

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO			
RAD:	11-181053- -7	FECHA:	2013-07-23 10:59:08
DEP:	2020 DIRECCION DE NUEVAS CREACIONES	EVE:	378 FASE NACIONAL INCLUIDO CAPITULO I
TRA:	11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS:	5
ACT:	519 PRESENTACION		

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
CARLOS REINALDO OLARTE GARCIA

Asunto: Radicación: 11-181053- -7
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 13234
 Folios: 5

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/EP2010/059008				
Fecha de Presentación Internacional	24/06/2010				

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 No	11-181053-00000-0000	29/Diciembre/2011
Reivindicaciones:	Radicado No	11-181053-00005-0000	29/Diciembre/2011
Secuencias	Radicado No	11-181053-00000-0000	29/Diciembre/2011
Dibujos:	Radicado No	11-181053-00000-0000	29/Diciembre/2011
Prioridad:	US 61 219 964		24/Junio/2009
Prioridad:	US 61 334 568		27/Mayo/2010

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. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero 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 47 (Radicado No 11-181053-00005-0000).

Título de la solicitud: “Antígeno de virus de Sincicio Respiratorio Recombinantes”.

El problema planteado en esta solicitud consiste en la necesidad de proporcionar, compuestos útiles para tratar y/o prevenir infecciones de RSV.

Para resolver este problema técnico la solicitud en estudio proporciona un antígeno de Virus de Sincicio Respiratorio que comprende un polipéptido de proteína F y una composición que lo contiene.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 39/155 // CO7K14/135

4. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- 1- La reivindicación 1 define el antígeno a partir de un resultado a alcanzar, se solicita definirlo por su secuencia específica de aminoácidos.
- 2- Las reivindicaciones 2 -11, 13, 16-22, 25, 28 29, 31-33, mencionan las modificaciones haciendo referencia solo al dominio sin definir las secuencias de las mismas. Se solicita definir el antígeno por su secuencia específica de aminoácidos y las secuencias de las modificaciones.
- 3- La reivindicación 1 se refiere a modificaciones que alteran glicosilación, lo cual no está sustentado de manera suficiente en la descripción.
- 4- La reivindicación 36 menciona las expresiones “3D-MPL, QS21, HRB, HRA” son términos imprecisos, se sugiere incluirlos por su denominación común y además se solicita definir la composición por sus características técnicas y no por su adyuvante únicamente.
- 5- En la reivindicación 5 no se define el aminoácido que es sustituido.
- 6- La reivindicación 15 no es concisa, no permite identificar el objeto que se busca proteger.
- 7- En la reivindicación 39, la expresión: “menos de” es relativa por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirla por un término o rango concreto.
- 8- La reivindicación 40 la expresión “adecuado para administrarse a un neonato” no permite definir al adyuvante. Se sugiere incluir las características opcionales o preferentes en una reivindicación dependiente.
- 9- La reivindicación 41, se refiere a un polipéptido de proteína G, lo cual no está

sustentada de manera suficiente en la descripción.

10-En la reivindicación 43 el ácido nucleico no está definido por a partir de la secuencia nucleótida ni se indica el número de la misma.

11-Las reivindicaciones 15 y 45 se refieren a un valor de 95% (de identidad de una secuencia) lo que no permite establecer la secuencia nucleótida exacta a la que se refiere.

12- En la reivindicación 45 se define el ácido nucleico en términos de secuencias de aminoácidos que codifica y por resultado a alcanzar como lo es que hibrida bajo condiciones astringentes sobre sustancialmente su longitud entera a SEQ ID NO 21, lo que no permite establecer la secuencia nucleótida del mismo. Se sugiere definirlo por su secuencia nucleótida indicando el número de la misma (SEQ ID No)

5. 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: Junio 24 de 2009 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	PNAS Vol 98 (Pág. 9859-9864) Luis Gonzalez Reyes Ruiz)	"Cleavage of the human respiratory syncytial virus fusion protein at to distinct sites is required for activation of membrane fusion "	14/Agosto/2001
D2	WO 0242326	"A method of expression and agents identified thereby"	30/Mayo/2002

El documento D1¹ divulga mutaciones en ambos sitios de corte de furina en la proteína F, en virus RSV. (Pág. 9859-9864, Figura 1).

El documento D1² divulga variantes de proteína F en virus RSV, que comprende mutaciones en secuencias del péptido y las variantes exhiben actividad funcional modulada. (Pág. 105, reivindicación 44).

6. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en *"un antígeno de virus de sincicio respiratorio (RSV) recombinante que comprende un polipéptido de proteína F soluble que comprende un dominio F2 y un dominio F1 de un polipéptido de proteína F de RSV, en donde el polipéptido de proteína F comprende por lo menos una modificación que altera la glicosilación."*

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

11<http://www.pnas.org/content/98/17/9859.full.pdf+html>

2[http://worldwide.espacenet.com/publicationDetails/biblio?](http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20020530&CC=WO&NR=0242326A1&KC=A1)

[DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20020530&CC=WO&NR=0242326A1&KC=A1](http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20020530&CC=WO&NR=0242326A1&KC=A1)

Características esenciales	D1	D2
Antígeno de virus de Sincicio respiratorio recombinante, que contiene proteína F.	Proteína del virus del sincicio respiratorio HRSV (Pág. 9859)	Producción de proteína F en virus de RNA. (resumen)
Modificaciones en secuencias de aminoácidos del péptido.	Mutaciones en dos sitios de corte de furina en proteína F (Pág. 9859-9864)	Mutaciones específicas en secuencias de aminoácidos de residuos de proteína F (Pág 6. , líneas 5-7 , reivindicación 44)

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación.

El documento D1 divulga mutaciones en ambos sitios de corte de furina en la proteína F, en virus RSV. (Pág. 9859-9864, Fig. 1).

Por su parte el documento D2 divulga variantes de proteína F en virus RSV, que comprende mutaciones en secuencias del péptido y las variantes exhiben actividad funcional modulada (Pág. 105, reivindicación 44).

La diferencia entre la invención, definida en la reivindicación 1 y el procedimiento de D1 consiste en que la proteína F, tiene modificaciones con el fin de alterar la glicosilación.

El efecto que logra el incluir modificaciones en secuencias de aminoácidos en proteína F es alterar procesos glicolíticos.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo proveer alteraciones en proteína F en virus RSV, el procedimiento conocido en D1 para alterar procesos glicolíticos”.

Sin embargo, las mutaciones en aminoácidos de proteína F para alterar actividades funcionales, ya está divulgada en el documento D2 que se refiere variantes de proteína F en virus RSV, que comprende mutaciones en secuencias del péptido y las variantes exhiben actividad funcional modulada (Pág. 105, reivindicación 44)

En consecuencia, el la persona del oficio normalmente versada en la materia, incluiría las variantes de proteína F del documento D5 en el procedimiento divulgado en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Conclusión:

La reivindicación 1 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

Las reivindicaciones dependientes 2 – 47 no mencionan alguna característica que cumlan on el requisito de nivel inventivo

7. Conclusión

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

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	PNAS VoL 98	1 –47	Nivel inventivo
D2	WO 0242326	1 – 47	Nivel inventivo

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 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.

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.

Finalmente, 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, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

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 2013-07-30

Elaboró: Edna Milena Bautista Rodriguez
Revisó: Jeny Paola Villate Becerra

Cleavage of the human respiratory syncytial virus fusion protein at two distinct sites is required for activation of membrane fusion

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Edited by Peter M. Howley, Harvard Medical School, Boston, MA, and approved May 14, 2001 (received for review February 27, 2001)

Preparations of purified full-length fusion (F) protein of human respiratory syncytial virus (HRSV) expressed in recombinant vaccinia-F infected cells, or of an anchorless mutant (F_{TM}-) lacking the C-terminal 50 amino acids secreted from vaccinia-F_{TM}-infected cells contain a minor polypeptide that is an intermediate product of proteolytic processing of the F protein precursor F0. N-terminal sequencing of the intermediate demonstrated that it is generated by cleavage at a furin-motif, residues 106–109 of the F sequence. By contrast, the F1 N terminus derives from cleavage at residue 137 of F0 which is also C-terminal to a furin recognition site at residues 131–136. Site-directed mutagenesis indicates that processing of F0 protein involves independent cleavage at both sites. Both cleavages are required for the F protein to be active in membrane fusion as judged by syncytia formation, and they allow changes in F structure from cone- to lollipop-shaped spikes and the formation of rosettes by anchorless F.

Human respiratory syncytial virus (HRSV) is the main cause of severe lower respiratory tract infections in infants and young children (1), and it is also a pathogen of considerable importance in the elderly (2). HRSV is an enveloped, nonsegmented negative-strand RNA virus, classified within the *Pneumovirus* genus of the *Paramyxoviridae* family. The virion has two main surface glycoproteins in the viral membrane: the attachment (G) protein, which mediates virus binding to the cell receptor (3), and the fusion (F) protein, which is responsible for fusion of the viral and cell membranes (4). A third small hydrophobic (SH) surface glycoprotein of unknown function is expressed abundantly at the surface of infected cells, but is incorporated only in small amounts in the virus particle (5).

Fusion of HRSV and cell membranes is thought to occur at the cell surface and is a necessary step for transfer of the viral ribonucleoprotein into the cell cytoplasm. The F protein, which mediates this process, also promotes fusion of infected cell membranes with those of adjacent cells, leading to syncytia formation. Whereas the G protein of HRSV shares neither sequence nor structural features with the attachment protein of related viruses (6), the F protein shares structural elements with its counterpart in other paramyxoviruses, and all of the F proteins have a low but significant level of sequence relatedness (7).

The F protein is synthesized as an inactive precursor of 574 amino acids (see Fig. 1A for a diagram of its primary structure) that is cleaved by furin-like proteases during transport to the cell surface to yield two disulfide-linked polypeptides, F2 from the N terminus and F1 from the C terminus (8). There are three hydrophobic sequences in the F polypeptide. The first is the signal peptide, located at the N terminus of the F2 chain. The second is the fusion peptide at the N terminus of the F1 chain, and the third is the transmembrane region, located near the C terminus of F1. Adjacent to the fusion peptide and transmembrane regions are two heptad repeat sequences, HRA and HRB,

that are predicted to form coiled-coil structures (9). HRA and HRB peptides form trimeric complexes in solution (10, 11), and x-ray crystallography of these complexes reveals an internal core of three HRA α -helices bounded by three antiparallel HRB α -helices packed in the grooves of the HRA coiled-coil trimer (11). This structure is very similar to that described for analogous peptides from the paramyxovirus SV5 fusion protein (12), confirming the structural similarities of the HRSV F with its counterparts in related viruses.

Recently, we described electron microscopy of the purified full-length F glycoprotein of HRSV and a truncated anchorless mutant lacking the C-terminal 50 amino acids (13), both expressed from recombinant vaccinia viruses. Full-length F, in the absence of detergent, formed rosettes that contained two distinct types of protein rods, one cone-shaped and the other lollipop-shaped. In contrast, the anchorless F mutant that was excreted by cells remained largely as individual cone-shaped rods, although a low proportion of rosettes containing lollipop-shaped rods was also observed. We proposed that these two types of rods might represent different structures that the F molecule may adopt before and after activation for its role in membrane fusion. Additional polypeptides to F1 and F2 that reacted with antibodies specific for the F protein were reported to be present in preparations of both full-length and anchorless F, and were particularly prominent in anchorless F (13). In this paper we have characterized these polypeptides as intermediates in F proteolytic processing. Analysis of their sequences indicate that the F precursor is cleaved at two sites, both preceded by motifs recognized by furin-like proteases. Cleavage at both sites is required for acquisition of membrane fusion potential by the F protein, and electron microscopy shows that, when cleavage is completed at both sites, there is a transition from cone-shaped to lollipop-shaped rods.

Materials and Methods

Cells and Viruses. All cells were grown in DMEM supplemented with 10% FCS. The Long strain of HRSV and recombinant vaccinia viruses were grown in HEp-2 cells as described (13). HRSV was purified from the culture supernatant of infected cells by precipitation with 6% polyethyleneglycol, centrifugation in a 20–60% sucrose gradient, and pelleting of the virus band through a 30% sucrose cushion (14).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: HRSV, human respiratory syncytial virus; F, fusion; SH, small hydrophobic; VAC, vaccinia.

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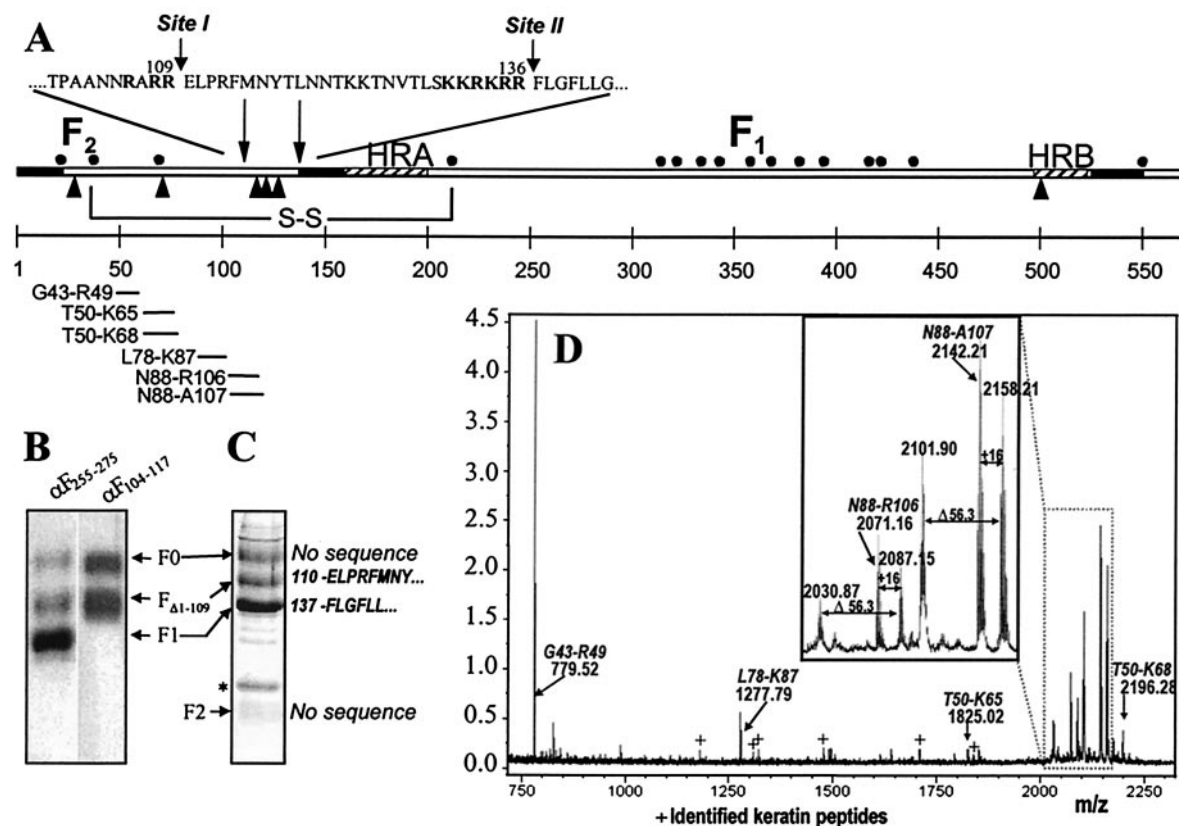


Fig. 1. Identification of two cleavage sites in anchorless F protein. (A) Diagram of the F protein primary structure denoting cysteine residues (●), potential glycosylation sites (▲), hydrophobic regions (■), heptad repeat sequences (▨) and cleavage sites by furin-like proteases (↓). Inserted is a partial amino acid sequence of the F protein where the furin-sequence motifs (boldface) and the cleavage sites I and II are indicated. (B) Western blot of purified F_{TM}- developed with anti-F₂₅₅₋₂₇₅ and anti-F₁₀₄₋₁₁₇ sera. The location of bands is shown at right (see text for explanation). (C) Coomassie blue-stained poly(vinylidene difluoride) membrane of purified F_{TM}- after SDS/PAGE. N-terminal sequences of F_{Δ1-109} and F₁ bands, determined by Edman degradation (see *Materials and Methods*), are shown at right. The band with the asterisk was identified as a contaminant of bovine apolipoprotein I by N-terminal sequencing. The F₀ and F₂ bands yielded no sequence. (D) The mass spectrum of tryptic peptides of the F₂ band denotes peaks with masses matching the expected values of peptides from F₂ (arrows). (Insert) Magnified spectrum of peptides N88-R106 and N88-A107 denoting by-products with oxidized methionines (+16 m/z) and metastable degradation products (Δ56.3 m/z) of these peptides. The location of the F₂ peptides in the F primary structure is shown in A, below the protein diagram.

Plasmids and Recombinant Vaccinia Viruses. pTM1-derived plasmids carrying inserts of the F gene (Long strain) with either the wild-type sequence (pTM1/F) or the sequence with the change I525Stop (ATC to TAA) that encodes the F_{TM}- protein (pTM1/F_{TM}-) have been reported (15). These plasmids have the F gene insert cloned under a T7 promoter. The F inserts were subcloned in the pRB21 plasmid (a gift of R. Blasco, Madrid, Spain) to generate vaccinia recombinants (VAC-F and VAC-F_{TM}-) of the vRB12 strain (15).

The following mutations were introduced in the F gene insert of plasmids pTM1/F, pTM1/F_{TM}-, pRB21/F and pRB21/F_{TM}-. R108N (AGA to AAC), R109N (AGA to AAC), R108N/R109N, K131Q (AAG to CAG), and Δ131-134 (deletion of nucleotides 404-415). The nucleotide changes for each mutant are shown between parentheses. Complementary oligonucleotides with the appropriate changes were synthesized and used in the Quik-Change site-directed mutagenesis kit (Stratagene) to generate the different mutants. pRB21-derived plasmids were used to generate vaccinia recombinants by the method of Blasco and Moss (16).

Antibodies and Peptides. Monoclonal antibodies raised against the F protein of HRSV (Long strain) were described by García-Barreno *et al.* (17). All these antibodies recognize epitopes in the F₁ chain. A polyclonal rabbit serum raised against a peptide of the F₁ chain, spanning residues 255-275, bound to keyhole

limpet hemocyanin (KLH) (anti-F₂₅₅₋₂₇₅) has been described (18). A peptide spanning residues 104-117 of the F sequence with an extra cysteine added at the C terminus was synthesized in an Applied Biosystems 430A synthesizer, by using fluorenylmethoxycarbonyl chemistry. The peptide was deprotected and cleaved off the resin with trifluoroacetic acid, purified by HPLC (19), coupled to KLH through the extra cysteine, and used to raise a rabbit serum (anti-F₁₀₄₋₁₁₇) as described (18).

Purification, Western Blotting, Sucrose Gradient Centrifugation, and Electron Microscopy of F Proteins. The full-length F protein was purified from extracts of HEp-2 cells infected with either Long virus or the vaccinia recombinant VAC-F, harvested 48 h after infection. The anchorless F protein was purified from the supernatant of HEp-2 cells infected with the vaccinia recombinant VAC-F_{TM}- and harvested 48 h after infection. Preparation of extracts and purification of the F protein by immunoaffinity chromatography have been described (13). Proteins were separated by SDS/PAGE, transferred to Immobilon membranes (Millipore), and blotted with the antibodies indicated in the figure legends.

Samples of F proteins (≈100 μg) were loaded onto preformed 10-25% sucrose gradients in PBS, and were centrifuged at 39,000 rpm for 15 h at 4°C in a Beckman SW40 rotor. Fractions (1 ml) were collected from the top of the tube, and 20-μl aliquots were analyzed

by Western blotting. Pools of fractions were concentrated and dialyzed against PBS in a Centricon-30 (Amicon).

F-protein samples in PBS were absorbed onto carbon films and stained with 1% sodium silicotungstate (pH 7.0). A JEOL 1200 electron microscope, operated at 100 kV, was used to view the samples. Micrographs were taken under minimum-dose, accurate defocus conditions to preserve details to ≈ 1.5 nm (20).

N-Terminal Sequencing and MS. Automated Edman degradation was performed on poly(vinylidene difluoride) (PVDF)-immobilized proteins after SDS/PAGE by using an Applied Biosystem Procise 494 sequenator.

Protein bands from Coomassie blue-stained gels were excised manually and processed automatically for trypsin digestion by using an Investigator ProGest Protein Digestion Station (Genomic Solutions, Cambridgeshire, U.K.), following the protocol of Schevchenko *et al.* (21). Briefly, after reduction and alkylation, protein bands were digested with sequencing-grade modified porcine trypsin (final concentration, 16 ng/ml; Promega) for 12 h at 37°C. Peptides were eluted with 25 mM ammonium bicarbonate/10% formic acid (vol/vol), and acetonitrile in a final volume of 100 μ l. A 0.5- μ l aliquot was mixed with α -cyano-4-hydroxy-*trans*-cinnamic acid matrix and processed for MS. Spectra were acquired by using a Bruker Reflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) equipped with delayed extraction and operated in the reflector mode. The equipment was first externally calibrated by employing protonated mass signals from a peptide mixture covering the 1000- to 4000-*m/z* range, and thereafter every spectrum was internally calibrated by using selected signals arising from trypsin autolysis. The measured tryptic peptide masses were transferred through MS BioTools program to Mascot Peptide Mass Fingerprinting search engine (Matrix Science, London) to search automatically the National Center for Biotechnology Information nonredundant database. No restrictions were placed on the species of origin of the protein and the allowed protein molecular mass was 1–200 kDa.

Syncytia Formation Assay. BSR-T7/5 cells (22), a BHK-derived cell line that expresses constitutively the T7 RNA polymerase (a gift of K.-K. Conzelmann, Munich, Germany), were transfected with 1.5 μ g of the plasmids indicated in the figure legends by the calcium phosphate precipitation method (MBS, mammalian transfection kit, Stratagene). Forty hours later, the cells were fixed with methanol/acetone and processed for indirect immunofluorescence by using anti-F monoclonal antibodies, as described (23).

Results

Identification of Two Processing Sites in the F Polypeptide. We have described before two vaccinia recombinant viruses that express either the full-length F glycoprotein of HRSV (VAC-F) or a truncated membrane anchorless mutant (VAC-F_{TM-}) lacking the C-terminal 50 amino acids (15). Full-length F is incorporated in the cell membrane; the F_{TM-} mutant is secreted into the cell culture medium. Both proteins were purified by immunoaffinity chromatography from either extracts of HEP-2 cells infected with VAC-F or culture supernatants of HEP-2 cells infected with VAC-F_{TM-} (13).

When the purified F_{TM-} protein was analyzed by SDS/PAGE and Western blotting by using an antiserum raised against a synthetic peptide of the F1 chain (residues 255–275 of the F polypeptide) three protein bands were observed (Fig. 1B). The smallest, and most abundant, band corresponds to the previously characterized F1 polypeptide (24), which lacks the C-terminal 50 amino acid of intact F1. The other two bands were not characterized in our previous work (13).

An antiserum was raised against a synthetic peptide containing residues 104–117 of the F sequence plus a cysteine added at the C terminus end for coupling to carrier proteins. When preparations of F_{TM-} were blotted with the anti-F_{104–117} serum, the two upper bands, but not F1, were detected (Fig. 1B).

To characterize the different polypeptides present in the preparations of purified F_{TM-}, we determined their N-terminal sequences (Fig. 1C). The largest band gave no sequence, suggesting a blocked N terminus. Analysis of tryptic peptides derived from this band by MALDI-TOF MS confirmed it contained sequences of F2 and F1, in agreement with its reactivity with anti-F_{104–117} and anti-F_{255–275} sera (Fig. 1B). Consequently, this band was identified as the uncleaved F0 precursor of the anchorless protein. The first eight Edman degradation cycles of the intermediate band indicated a sequence that matched residues 110–117 of the F polypeptide (Fig. 1C). Because this band also contained sequences of the F1 chain—inferred from its reactivity with the anti-F_{255–275} serum (Fig. 1B) and from tryptic peptide mass analysis—it was identified as a polypeptide starting at residue 110 of the F sequence and ending at the C terminus of F1_{TM-}. This band was named F _{Δ 1–109}. Finally, the sequence of the six N-terminal amino acids of the F1 band matched the first residues of the fusion peptide (Fig. 1C), confirming that it is the mature F1 chain generated by cleavage of the F precursor after amino acid 136. These results, therefore, demonstrated that the F-protein precursor could be cleaved at two sites (Fig. 1A): site I, after residue 109, and site II, after residue 136. Both sites are preceded by sequence motifs recognized by furinlike proteases.

N-terminal sequencing of a diffuse band of F_{TM-} near the bottom of the gel was also attempted (Fig. 1C) but no amino acid sequence was detected. Peptide mass fingerprinting of the band showed that it corresponded to the F2 chain (Fig. 1D). Peptides derived from the F sequence between cleavage sites I and II, described above, were not detected in the mass spectrum of the F2 band. However, tryptic peptides with masses corresponding to those preceding amino acid 109 were clearly identified (Fig. 1D *Inset*). Among these peptides there was one, N88-A107, which is unlikely to be generated by the *in vitro* trypsin digestion of the F2 band, implying that it was generated during the *in vivo* processing of the anchorless F protein.

To determine whether the F _{Δ 1–109} polypeptide was present in preparations of the F molecule, in addition to F_{TM-}, full-length F protein purified from extracts of HEP-2 cells infected with either VAC-F or HRSV (Long strain) or from purified HRSV virions were analyzed by Western blotting with anti-peptide sera (Fig. 2). The anti-F_{255–275} serum recognized an F1 band in all preparations containing full-length F protein that migrated slightly less than the F1 band of F_{TM-}, as expected by their size differences (Fig. 2A). The anti-F_{104–117} serum reacted with a band, corresponding to F _{Δ 1–109}, in the preparations that contained full-length F protein (Fig. 2B). Relative to F1, the amounts of F0 and F _{Δ 1–109} depended on the origin of each protein sample. It has been reported that processing of F protein is less efficient in cells infected with VAC-F than with HRSV (25). Accordingly, F0 and F _{Δ 1–109} were less abundant in preparations of virus or full-length F protein obtained from HRSV infected HEP-2 cells than in preparations of F protein from VAC-F-infected cultures. The amounts of F0 and F _{Δ 1–109} were highest in the preparation of anchorless F protein. This abundance may reflect the different intracellular pathway that this form of F protein follows in its transport to the extracellular medium.

The F Protein Precursor Is Cleaved Independently at Sites I and II. To investigate whether or not cleavage of F0 at site I was required for cleavage at site II, mutants of anchorless F protein and full-length F protein were generated at both sites. HEP-2 cells

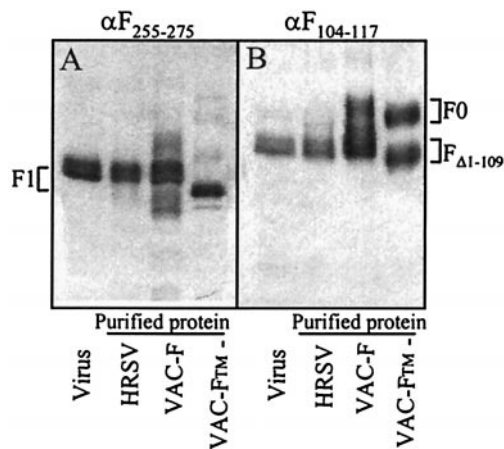


Fig. 2. Immunoblots of full-length and anchorless F protein preparations. (A) Purified HRSV virus or F protein purified from extracts of HEp-2 cells infected with either HRSV or VAC-F or F_{TM-} protein purified from the supernatant of HEp-2 cells infected with VAC- F_{TM-} were blotted with the anti-F₂₅₅₋₂₇₅ serum. The amount of each sample was standardized to yield F1 bands of similar intensity. (B) The same protein samples from A were blotted with the anti-F₂₁₀₄₋₁₁₇ serum. The amount of each sample loaded in the gel relative to the blot of part A was as follows: purified virus, 10:1; F(HRSV), 2:1; F(VAC-F), 1:1; F(VAC- F_{TM-}), 1:1.

were infected with vaccinia recombinants expressing either the anchorless F protein or the anchorless F mutants R108N, R109N, or R108N/R109N. After labeling with either [³⁵S]methionine (Fig. 3A) or [³H]glucosamine (Fig. 3B), proteins were immunoprecipitated from the culture supernatants with a monoclonal antibody specific for the F1 chain. Whereas the F0, $F_{\Delta 1-109}$, and F1 polypeptides were observed in the immunoprecipitates of anchorless F, the $F_{\Delta 1-109}$ band was absent in the three mutants, indicating that cleavage at site I was inhibited in these cases. In the same gels, a diffuse band corresponding to the F2 chain of anchorless F protein was less intense in the single mutants and totally absent in the immunoprecipitate of the double mutant. Very diffuse bands that migrated less than F2 ($F2^*$) were observed in the immunoprecipitates of the three mutant proteins (more clearly seen in the samples labeled with ³H-glucosamine). Absence of $F_{\Delta 1-109}$ and F2 and presence of $F2^*$ chains was also observed in immunoprecipitates of cell extracts infected with vaccinia recombinants expressing full-length F gene with the mutations R108N, R109N, or R108N/R109N (Fig. 3C). These results, therefore, demonstrated that mutations in the furin-target sequence preceding site I inhibited cleavage after residue 109 of the F polypeptide. However, inhibition of that cleavage did not prevent processing at site II, after residue 136, to generate the F1 chain.

Mutations K131Q and $\Delta 131-134$, which alter the sequence preceding site II, were introduced in the full-length F gene of pTM1-derived plasmids. HEp-2 cells were infected with the recombinant vaccinia MVA-T7 (26), which expresses the T7 RNA polymerase, and transfected with pTM1 plasmids carrying the different F gene constructs. Extracts of the cells were subjected to SDS/PAGE and blotted with a monoclonal antibody specific for the F1 chain. Similar amounts of the F1 polypeptide were detected in the extracts containing the wild-type F or the mutant K131Q (Fig. 3D), but the amount of F1 was clearly reduced in the case of mutant $\Delta 131-134$. An increase in the amount of $F_{\Delta 1-109}$ was observed for mutant $\Delta 131-134$, indicating that inhibition of cleavage at site II did not prevent cleavage at site I. Thus, F0 is cleaved at both sites after the furin motifs and cleavage at one site is not required for cleavage at the other site.

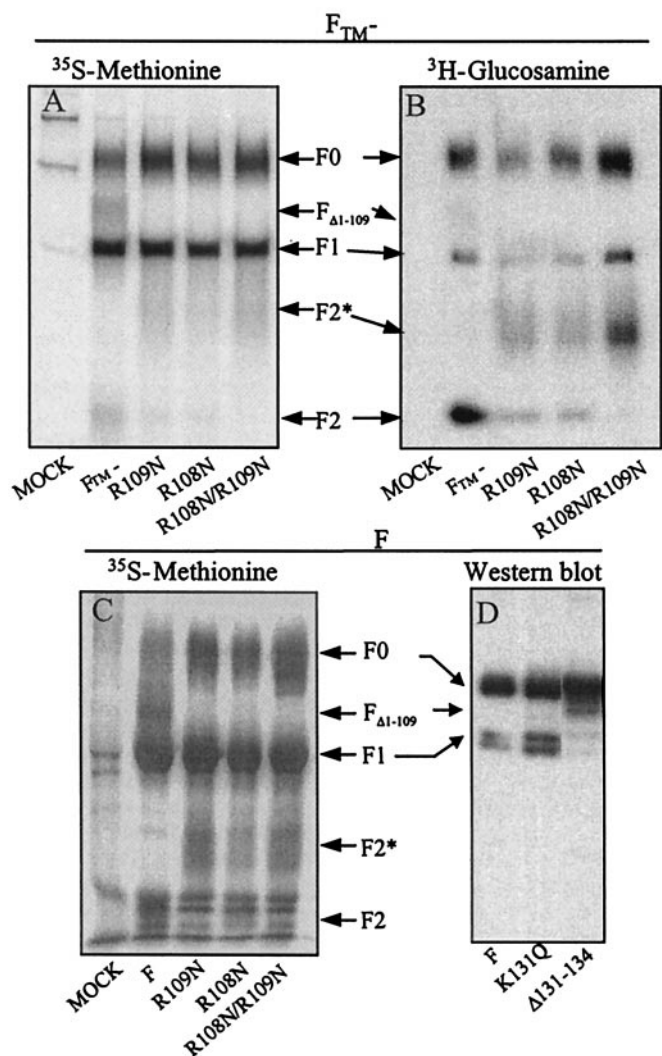


Fig. 3. Analysis of F-protein mutants with changes at sites I or II. (A) HEp-2 cells were infected with vaccinia recombinants (multiplicity of infection, 5 plaque-forming units per cell) expressing the F_{TM-} protein with the wild-type sequence or with the mutations R109N, R108N, or R108N/R109N. Cells were labeled from 4 to 24 h after infection with [³⁵S]methionine (100 μ Ci/ml). Cell supernatants were harvested at the end of the labeling period and aliquots were immunoprecipitated with a monoclonal antibody (47F) specific for the F1 chain (17). Proteins were resolved by SDS/PAGE and visualized by fluorography. (B) Immunoprecipitate of cell culture supernatants from cells infected as indicated in A, but labeled with [³H]glucosamine (100 μ Ci/ml) from 4 to 24 h after infection. (C) HEp-2 cells were infected with vaccinia recombinants expressing either wild-type F or the same mutants from A but in the full-length protein. Cells were labeled from 4 to 24 h after infections with [³⁵S]methionine, and extracts were immunoprecipitated as in A. (D) HEp-2 cells were infected with the vaccinia virus MVA-T7 (26) and transfected with pTM1 plasmids carrying either the full-length F gene or mutants K131Q or $\Delta 131-134$, by using Lipofectin (23). Extracts were made 48 h after infection and analyzed by SDS/PAGE and Western blotting with monoclonal antibody 47F.

Structural Changes Associated with Proteolytic Processing of the F Protein. Purified anchorless F protein was incubated with increasing amounts of trypsin (Figs. 4A and B) and then analyzed by Western blotting with anti-peptide sera. Whereas F0 and $F_{\Delta 1-109}$ were progressively digested with increasing doses of trypsin, the F1 band remained essentially unchanged, although some faster migrating bands reacting with anti-F₂₅₅₋₂₇₅ antibodies were observed with the highest amounts of trypsin (Fig. 4A).

In agreement with our previous results (13), the anchorless F

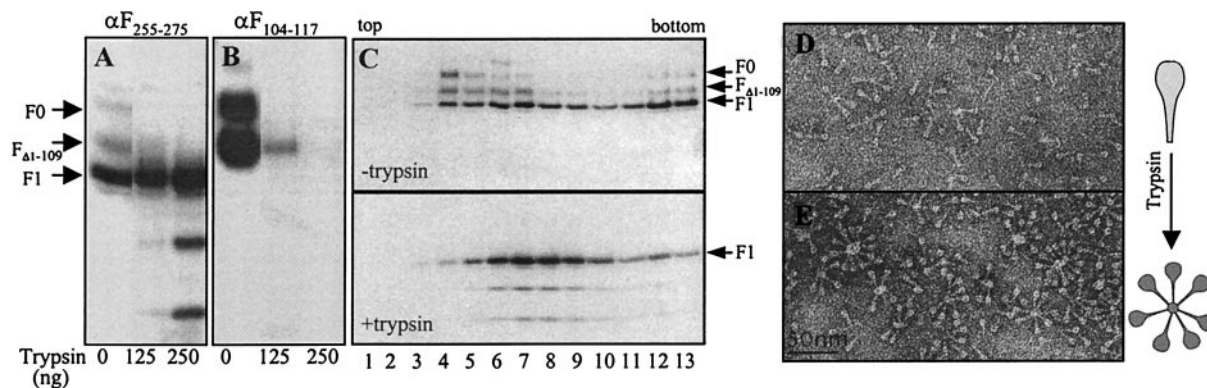


Fig. 4. Changes associated with trypsin digestion of purified F protein. Aliquots of purified F_{TM} (1 μ g) were incubated with the indicated amounts of trypsin for 60 min at 37°C. Subsequently, a large excess of sample buffer was added and the proteins were resolved by SDS/PAGE and blotted with the anti-F₂₅₅₋₂₇₅ (A) or anti-F₁₀₄₋₁₁₇ (B) sera. (C) Samples of F_{TM} ($\approx$ 100 μ g) were digested with trypsin in PBS (equivalent to the conditions of the 250-ng lane of A), or mock-digested. Then, one-fifth volume of a trypsin inhibitor (complete miniEDTA, Roche Diagnostics) was added and the samples were loaded on top of a 10–25% sucrose gradient and centrifuged as indicated in *Materials and Methods*. Fractions (1-ml) were analyzed by Western blotting with the anti-F₂₅₅₋₂₇₅ serum. Pools of fractions from the gradients shown at the top (fractions 4–7) or at the bottom (fractions 6–9) of C were observed under the electron microscope by negative staining (D and E, respectively). A diagram of the changes observed in F_{TM} molecules is shown at right.

protein, mock-treated with trypsin, sedimented in a 10–25% sucrose gradient as a broad band (Fig. 4C), with a peak near the top of the gradient (fractions 4–7); some aggregated forms sedimented toward the bottom of the gradient. F0 and $F_{\Delta 1-109}$ were more prominent in the fractions near the top of the gradient. Densitometric quantitation of the proteins in the gradient fractions yielded the following figures: the material in fraction 4 was 53% F1 and 47% F0 plus $F_{\Delta 1-109}$; fraction 9, 91% was F1 and 9% F0 plus $F_{\Delta 1-109}$. Samples of anchorless F protein, incubated with trypsin under conditions in which F0 and $F_{\Delta 1-109}$ were fully digested, sedimented toward the middle of the gradient (Fig. 4C). This change in the sedimentation profile of anchorless F protein after trypsin treatment was accompanied by changes in the aggregation state of the molecules in electron microscopy. Although the mock-treated sample contained a majority of individual, unassociated molecules with <25% in rosettes in the top fraction (13) and about 47% in pooled fractions 4–7 (Fig. 4D), most of the molecules (86%) in pooled fractions 6–9 of the gradient of the trypsin-treated sample were in rosettes (Fig. 4E). The rosettes of the trypsin-treated sample also contained more spikes than those of the untreated anchorless F protein. These results indicate that fully processed anchorless F protein forms rosettes in aqueous media. In agreement with our previous observations, the anchorless F molecules in rosettes are lollipop-shaped compared with unassociated molecules, which are cone-shaped.

Cleavage of the F Protein at Sites I and II Is Needed for Membrane Fusion. The membrane fusion capacity of full-length F proteins, and mutants derived from it, was evaluated in a syncytia formation assay. BRS-T7/5 cells (22) transfected with F-containing plasmids were fixed 40 h after transfection and stained with a monoclonal antibody specific for the F1 chain. Large syncytia, containing several nuclei in the same cytoplasm, were clearly visible in cultures expressing either the wild type or the K131Q mutant (Fig. 5). In contrast, BSR-T7/5 cells transfected with plasmids carrying the F genes of the double mutant R108N/R109N, which is not processed at site I, or the mutant $\Delta 131-134$, which is not processed at site II, showed individual cells stained with the anti-F1 antibody but no syncytia. These results indicate that inhibition of F-protein cleavage at either site removes the capacity of the F protein to promote membrane fusion.

Discussion

The results presented here demonstrate that cleavage of the biosynthetic precursor F0 of HRSV after two furin-recognition sequence motifs is required for membrane fusion activity. The fate of the F segment located between the two sites is not known. The F segment was not found in immunoprecipitates of either full-length or anchorless F proteins following processing, suggesting that it is released from the processed molecule. An analogous segment is present in the F protein of the closely related bovine respiratory syncytial virus; however, the F proteins of other pneumoviruses and paramyxoviruses lack the sequence between the two cleavage sites (9).

The mechanism of activation of membrane fusion by paramyxoviruses is unknown. Enveloped viruses, such as influenza virus (27), which are endocytosed after binding of the attachment protein to specific receptors present on the surface of target cells, are activated at endosomal pH. In contrast,

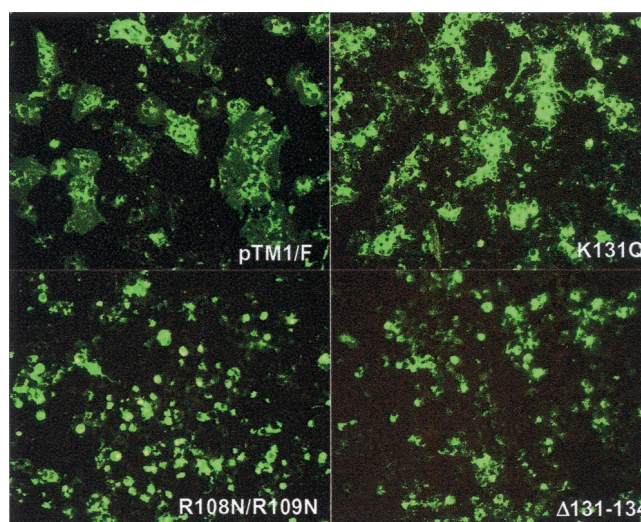


Fig. 5. Syncytia formation in BSR-T7/5 cells transfected with pTM1 plasmids carrying wild type or mutant full-length F genes. Cells were transfected with the indicated plasmids and processed for immunofluorescence 40 h later with an antibody specific for the F1 chain, as indicated in *Materials and Methods*. (Magnification, $\times 200$.)

paramyxoviruses fuse their membranes to those of target cells at the cell surface. It has been postulated that the interaction of paramyxovirus attachment protein with the cell receptor triggers a conformational change in the F protein that promotes membrane fusion (28). It has also been reported that the G and SH glycoproteins of HRSV enhance the fusion activity of F proteins when coexpressed in transfected cells (29). However, a deletion mutant of HRSV lacking both the G and SH genes is able to replicate efficiently in certain cell lines and to produce syncytia (30). In addition, the expression of the F glycoprotein alone is sufficient to induce formation of syncytia in transfected cells (Fig. 5). Thus, the question arises, is cleavage at the two furin-motif sites of HRSV F the trigger for membrane fusion? If this were so, by analogy with viruses that can be inactivated by triggering at low pH in the absence of a target membrane, inactivation of F might occur before its expression at the cell surface. Perhaps the process of activation occurs slowly after cleavage, ensuring that, for a significant time, some noninactivated molecules survive to maintain virus infectivity. Perhaps activation after processing might involve the slow release of the intervening peptide and perhaps the rate of activation might be enhanced by, for example, recognition of target cells by F2 or some other region of F.

The anchorless F mutant described here remains mainly as single trimeric molecules when a significant proportion of the molecules are not cleaved at either site I to generate F_{Δ1-109} or sites I and II to generate F1 + F2. On trypsin digestion of F_{TM-}, under conditions in which F_{Δ1-109} and F0 are digested, the protein forms rosettes and changes its shape from cone-shaped to lollipop-shaped. There are reports from other paramyxovirus that cleavage of F0 results in exposure of a new

hydrophobic region (31) and in accessibility to site specific antibodies (32). Rosettes of anchorless F proteins of HRSV are assumed to form as a result of interactions between exposed fusion peptides, which are the major conserved hydrophobic regions remaining in the F_{TM-} molecules. If this is so, processing of F0 at both sites might be needed for fusion peptide exposure. Because cleavage at both sites is also required for the F protein to acquire its potential to induce syncytia, the change in shape of F, its ability to form rosettes, and the capacity to fuse membranes may be directly related.

In the case of some influenza and parainfluenza viruses, cleavability of the fusion protein has been clearly shown to correlate with pathogenicity (for a review, see ref. 33). For avian influenza hemagglutinins, for example, insertions at the site of cleavage increase cleavability and virulence for chickens (34). By analogy, the sequences located between sites I and II may confer unique pathogenic properties to human and bovine respiratory syncytial viruses. The feasibility of constructing mutant viruses (35) should enable the relevance of sites I and II for pathogenicity and attenuation to be assessed.

We are grateful to Rafael Blasco (Madrid) for the pRB21 plasmid and for the vRB12 vaccinia virus, and to Klaus-K. Conzelmann (Munich) for the BSR-T7/5 cells. This work was supported in part by Grants PM99-0014 from Ministerio de Ciencia y Tecnología and QLK2-CT-1999-00443 from the European Union (to J.A.M.), by the Medical Research Council (to J.J.S. and L.C.) and by the Howard Hughes Medical Institute. D.C.W. is an Investigator of the Howard Hughes Medical Institute. L.G.-R. was recipient of a predoctoral fellowship from Ministerio de Educación y Cultura (Spain) and M.B.R.-A. was recipient of a postdoctoral fellowship from the Comunidad de Madrid (Spain).

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