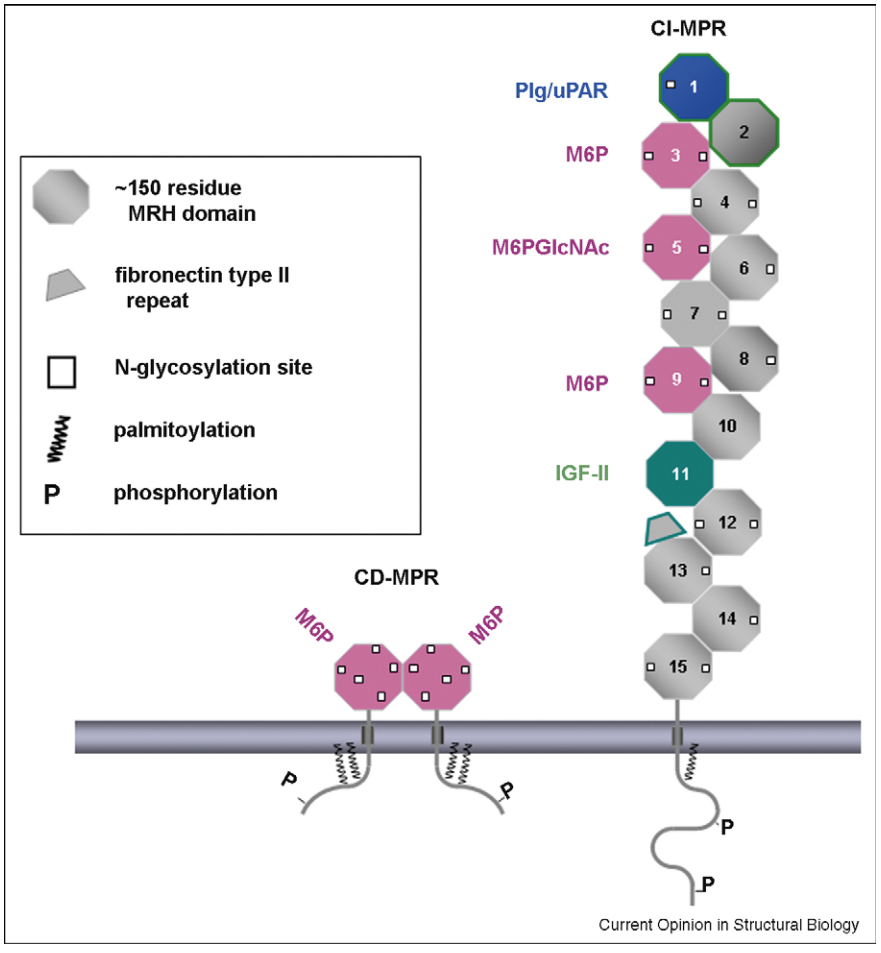
In addition to the two MPRs, several proteins have been identified as containing mannose-6-phosphate receptor homology (MRH) domains [27], including erlectin (a luminal ER protein implicated in the regulation of glycoprotein trafficking) [28], the β-subunit of glucosidase II (also in ER), and GlcNAc-phosphotransferase γ-subunit (in Golgi), all of which are implicated in *N*-glycan recognition [29]. However, studies on their recognition of specific glycans are only beginning to emerge. Recently, human OS-9, which is involved in ER protein quality control, has been shown to interact with mannose-trimmed *N*-glycans [30•]. However, studies will be needed to verify that the MRH domains of these proteins are structurally related to the MPRs.

This review will focus on recent structural studies of the CD-MPR and CI-MPR, with emphasis on how these receptors interact with phosphomannosyl residues. For other aspects of MPRs and related proteins, the reader is referred to articles by Ghosh *et al.* [31•], Gary-Bobo *et al.* [32••], Dahms *et al.* [33••], and Brown *et al.* [34••].

Structures of the CD-MPR and CI-MPR

The MPRs are type 1 transmembrane glycoproteins (Figure 2). The CD-MPR forms a stable homodimer and the CI-MPR exists most likely as a dimer [35]. The bovine CD-MPR is composed of a 28-residue amino-terminal signal sequence, a 159-residue extracytosolic domain, a 25-residue transmembrane region, and a 67-residue carboxyl-terminal cytosolic domain. The six cysteine residues in the extracellular region of the CD-MPR form three disulfide linkages that play an important role in the folding of the molecule. The bovine CI-MPR contains a 44-residue signal sequence, a 2269-residue extracytosolic region, a 23-residue transmembrane region,

Figure 2

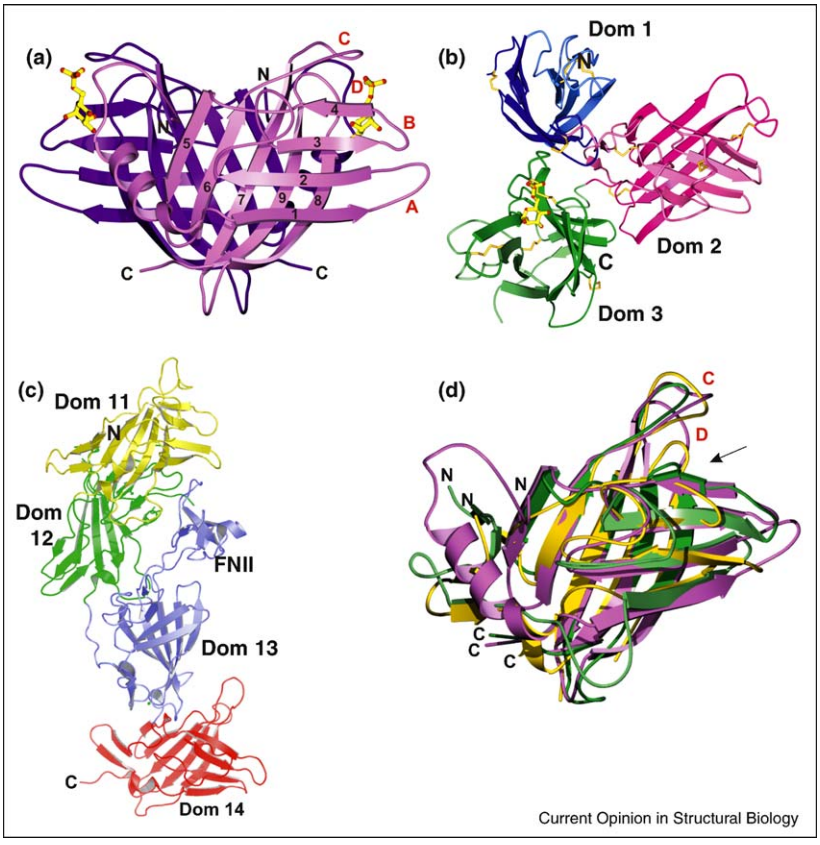


Schematic representation of the CD-MPR (left) and CI-MPR (right). The MPRs are transmembrane glycoproteins. Various post-translational modifications are indicated, including palmitoylation and phosphorylation. The CD-MPR is shown as a dimer with each subunit having one Man-6-P binding site. The 15 repeating domains of the CI-MPR are numbered sequentially from the N-terminus to C-terminus. Domains 3, 5, and 9 bind Man-6-P with domain 5 preferentially binding to phosphodiesters. Domain 1 is known to bind to urokinase plasminogen activator receptor (uPAR) and plasminogen (Plg). Domain 11 binds IGF-II and domain 13 has a fibronectin II insert.

and a 163-residue cytosolic domain. The large extracytosolic region consists of 15 contiguous domains, each of which, when compared to CD-MPR and to each other, has a similar size and significant amino acid sequence identity (14–38%) including cysteine distribution, strongly suggesting that they have similar tertiary structures. Indeed, the crystal structures of the extracytosolic domain of the CD-MPR and domains 1, 2, 3, 11, 12, 13, and 14 of the CI-MPR all have the same fold (Figure 3) [36,37••,38,39] (see below).

The structure of the extracytosolic domain of the CD-MPR in complex with Man-6-P and Mn^{2+} was first determined over 10 years ago [40,41] and has established the overall polypeptide fold of each domain in the P-type lectin family (Figure 3A). The molecule was found to be a dimer, consistent with the predominant oligomeric form of the CD-MPR found in membranes. The overall fold of the CD-MPR monomer is that of a flattened β-barrel consisting of two antiparallel β-sheets, one with four β-strands and the other with five strands, with strand nine interjecting between strands 7 and 8. The dimer interface is formed by two five-stranded β-sheets. The structure of domains 1–3 (the N-terminal 432 residues) of the CI-MPR has been determined in complex with Man-6-P [36,42], confirming that the overall fold of each domain in the CI-MPR is the same as that of the CD-MPR (Figure 3). While the CD-MPR forms a dimer, the N-terminal three domains of the CI-MPR exist as a monomer. The three domains of the CI-MPR form a compact tri-lobed disk and have considerable contact with one another (contact areas between domains range from 8 to 22%). These extensive interactions suggest that the observed three-domain arrangement forms a structural unit within the entire CI-MPR molecule [36], and that the interactions of domain 3 with the loop between

Figure 3



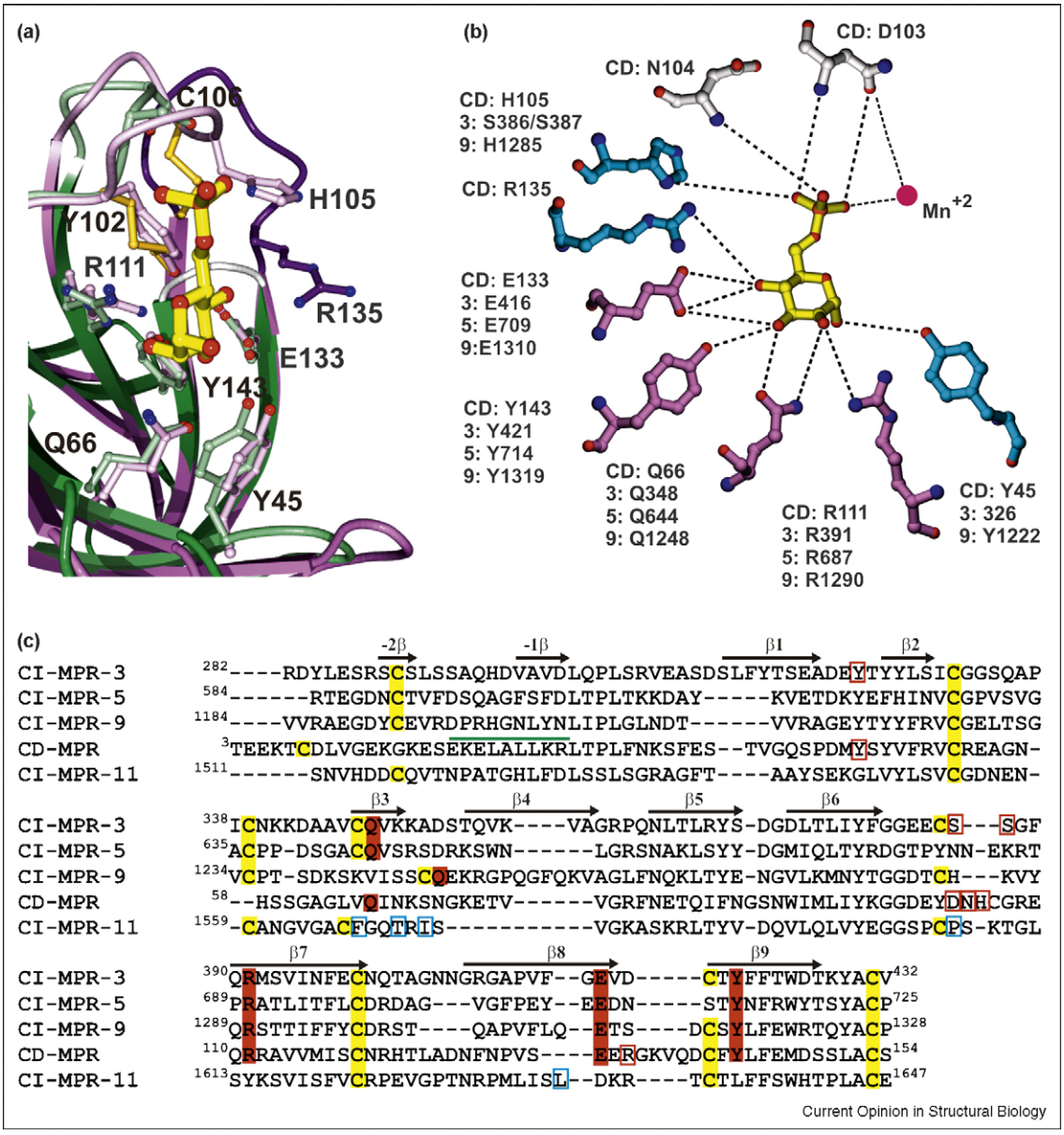
A collage of structures of the CD-MPR and CI-MPR. **(A)** Dimer of the CD-MPR. β-Strands are numbered from N-terminus to C-terminus and loops between strands are labeled in alphabetic order. **(B)** Structure of domains 1–3 of CI-MPR, with bound Man-6-P in domain 3. **(C)** Structure of domains 11–14 of the CI-MPR. N-termini and C-termini are indicated. FNII denotes the fibronectin II insert in domain 13. **(D)** Superposition of the structures of the CD-MPR (monomer, purple) and domains of 3 (green) and 11 (gold) of the CI-MPR, demonstrating that they all have a similar polypeptide fold. For clarity, the structures of domains 1, 2, 12, 13, and 14, whose structures have been determined to have the same fold, are not included in the overlay. The ligand-binding site is indicated with an arrow.

domains 1 and 2 are required for maintaining the architecture of its sugar-binding site.

Comparison of Man-6-P binding pockets in the CD-MPR and CI-MPR

The Man-6-P binding site in the CD-MPR is located at the C-terminal opening of the β-barrel (Figures 3 and 4). One side of the binding pocket is walled off by two loops (loop C, residues G98–R108; and loop D, E134–C141) and the other side is exposed to the solvent, to provide room for the rest of the ligand molecule. The pocket is lined with residues Y45, D103, N104, H105, R135, E133, Y143, Q66, and R111, the latter four of which are found to be essential for Man-6-P binding by mutagenesis studies of CD-MPR [43–45] and domains 3, 5, and 9 of the CI-MPR [46,47•] (Figure 4B). These four signature residues in both the CD-MPR (E133, Y143, Q66, and R111) and domain 3 of CI-MPR (E416, Y421, Q348, and R391) are absolutely conserved (Figure 4C) and interact with the Man-6-P ligand in the same manner, strongly suggesting that the sugar-binding pockets in domains 5 and 9 are also very similar to those of CD-MPR and domain 3 of the CI-MPR (Figure 4A). Indeed, the Man-6-P binding pocket located in domain 3 is essentially the same as that of the CD-MPR, with one exception: the absence of Mn^{2+} which accounts for the metal independence of the CI-MPR. In addition, solution structures of domain 5, which preferentially binds phosphodiesters, with and without a phosphodiester ligand have been determined (L Olson, abstract 216 in Glycobiology 18, 993, 2008). Preliminary results indicate that the overall fold of the isolated domain 5 is similar to that of the other CI-MPR domains and CD-MPR, and mutagenesis studies have demonstrated the essential role of these four signature residues in the lectin activity of domain 5 [47•]. It is interesting to note that the IGF-II binding pocket is located in the homologous position in domain 11 as the Man-6-P binding pocket in domain 3 [37••] (Figure 3D).

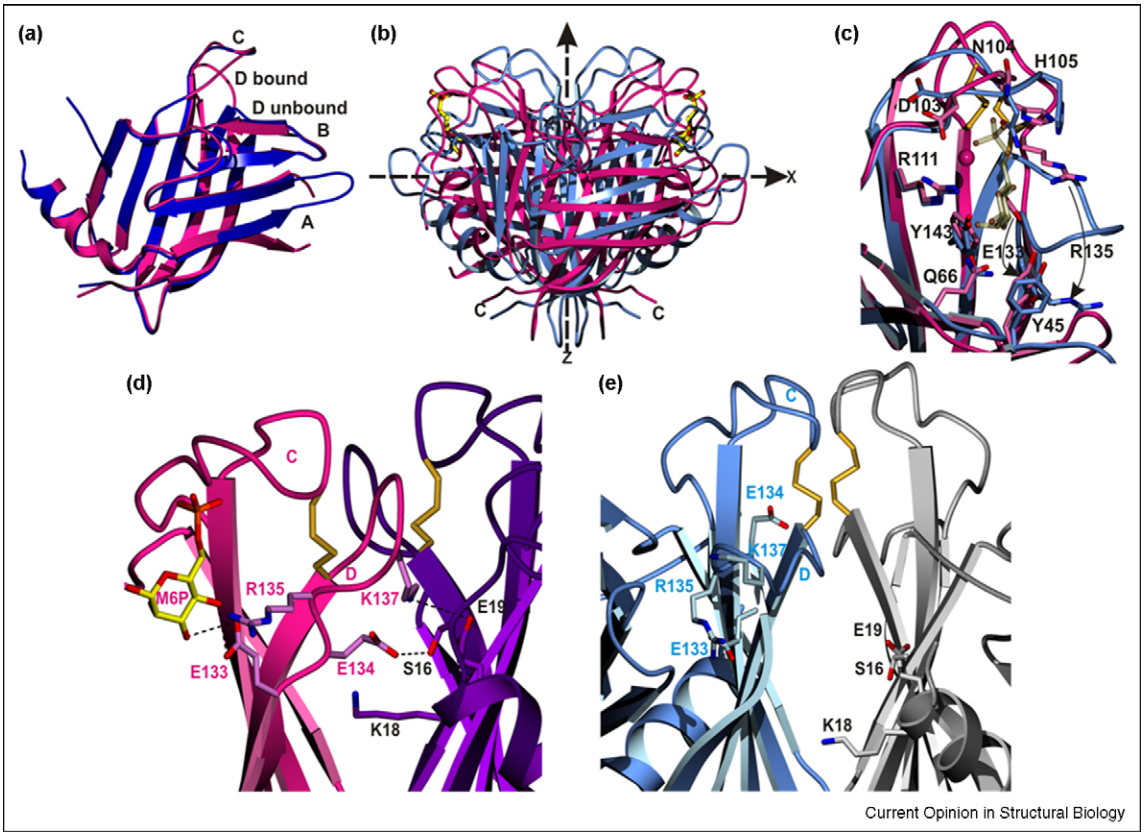
Figure 4



Conservation of the Man-6-P binding site and essential residues for carbohydrate binding. **(A)** Superposition of the Man-6-P binding sites of the CD-MPR (purple) and domain 3 of the CI-MPR (green). The architecture of both binding pockets is essentially the same with the exception of loop D (dark purple, CD-MPR; gray, domain 3), which is shorter in domain 3. **(B)** A schematic drawing showing interactions between Man-6-P and residues in the CD-MPR and their homologous residues in domains 3, 5, and 9 of the CI-MPR. Dotted lines indicate potential hydrogen bonds. Mutational studies have shown that the four residues shown in purple are essential for Man-6-P binding and that mutation of the residues shown in blue partially decreased Man-6-P binding affinity. The two residues in gray have not been tested (N104) or decreased the cation dependency (D103). **(C)** Sequence alignment of domains 3, 5, 9, and 11 of the CI-MPR and the extracytosolic domain of the CD-MPR. The conserved cysteines are highlighted in yellow and the four residues that are essential for Man-6-P binding are highlighted in red. Residues that are within hydrogen bonding distance of Man-6-P in the crystal structures of CD-MPR and domains 1–3 of the CI-MPR, but found to be not essential for Man-6-P binding are boxed in red. Secondary structural elements are indicated with arrows and the single α-helix found in the CD-MPR is indicated with a green cylinder. Residues involved in IGF-II binding in domain 11 of the CI-MPR are boxed in blue.

Inhibition studies using chemically synthesized oligomannosides or neoglycoproteins have shown that the presence of Man-6-P at a terminal position is the major determinant of receptor binding. Furthermore, linear glycans which contain a terminal Man-6-P linked α1,2 to the penultimate mannose were shown to be the most potent inhibitors [48,49]. Recent studies using a novel phosphorylated glycan microarray demonstrate that the CD-MPR demonstrates a preference for glycans containing two phosphomonoesters, whereas the CI-MPR shows

Figure 5



Comparison of the ligand-bound and ligand-free structures of CD-MPR. **(A)** Superposition of the monomer structures bound (pink) and unbound (blue) structures. Note the difference in the conformations of loop D. **(B)** The dimer structures, with the same color scheme as in (A). The molecule is scissoring along its molecular twofold axis (*z*-axis) and twisting along the *x*-axis. The ligand, Man-6-P, is shown with ball-and-sticks. **(C)** Superposition of the ligand-binding sites of the bound (red) and unbound (blue) structures. Loop D in the bound structure forms the side of the binding pocket, while in the unbound structure loop D folds down and occupies the Man-6-P binding site. Mn^{+2} ion in the bound state is denoted as a red ball. The movements of E133 and R135 are indicated (curved arrows). Intersubunit interfaces are shown, including the N-terminus and loop D of the bound (panel **(D)**) and unbound (panel **(E)**) structures. Electrostatic interactions found between the two subunits in the bound structure are disrupted in the unbound structure, resulting in a weaker dimer interface. Disulfide bonds are shown in yellow connecting loops C and D.

little difference in affinity toward glycans containing one or two phosphomonoesters [55].

The CD-MPR is dynamic — comparison of the ligand-bound and ligand-free CD-MPR

The MPRs travel between various cellular compartments during the transportation of their ligand, from TGN (where they bind the ligand) to late endosomes (where they release the ligand). Unlike the CI-MPR, the CD-MPR does not bind ligands at the cell surface. It has been shown that this loading/unloading is facilitated by changes in the pH of each compartment [50]. In order to understand the mechanism of the pH dependence of the ligand binding/release, crystal structures of the CD-MPR have been obtained at various pHs and in the presence and absence of bound Man-6-P [51,52••]. Unexpectedly, regardless of pH, the receptor molecule adopts two conformations: an 'open' conformation found in all structures with bound Man-6-P and a 'closed' conformation observed in all structures without bound ligand (Figure 5). There are three major structural differences between these two states. First, in the open state (i.e. the bound state), the two ligand-binding sites of the dimeric molecule are ~35 Å apart, whereas in the closed (unliganded) state, they are ~26 Å apart. Second, the architecture of the ligand-binding pocket in the closed form differs significantly from the open form (Figure 5C). When the ligand is bound, loop D (residues E134–C141) forms one wall of the binding pocket. When the ligand is absent, loop D folds into the binding pocket, occupying the same space where the sugar molecule binds and thereby keeps the position of the sugar-binding residues unchanged. Lastly, this loop D movement disrupts electrostatic interactions between the two subunits, releasing intersubunit salt bridges, causing the two subunits to slide and twist at their interface which results in the 'closed' form (Figure 5B and C). Thus, the movements involved in the bound-to-unbound transition of the

CD-MPR are reminiscent of those in the oxy-to-deoxy transition of hemoglobin. However, whether the CD-MPR has ligand-binding cooperativity has not been studied. The overall movement of the bound-to-unbound transition can be described as a 'scissoring and twisting' motion between the two subunits at the dimer interface. The implication of the existence of these two states is that the CD-MPR must be able to readily transition between these two conformations as it travels to the different cellular compartments, with the unique environment of each compartment (e.g. Golgi, cell surface, endosome) impacting the equilibrium between the two states.

Mechanisms of ligand binding and release by the CD-MPR

From the above structural studies, plausible mechanisms for the dissociation of lysosomal enzymes in the acidic endosome (pH <6.0) and at the cell surface (pH 7.4) have been proposed [52••]. The structure of the bound form of CD-MPR has three intersubunit ion pairs that 'tie' loop D of one subunit to the other subunit (K18 and E19 of one subunit to E134 and K138 of the other subunit, respectively) (Figure 5D). In the acidic environment of the endosome, the disruption of intersubunit electrostatic interactions may trigger the ligand release by protonation of the carboxyl groups of the glutamates and aspartate (Figure 5D and E). In addition, E133, one of the essential residues for binding of Man-6-P, might also be protonated in the endosome and weaken its binding to Man-6-P, promoting release of the bound ligand (Figure 5D). On the other hand, at the cell surface, deprotonation of H105 and the phosphate moiety of Man-6-P (both of which interact with each other in the bound conformation) are most likely responsible for the release of the ligand (Figures 4 and 5C). To probe directly the role of the electrostatic interactions between the two subunits, specifically the salt bridge between E19 and K137, a double mutant (E19Q/K137M) has been analyzed by surface plasmon resonance. The mutant CD-MPR lacking the intersubunit salt bridge binds lysosomal enzymes with ~100-fold lower affinity, clearly demonstrating the critical role of the ionic interactions between the two subunits in stabilizing the bound conformer of the CD-MPR.

Conclusions

Although we have accumulated considerable knowledge on the MPRs regarding the structural basis for their phosphomannosyl recognition and the mechanisms of the cargo loading and unloading for the CD-MPR, there still remain many outstanding questions. It is interesting to note that all ligand-binding sites of CI-MPR are located at odd-numbered domains, that is, domains 3, 5, and 9 for Man-6-P binding and domain 11 for IGF-II, as well as domain 1 which is found to interact with plasminogen and uPAR. What are the roles of the remaining domains? Do even-numbered domains function only as spacers and/or support for the functional domains? What are the mechanisms of ligand binding and release for the CI-MPR, which must be very different from those of CD-MPR? Answers to these questions must await further biochemical and structural studies of the CI-MPR including fragments containing overlapping ranges of domains. It is also intriguing that the CI-MPR complements the two enzymes that make the phosphomannosyl tag by being able to bind to the products of both the first and the second enzymes of the two-step process. Is this purely for redundancy? Furthermore, the mechanisms that govern the MPRs' trafficking to different subcellular compartments are not clearly known. MPRs are present in TGN, endosomes, and plasma membrane, but absent in lysosomes. They cycle constitutively between these compartments and this trafficking is directed by sorting signals that reside in the cytosolic tails of the receptors. Currently, only the structures of peptides (12 or 13 residues) that contain the di-leucine motif in the cytosolic tails of CD-MPR and CI-MPR, in complex with the VHS domain of human GGA (Golgi-localized, γ-ear-containing, ADP-ribosylating-factor-binding) proteins are known [53,54]. Thus, in order to gain a clearer picture of the factors that regulate the interaction of the MPRs' cytoplasmic region with numerous cytosolic adaptor proteins, further structural analyses are needed.

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