Proteolytic Activation of Respiratory Syncytial Virus Fusion Protein

CLEAVAGE AT TWO FURIN CONSENSUS SEQUENCES*

Gert Zimmer, Linda Budz, and Georg Herrler‡

From the Institut für Virologie, Tierärztliche Hochschule Hannover, Bünteweg 17, D-30559 Hannover, Germany

The F (fusion) protein of the respiratory syncytial viruses is synthesized as an inactive precursor F₀ that is proteolytically processed at the multibasic sequence KKKRRKR₁³⁶ into the subunits F₁ and F₂ by the cellular protease furin. This maturation process is essential for the F protein to gain fusion competence. We observed that proteolytic cleavage additionally occurs at another basic motif, RARR₁⁰⁹, that also meets the requirements for furin recognition. Cleavage at both sites leads to the removal from the polypeptide chain of a glycosylated peptide of 27 amino acids. When the sequence RARR₁⁰⁹ was changed to NANR₁⁰⁹ or to RANN₁⁰⁹ by site-directed mutagenesis, cleavage by furin was completely prevented. Although the mutants were still processed at position Arg₁³⁶, they did not show any syncytia formation. Proteolytic cleavage of the modified motifs was achieved by treatment of transfected cells with trypsin converting the F mutants into their fusogenic forms. Our findings indicate that both furin consensus sequences have to be cleaved in order to activate the fusion protein.

Endoproteolytic cleavage is a common post-translational modification of membrane and secretory proteins on the exocytotic route. Precursors of peptide hormones, neuropeptides, growth factors, coagulation factors, serum albumin, cell surface receptors, and adhesion molecules are converted to their biological active form by an endoproteolytic cleavage usually at the C-terminal end of an arginine residue (1). Likewise, many viral membrane proteins involved in the fusion process between viral and cellular membranes depend on proteolytic activation (2, 3). The cleavage of these proteins usually results in the exposition of a hydrophobic fusion peptide at the N terminus of the membrane-anchored fragment. The fusion peptide is supposed to initiate the fusion process by direct interaction with the lipid bilayer of the host membrane. Cleavage of the fusion proteins is therefore essential for virus infectivity. The majority of the viral fusion proteins contain a multibasic cleavage motif of the consensus sequence RX(K/R)R. This sequence is recognized and cleaved by furin, an ubiquitous subtilisin-like endoprotease localized in the trans-Golgi network (2, 3). The ubiquitous expression of furin has important consequences for virus pathogenicity as all cells produce infectious virus with activated fusion proteins allowing the rapid spread of infection. Some viral glycoproteins contain a monobasic cleavage site that is not recognized by furin. Viral glycoproteins of this type are activated by trypsin-like proteases that are secreted by a restricted subset of host cells or by co-infecting bacteria (4, 5). As a consequence, many cells will produce virus that is not proteolytically activated and therefore not infectious. Viruses with a monobasic cleavage motif in their fusion proteins usually cause localized infections and are unable to spread to different organs or tissues. Thus, the cleavage site is an important determinant of virus pathogenicity. This concept has been well established for Newcastle disease virus, Sendai virus, and avian influenza viruses (2, 3).

Human respiratory syncytial virus (HRSV)¹ and bovine respiratory syncytial virus (BRSV) are closely related members of the genus Pneumovirus within the family Paramyxoviridae. HRSV is the most important viral agent of pediatric respiratory tract disease worldwide causing bronchiolitis and pneumonia (6). A very similar disease is caused by BRSV in calves (7–10). The importance of HRSV as a respiratory pathogen makes development of a safe and effective vaccine a demand of high priority. The envelope of the respiratory syncytial viruses contains three glycoproteins, designated F, G, and SH, respectively. The presence of both the G and SH protein is non-essential for virus replication in cell culture (11–13). The F protein is a highly conserved molecule with a homology of 80% or more between different serotypes of HRSV and BRSV and it is the major virus antigen inducing neutralizing antibodies (14–16). The F protein plays a central role in virus entry. It mediates fusion between the viral and cellular membrane thereby allowing the nucleocapsid to enter the cytoplasm of the host cell. In addition, cells infected with RSV can fuse with adjacent cells resulting in giant, multinucleated syncytia. Syncytia formation can also be observed with cells transfected with the F gene, although coexpression of F together with G and/or SH protein has been reported to enhance fusion activity (17, 18). Recent studies suggest that certain glycosaminoglycans of the cell surface are required for HRSV infection (19–22). The G as well as the F protein have been demonstrated to bind to these carbohydrate structures (19, 23, 24).

The F protein is a type I integral membrane protein that is synthesized as a precursor F₀ of 70 kDa which is post-transla-

---

* The work was supported Deutsche Forschungsgemeinschaft Grant HE 1168/11-2/1 (to G. H.) and European Community Grant QLK2-CT-1999-00443. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This publication is dedicated to Prof. Dr. R. Rott on the occasion of his 75th birthday.

‡ To whom correspondence should be addressed: Institut für Virologie, Tierärztliche Hochschule Hannover, Bünteweg 17, D-30559 Hannover, Germany. Fax: 49-511-953-8898; E-mail: Georg.Herrler@tiho-hannover.de.

¹ The abbreviations used are: HRSV, human respiratory syncytial virus; BRSV, bovine respiratory syncytial virus; RSV, respiratory syncytial virus; FCS, furin consensus sequence; EMEM, Earle's minimal essential medium; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
titionally cleaved by a cellular protease at a multibasic sequence into two disulfide-linked subunits F₂ (20 kDa) and F₁ (50 kDa) (25, 26). The cleavage site preceding the hydrophobic fusion peptide is composed of six basic amino acids (KKRRKKR) and is strictly conserved in all HRSV and BRSV isolates. This sequence meets the requirements for furin recognition but with respect to the number of basic amino acid residues, it differs from those of all other paramyxoviruses that usually have tri- or tetrabasic motifs (2, 3). Pulse-chase experiments using various drugs that inhibit the vesicular transport between the endoplasmic reticulum and Golgi compartments suggested that the F₀ precursor is cleaved in the Golgi or trans-Golgi network (26, 27). Proteolytic activation of the F protein was also inhibited when RSV-infected cells were treated with a peptidyl chloromethylketone inhibitor containing the furin target sequence (27). In addition, Lovo cells that do not express furin because of a genetic defect did not efficiently cleave the F₀ precursor protein (27). These data provide evidence that furin is responsible for RSV F protein activation. Interestingly, in neither case the uncleaved precursor F₀ was detected at the cell surface suggesting that only the proteolytically cleaved fusion protein is transported to the plasma membrane (26, 27). In this respect, RSV differs from other paramyxovirus fusion proteins that are transported to the cell surface also in their uncleaved form (28). The F₀ of RSV contains another conserved furin consensus sequence (FCS-2) that is separated from the furin motif FCS-1 immediately upstream of the fusion peptide by a stretch of 27 amino acids (Fig. 1). This peptide, pep27, contains one potential N-glycosylation site in the case of BRSV and two to three N-glycosylation motifs in the case of HRSV. In this report, we investigated the role of FCS-2 in the proteolytic activation of the RSV fusion proteins by site-directed mutagenesis. We provide evidence that both furin consensus sequences have to be cleaved to obtain an active fusion protein.

EXPERIMENTAL PROCEDURES

Cells and Virus—BSR-T7/5 cells were a generous gift of Dr. Conzelmann (Max-von-Pettenkofer-Institut, München, Germany). The cells were grown in Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal calf serum, non-essential amino acids, and 0.5 mg/ml G418 sulfate (Calbiochem-Novabiochem, Bad So- dden, Germany). Vero cells and primary chicken fibroblasts were main- tained in Dulbecco’s modified Eagle medium with 5 and 10% fetal calf ' medium and 0.5 mg/ml G418 sulfate (Calbiochem-Novabiochem, Bad So- dden, Germany). The cells were grown in Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal calf serum, non-essential amino acids, and 0.5 mg/ml G418 sulfate (Calbiochem-Novabiochem, Bad So- dden, Germany). Vero cells and primary chicken fibroblasts were main- tained in Dulbecco’s modified Eagle medium with 5 and 10% fetal calf serum, respectively. RSV differs from other paramyxovirus fusion proteins with respect to the number of basic amino acid residues, it differs from those of all other paramyxoviruses that usually have tri- or tetrabasic motifs (2, 3). Pulse-chase experiments using various drugs that inhibit the vesicular transport between the endoplasmic reticulum and Golgi compartments suggested that the F₀ precursor is cleaved in the Golgi or trans-Golgi network (26, 27). Proteolytic activation of the F protein was also inhibited when RSV-infected cells were treated with a peptidyl chloromethylketone inhibitor containing the furin target sequence (27). In addition, Lovo cells that do not express furin because of a genetic defect did not efficiently cleave the F₀ precursor protein (27). These data provide evidence that furin is responsible for RSV F protein activation. Interestingly, in neither case the uncleaved precursor F₀ was detected at the cell surface suggesting that only the proteolytically cleaved fusion protein is transported to the plasma membrane (26, 27). In this respect, RSV differs from other paramyxovirus fusion proteins that are transported to the cell surface also in their uncleaved form (28). The F₀ of RSV contains another conserved furin consensus sequence (FCS-2) that is separated from the furin motif FCS-1 immediately upstream of the fusion peptide by a stretch of 27 amino acids (Fig. 1). This peptide, pep27, contains one potential N-glycosylation site in the case of BRSV and two to three N-glycosylation motifs in the case of HRSV. In this report, we investigated the role of FCS-2 in the proteolytic activation of the RSV fusion proteins by site-directed mutagenesis. We provide evidence that both furin consensus sequences have to be cleaved to obtain an active fusion protein.

Cell Surface Biotinylation—BSR-T7/5 cells were infected with MVA-T7 and transfected with recombinant pTM1 plasmid as described in the previous section. Twenty hours post-transfection, the cells were washed three times with PBS and incubated for 1 h at 37 °C either with EMEM containing 1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at a concentration of 1 μg/ml or with EMEM alone. Thereafter, the cells were washed with PBS and incubated for 20 min at 4 °C with 0.5 ml of 50 μM ¹²⁵I labeled protein A-Sepharose (Sigma, Deisen- hofen, Germany) and 2.5 μl of the RSV3216 monoclonal antibody di- rected toward the HRSV F protein (Sorotec, Oxford, United Kingdom). After agitation for 90 min at 4 °C, the immunoprecipitates were col- lected by centrifugation, washed four times with Nonidet P-40 lysis buffer, and eluted by boiling the beads in 2-fold concentrated sodium dodecyl sulfate (SDS) sample buffer. The immunoprecipitates were run on a 10% acrylamide gel, transferred to nitrocellulose membranes, and probed with a 1:1000 dilution of the monoclonal antibody 1H9262 (ICN, Eschwege, Germany). In some experiments, the furin inhibitor decanoyl-RVKR-chloromethylketone (50 μM; dec-RVKR-cmk; Bachem, Heidelberg, Germany) was added at the start of starvation period and maintained in the medium during radiolabeling of the cells. For pulse-chase experiments, the cells were labeled for 5 min with 100 μCi of [³⁵S]methionine/cysteine in 50 μl of starvation medium and chased for different time intervals in 1 ml of normal EMEM containing 10% fetal calf serum. The cells were lysed in 1 ml of Nonidet P-40 lysis buffer (50 mm Tris/HCl, pH 7.5, 150 mm NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, and protease inhibitor mixture) and insoluble material was removed by centrifugation. To 500 μl of each supernatant were added 50 μl of a 50% slurry of protein A-Sepharose (Sigma, Deisen- hofen, Germany) and 2.5 μl of the RSV3216 monoclonal antibody di- rected toward the HRSV F protein (Sorotec, Oxford, United Kingdom). After agitation for 90 min at 4 °C, the immunoprecipitates were col- lected by centrifugation, washed four times with Nonidet P-40 lysis buffer, and eluted by boiling the beads in 2-fold concentrated sodium dodecyl sulfate (SDS) sample buffer containing 1% acetic acid, 40% methanol, or 20 min, incubated in Amplify enhancer reagent (Amersham Pharmacia Biotech, Freiburg, Germany) for another 30 min and dried. The gels were ex- posed for 12 h, in case of the pulse-chase experiment for up to 10 days, at −80 °C to Bio-Max autoradiography film (Kodak, Rochester, NY). For treatment with N-glycosidase F, the immunoprecipitates were eluted in 50 μl of 1% SDS in 50 mM phosphate buffer, pH 7.0, by heating the samples at 100 °C for 10 min. The protein A-Sepharose was pelleted by centrifugation and the eluted F protein was recovered from the supernatant. To 10 μl of the supernatant, 90 μl of 50 mM phosphate buffer containing 1% octylglucoside (Sigma), 1 mM EDTA, and 1 μg of each of the protease inhibitors leupeptin, pepstatin A, and Pefabloc (Roche Molecular Biochemicals) were added. A 50-μl aliquot of this solution was incubated for 3 h at 37 °C with 6 units of N-glycosidase F (Roche Molecular Biochemicals), while the control received no enzyme. The reaction was stopped by addition of 50 μl of 2-fold concentrated SDS sample buffer containing 200 mM dithiothreitol. The samples were analyzed by Tricine-SDS 10% polyacrylamide gel electrophoresis as described above. The dried gels were exposed to Bio-Max autoradiography film.

Immunofluorescence Analysis—BSR-T7/5 cells grown on 24-well tissue culture plates to 80% confluence were infected with recombinant vaccinia virus MVA-T7 and transfected with recombinant pTM1 plasmid as described in the previous section. Twenty hours post-transfection, the cells were washed three times with PBS and incubated for 1 h at 37 °C either with EMEM containing 1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at a concentration of 1 μg/ml or with EMEM alone. Thereafter, the cells were washed with PBS and incubated for 20 min at 4 °C with 0.5 ml of PBS containing 0.5 mg/ml Sulfo-NHS-biotin (Pierce, Rockford, IL). The cells were washed once with 0.1 mM glycine in PBS and incubated in the same solution for 15 min at 4 °C in order to quench excess biotinylation reagent. The cells were solubilized in Nonidet P-40 lysis buffer and F protein was immunopre- cipitated as described above. The immunoprecipitates were run on an SDS-10% polyacrylamide gel under reducing or nonreducing conditions and transferred to nitrocellulose by the semi-dry blotting technique (34). The membrane was incubated with blocking reagent (Roche Mo- lecular Biochemicals) overnight at 4 °C, washed three times with PBS containing 0.1% Tween 20, and incubated with streptavidin-peroxidase (1:1000, Amersham Pharmacia Biotech) for 1 h at room temperature. The nitrocellulose was washed as described above and incubated for 1 min with a chemoluminescent peroxidase substrate (BM Chemolumi- nescence blotting substrate, Roche Molecular Biochemicals). The result- ing light emission was visualized by short exposure of the membrane to Bio-Max autoradiography film.
fixed with 3% paraformaldehyde for 20 min at room temperature and excess paraformaldehyde was quenched with 0.1 M glycine in PBS for 5 min. The cells were incubated with the RSV3216 monoclonal antibody for 1 h at room temperature, washed three times with PBS, and subsequently incubated with a fluorescein isothiocyanate-conjugated antibody directed to mouse immunoglobulin (Amersham Pharmacia Biotech). Both antibodies were used at a dilution of 1:200. Conventional epifluorescence was performed with a Zeiss Axiosplan 2 microscope. Digital photographs were taken using a digital video camera (INTAS focus imager, INTAS, Göttingen, Germany).

RESULTS

The RSV fusion protein contains two conserved FCS, FCS-1 is located immediately upstream of the fusion peptide while FCS-2 is separated from the fusion peptide by a stretch of 27 amino acids, designated pep27 (Fig. 1). In analogy to other paramyxoviruses it has been assumed that proteolytic activation of the RSV fusion protein occurs by cleavage at only FCS-1 resulting in an F1 subunit with the hydrophobic fusion peptide at the very N terminus. If cleavage would occur at FCS-2 rather than at FCS-1, a shorter F2 subunit would be expected, while the amino acid stretch of pep27 would be located upstream of the fusion peptide at the N terminus of F1. If cleavage occurs at both FCS-1 and FCS-2, pep27 will be absent from the mature F protein.

In the latter case, if cleavage is experimentally restricted to either FCS-1 or FCS-2, pep27 will be part of either the F1 or the F2 subunit, respectively. In order to determine the site(s) of proteolytic activation, we abolished the furin recognition motif (RX/R/K)R at FCS-1 or FCS-2 in the HRSV fusion protein by site-directed mutagenesis. The FCS-1 sequence RKRR

\[ \text{RKRR}^{136} \text{ was changed to RRKR}^{136} \text{ (mutant hF:R136K, } \text{and the FCS-2 sequence RARR}^{109} \text{ was modified to RANN}^{109} \text{ (hF:R108N/R109N). The parental and mutated F protein genes were cloned into the pTM1 plasmid, a vector designed for gene expression under control of the T7 promoter. This plasmid also contains the internal ribosomal entry site from encephalomyocarditis virus to allow cap-independent translation of the transcripts (29). Transient expression of the F protein was achieved by transfection of BSR-T7/5 cells, a cell line that stably expresses the T7-RNA polymerase (31). In addition to transfection, the cells were infected with a recombinant vaccinia virus encoding the T7-RNA polymerase gene (31). In addition to transfection, the cells were infected with a recombinant vaccinia virus.}

The transfected cells were metabolically labeled with \[^{35}S\]methionine/cysteine and the recombinant F protein was immunoprecipitated from the cell lysates by the monoclonal antibody RSV3266. The epitope recognized by this antibody is located on the F1 subunit and has been mapped to amino acids 255–279. The immunoprecipitates were analyzed by Tricine-SDS-PAGE under reducing conditions (Fig. 2A). The parental F protein (lane a) appeared as several distinct bands. In addition to the uncleaved precursor F0 (70/72 kDa), the large subunit F1 (50 kDa), and the small subunit F2 (22 kDa), we also detected a 58-kDa form of the fusion protein. We propose that this latter band represents an intermediate product that most likely originates from a fusion protein that was proteolytically processed at FCS-2 but not at FCS-1. This form was designated F1+ and probably contains pep27 attached to the F1 subunit. In accordance with our view, F1+ disappeared when cleavage at FCS-2 was prevented by site-directed mutagenesis of the furin motif (mutant hF:R108R/N109N, lane c). Concomitantly, the F2 subunit was almost completely transformed into a 38-kDa form that probably represents pep27 attached to F2 (designated F2+). When we changed the furin motif of FCS-1 but kept FCS-2 intact (mutant hF:R136K, lane e), most of F1 was converted to F1+, but not to F2, as it would be expected if only a single furin cleavage site exists. Although the furin recognition motif of FCS-1 was abolished in mutant hF:R136K, some F1 was still detected indicating that the basic motif KKKKRK

\[ \text{K}^{136} \text{ either represents a suboptimal furin recognition motif or is cleaved by an endogenous trypsin-like enzyme. In order to prevent cleavage at both FCS-1 and FCS-2, the furin inhibitor decanoyl-RVKR-chloromethylketone (dec-RVKR-cmk) was applied in the experiment. The inhibitor at a concentration of 50 μM was added 1 h before the transfected cells were labeled with \[^{35}S\]methionine/cysteine and was maintained in the medium until cell lysis. Under these conditions, the major band detectable was the precursor F0 (lanes b, d, and f). Minor bands of F1 and F2 indicate that some F protein was cleaved despite the presence of the inhibitor. As a control for the efficiency of the inhibitor, the hemagglutinin HA of fowl plague virus, influenza A/FPV/Rostock/34 virus (H7N1), was expressed in BSR-T7/5 cells (Fig. 2B). This viral glycoprotein contains a cleavage motif, KKKEKR, that is similar to that of the RSV fusion protein. In the absence of the furin inhibitor, in addition to the cleavage products HA1 and HA2, a substantial amount of uncleaved hemagglutinin (HA0) was detected (Fig. 2B, lane a) indicating that the influenza HA was not cleaved as efficiently in BSR-T7/5 cells as was the RSV F protein (compare lanes a from Fig. 2, A and B). Only faint bands of cleavage products HA1 and HA2 are visible in the presence of the inhibitor dec-RVKR-cmk (Fig. 2B, lane b). This finding suggests that the F protein of RSV is much more sensitive to the action of furin than is the hemagglutinin of fowl plague virus. Proteolytic cleavage of both HA and F is sensitive to the furin inhibitor. Taken together, these data provide the first evidence that the RSV fusion protein is proteolytically processed at two furin consensus sequences.

The difference in the apparent molecular mass of about 16 kDa between F2 and F1+, on the one hand and between F1 and F1+, on the other hand is too large to be solely attributed to pep27 that corresponds to only 3 kDa. Pep27 of the HRSV

\[ F_{\text{CS-1}} \text{ fusion peptide} \]

\[ F_{\text{CS-2}} \text{ pep27} \]

\[ F_{\text{F1}}^+ \]

\[ F_{\text{F2}}^+ \]

\[ F_{\text{F0}} \]

\[ F_{\text{F1}} \]

\[ F_{\text{F2}} \]

\[ F_{\text{F1+}} \]

\[ F_{\text{F2+}} \]

\[ F_{\text{F0+}} \]

\[ F_{\text{F1+}} \]

\[ F_{\text{F2+}} \]

\[ F_{\text{F0+}} \]
fusion protein (strain Long) contains three potential N-glycosylation sites, Asn\(^{116}\), Asn\(^{120}\), and Asn\(^{126}\). To analyze whether oligosaccharides attached to these sites are responsible for the strikingly high molecular weight of F\(_2\), we immunoprecipitated the parental F protein as well as the mutants hF:R133K/R136K and hF:R108N/R109N from transfected, metabolically labeled cells and treated them with N-glycosidase F, an enzyme that removes all N-linked carbohydrates. The samples were run on a Tricine-SDS-polyacrylamide gel to separate the different F\(_2\) and F\(_3\) forms (indicated by arrowheads in Fig. 3A). When the parental protein was treated with N-glycosidase F, the 22-kDa F\(_2\) band (lane a) shifted to its deglycosylated form with an apparent molecular mass of about 10 kDa (lane b). This change in the electrophoretic mobility is due to the removal of two N-linked oligosaccharides attached to the N-glycosylation sites at Asn\(^{27}\) and Asn\(^{70}\) (35). A similar result was obtained with the FCS-1 mutant hF:R133K/R136K (lanes c and d). In contrast, deglycosylation of the 38-kDa F\(_2\) band of the FCS-2 mutant hF:R108N/R109N (lane e) resulted in a 13-kDa band (lane f). The difference in the molecular mass of 3 kDa between deglycosylated F\(_2\) and deglycosylated F\(_3\) corresponds to the size of pep27. This experiment also shows that about two-thirds of the molecular weight of F\(_2\) can be attributed to N-linked carbohydrates. Assuming that a single N-glycan corresponds to 3 to 6 kDa, we suppose that oligosaccharides are attached to at least to two of the three potential N-glycosylation sites. There appears to be some variation in the glycosylation pattern of pep27. In addition to F\(_2\) (38 kDa), a 28-kDa band was detected that may represent an underglycosylated form of F\(_3\) (lane c, see arrow). The primary sequence of the BRSV (strain ATue51908) fusion protein also contains two furin consensus sequences that are located at the same positions as FCS-1 and FCS-2 of the HRSV homologue (Fig. 1). However, the intervening peptide between the two cleavage sites shows only little homology with its counterpart from HRSV and contains only one potential N-glycosylation site located at Asn\(^{120}\). In case that this peptide is released from the BRSV fusion protein due to a similar mechanism as observed with the HRSV fusion protein, it should differ from its HRSV counterpart with regard to glycosylation and molecular weight. In order to get information about the proteolytic processing of the fusion protein of BRSV, the F protein of this virus was analyzed by radioimmunoprecipitation and enzymatic deglycosylation as shown above for the HRSV F protein. Fig. 3B shows that the F\(_2\) subunit of the parental F protein migrates as a 17-kDa band (lane a) that is converted to a 10-kDa form when treated with N-glycosidase F (lane b). The smaller size of bF\(_2\) of this BRSV strain compared with the F2 subunit of HRSV (Fig. 3A, lane a) is due to the lack of a potential N-glycosylation site at Asn\(^{70}\) that is present in HRSV and in most other BRSV isolates. We assumed that the BRSV bF\(_2\) like its human homologue is cleaved at both FCS-1 and FCS-2. As a consequence, the N-linked oligosaccharide at Asn\(^{120}\) would be removed together with pep27 and cannot be detected in the mature protein. To provide experimental evidence for this view, we replaced Asn\(^{120}\) with a glutamine residue and thus abolished the N-glycosylation motif. The F\(_2\) subunit of this mutant (bF:N120Q, lane c) showed the same apparent molecular weight as the parental bF\(_2\). Therefore, the decrease in size after treatment with N-glycosidase F is only due to the removal of the single N-glycan at Asn\(^{27}\) (lanes b and d). To prevent the cleavage at FCS-2 by furin, we changed the motif RAKR\(^{109}\) to RANN\(^{109}\). The bF\(_2\) subunit of this mutant (bF:N120Q, lane c) was converted to a 26-kDa form that was reduced to a 13-kDa band upon treatment with N-glycosidase F (lane f). When we introduced into this mutant the N120Q mutation to give the triple mutant bF:K108N/R109N/N120Q (lane g), we obtained a F\(_2\) subunit of about 20 kDa that migrated as a 13-kDa band after deglycosylation (lane h). These data show that the difference in molecular mass between the parental bF\(_2\) and the 26-kDa form can be attributed to the presence of pep27 (3 kDa) that contains a single N-linked oligosaccharide at Asn\(^{120}\). In analogy to HRSV F\(_2\), the 26-kDa form was designated bF\(_{2-}\).
**FIG. 3. Detection of N-glycans attached to pep27.** Parental or mutant F proteins were immunoprecipitated from transfected, metabolically labeled BSR-T7/5 cells and were either treated with N-glycosidase F or left untreated as indicated on the top. The samples were applied to Tricine-SDS 10% polyacrylamide gel and electrophoresed under reducing conditions. A, analysis of HRSV F; lanes a and b, parental F protein; lanes c and d, hF:R136K; lanes e and f, hF:R108N/R109N; lanes g and h, hF:R108N/R109N/N120Q. The arrowheads indicate the positions of the different F2 and F1 subunit forms. The arrow points to a band that presumably represents an underglycosylated form of F2.

The biotinylation approach was also applied to three mutants with a modified FCS-1 motif (Fig. 4A), i.e. hF:R136K (lane i), hF:R133K/R136K (lane k), and hF:R135S (lane m). Under reducing conditions (lower panel), the three mutants showed the characteristic F1 band that represents pep27 attached to the F1 subunit. Some F1 was also observed indicating that the mutations introduced into FCS-1 did not completely block cleavage by furin. In particular, Arg135 appears to be less accessible to biotinylation as is the parental F subunit.

Under nonreducing conditions (upper panel), the FCS-2 mutants revealed a higher molecular weight than the parental protein. The difference is explained by the glycosylated pep27 that remained attached to F2. To account for the presence of both F1 and F2, this complex was designated F1,2. While the mutant hF:R106N/R108N (lane e) showed only the F1,2 form, a small amount of the mutant proteins hF:R106N (lane c) and hF:R106K/R109K (lane g) was cleaved and converted into F1,2. When the cells were treated with trypsin prior to biotinylation, all three mutants were completely converted into F1,2 demonstrating that the modified FCS-2 motifs represent suitable substrates for this protease (lanes d, f, and h).

Other authors had noticed that the uncleaved precursor F0 is not detected at the cell surface suggesting that only the proteolytically cleaved fusion protein is transported to the plasma membrane (26, 27). We asked whether the cleavage products F1,2 produced by the FCS-1 and FCS-2 mutants, respectively, would be found at the cell surface. For this purpose, we labeled transfected cells with sulfo-NHS-biotin at 4°C. This reagent does not penetrate the plasma membrane and therefore reacts only with proteins at the cell surface (36). In parallel experiments, the cells were treated with 1 μg/ml trypsin prior to biotinylation. With this approach we tried to answer the question whether the modified FCS motifs would be cleaved at the cell surface by exogenous trypsin at one of the basic amino acids still present in the motifs. Following the biotinylation procedure, F protein was immunoprecipitated, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The biotin label was used for detection of the cell surface F protein by a streptavidin-peroxidase complex (Fig. 4). When the samples were treated with dithiothreitol to reduce disulfide bonds (lower panel), only the F1,2 but not the F2 subunit of the parental fusion protein was recognized (lane a). The F2 subunit appears not to be accessible to biotinylation. No evidence for the presence of the uncleaved precursor F0 on the cell surface was obtained by the biotinylation approach. Under non-reducing conditions (upper panel), the biotinylated F protein migrated as a 72-kDa band indicating that the F2 and F1 subunits formed a disulfide-linked complex (F1,2) that was expressed at the cell surface. With the FCS-2 mutants hF:R106N (lane c), hF:R106N/R108N (lane e), and hF:R106K/R109K (lane g) only the F1 subunit was detected under reducing conditions. The characteristic F2 form found with these mutants in the immunoprecipitation analysis (see Fig. 2, lane c) appears to be as inaccessible to biotinylation as is the parental F2 subunit.
FIG. 4. Effect of trypsin on HRSV F mutants at the cell surface. A, BSR-T7/5 cells were transfected in duplicate with recombinant pTM1 plasmids (lanes a and b, parental F; lanes c and d, hF: R106N; lanes e and f, hF:R106N/R108N; lanes g and h, hF:R106K/R109K, lanes i and j, hF:R136K; lanes k and l, hF:R133K/R136K; lanes m and n, hF:R135S). One duplicate of each transfection was treated with 1 μg/ml trypsin for 1 h while the other duplicate remained untreated as indicated on top of the gel. The cells were labeled with sulfo-NHS-biotin at 4°C and the F protein was immunoprecipitated from the cell lysates using a monoclonal antibody. The immunoprecipitates were separated by conventional SDS-PAGE under reducing conditions (lower panel) or nonreducing conditions (upper panel), transferred to nitrocellulose membranes, and probed with streptavidin peroxidase. B, transfected BSR-T7/5 cells were metabolically labeled with [35S]methionine/cysteine for 3 h and were either treated for 1 h with 1 μg/ml trypsin or remained untreated as indicated. F protein was immunoprecipitated from the cell lysates, and the immunoprecipitates were separated by Tricine-SDS 10% polyacrylamide gel electrophoresis under reducing conditions (lanes a and b, parental F; lanes c and d, hF: R106N; lanes e and f, hF:R106N/R108N; lanes g and h, hF:R106K/R109K, lanes i and j, hF:R108N/R109N). The different F protein forms are indicated on the right. The relative positions of standard proteins of the indicated molecular masses (in kDa) are shown on the left.

Proteolytic Activation of RSV Fusion Proteins

A

\[
\begin{array}{cccccccccccc}
\text{parental} & \text{NARR} & \text{NARR} & \text{KARK} & \text{KARK} & \text{KKRK} & \text{KKRK} & \text{RKKR} & \text{RKKR} \\
\text{a} & \text{b} & \text{c} & \text{d} & \text{e} & \text{f} & \text{g} & \text{h} & \text{i} & \text{j} & \text{k} & \text{l} & \text{m} & \text{n} \\
- & + & - & - & + & + & + & + & + & + & + & + & + & + \\
\end{array}
\]

\[
\text{Trypsin} \\
\text{F}_{1,2} \\
\text{F}_{1,2} \text{+ DTT} \\
\text{F}_{1,2} \text{+ DTT} \\
\text{F}_{1,2} \text{+ DTT} \\
\]

B

\[
\begin{array}{cccccccccccc}
\text{parental} & \text{NARR} & \text{KARK} & \text{KARK} & \text{RANK} & \text{RANK} \\
\text{a} & \text{b} & \text{c} & \text{d} & \text{e} & \text{f} & \text{g} & \text{h} & \text{i} & \text{j} & \text{k} & \text{l} & \text{m} \\
- & + & + & + & - & - & - & - & + & + & + & + & + & + \\
\end{array}
\]

\[
\text{Trypsin} \\
\text{F}_{0 } \\
\text{F}_{1 } \\
\text{F}_{2 } \\
\text{F}_{2,+} \\
\]

\[
\text{F}_{2} \\
\]

important for furin recognition (lane m). Under nonreducing conditions (upper panel), the mutants migrated as a disulfidelinked complex composed of F$_{1,+}$ and F$_2$ that was designated F$_{1,2}$. It could not be distinguished from the F$_{1,2}$ complex of the FCS-2 mutants (lanes c, e, and g) on basis of electrophoretic mobility. Partial cleavage at the modified FCS-1 by furin explains the appearance of the F$_{1,2}$ bands (lanes i, k, and m). When the transfected cells were treated with trypsin, FCS-1 mutants hF:R136K (lane j) and hF:R133K/R136K (lane l) were almost completely degraded and partial decomposition was observed with mutant hF:R135S (lane n). This result suggests that the changes made in the FCS-1 motifs caused the fusion protein to adopt a different conformation with domains being exposed that are highly sensitive toward trypsin. In this respect, FCS-1 differs significantly from FCS-2, since changes made in the latter motif did not result in degradation by trypsin. Corresponding mutations were also introduced into the FCS-1 and FCS-2 of the BRSV fusion protein. The results were essentially the same (not shown). The mutations R106N, R106N/K108N, and K108N/R108N rendered FCS-2 quite resistant toward furin but allowed efficient cleavage of the modified motif at the cell surface by trypsin. Mutations in the FCS-1 on the other hand changed the conformation of the protein resulting in reduced cell surface expression and degradation by trypsin.

There are two possible explanations for the behavior of the FCS-1 mutants. First, the amino acid changes by themselves may interfere with the correct folding of the protein. Alternatively, the F protein may require cleavage at FCS-1 in order to adopt a more stable conformation. In the latter case, the order of cleavage at the furin consensus sequences may be important for the generation of a correctly folded and fusogenic F$_{1,2}$ complex. In order to find out whether cleavage at FCS-1 and FCS-2 occur in a sequential order, the fusion protein of HRSV was transiently expressed in BSR-T7/5 cells, and the course of protein maturation was followed by pulse-chase analysis (Fig. 5). The precursor F$_0$ was detected as a band of 72 kDa which showed an increasing intensity from 0 to 15 min of chase. During this time, the protein was subject to glycosylation and folding which possibly resulted in an improved presentation of the antigenic epitope used for immunoprecipitation. With chase times longer than 15 min, the intensity of the F$_0$ band continuously decreased. This was paralleled by an increase in the 50-kDa F$_1$ subunit. Detectable amounts of F$_2$ and F$_{2,+}$ were visible after 20 min of chase. A faint band of about 32 kDa (below the F$_{2,+}$ band) was detected from 0 to 30 min of chase with more or less constant intensity. This band presumably represents a fragment of the F protein that was generated by proteolytic attack during immunoprecipitation. The detection of the F$_{1,+}$ form was complicated by the presence of another band which showed the same mobility in the gel as F$_1$. This band was already detected after the 5-min pulse (0 min) but, in contrast to F$_0$, did not show an increase in its intensity even after a chase of 10 min. However, starting with 15 min of chase, parallel to the appearance of F$_1$, the amount of this band increased with time and showed a drop after 30 min. These data indicate that after a chase of 15 min F$_1$ has entered the trans-Golgi network compartment where it was cleaved by furin at FCS-1 and FCS-2. The detection of small amounts of both intermediates F$_{1,+}$ and F$_{2,+}$ suggests that cleavage at
FCS-1 does not depend on previous cleavage at FCS-2 and vice versa.

In the previous experiments, we have shown that the RSV fusion proteins are processed at two furin consensus sequences resulting in the removal of the glycosylated peptide pep27. We wanted to determine whether cleavage at FCS-1 (adjacent to the fusion peptide) is sufficient to render the F protein fusion-active or whether cleavage at FCS-2 is also required for fusion competence. For this reason, the ability of HRSV FCS-1 and FCS-2 mutants to form syncytia in transfected BSR-T7/5 cells was analyzed by immunofluorescence (Fig. 6). The parental F protein (row 1, left column) was found to induce several large syncytia, whereas the FCS-2 mutant hF:R106N (row 2, left column) did not show a significant fusion activity. Rarely, some small syncytia were observed that were probably caused by small amounts of the mutant proteins that were cleaved despite the mutations introduced into the furin motif (compare Fig. 4A). The mutant hF:R106K/R109K (not shown) revealed a similar phenotype as hF:R106N. With mutants hF:R108N/R109N (row 3, left column) and hF:R106N/R108N (not shown), single fluorescent cells, but no syncytia were observed. In accordance with this phenotype, both mutants were not cleaved at the single basic amino acids of their modified FCS-2 (see Fig. 4A, lane e; Fig. 2A, lane c). All the FCS-2 mutants were processed at FCS-1. However, our results suggest that this is not sufficient for the F protein to be fusion-active. From the previous experiment (see Fig. 4, A and B) we knew that trypsin is capable of cleaving at the modified FCS-2. Therefore, the transfected cells were treated with trypsin and cultured thereafter for 20 h to allow syncytia to form. With no exception, the FCS-2 mutants revealed numerous, large syncytia indistinguishable from those formed by the parental F protein (rows 1–3, right column). Thus, the fusion protein has to be cleaved at FCS-2 to be converted to its active form. Similar results were obtained with the corresponding FCS-2 mutants of BRSV hF:R106N, hF:R106N/R108N, and hF:R106N/R109N (not shown). We performed a complementary experiment using HRSV FCS-1 mutants hF:R136K (row 4) and hF:R133K/R136K (not shown). The biotinylation approach has shown that these mutants are inefficiently cleaved by furin and largely degraded by exogenous trypsin (see above, Fig. 4A). In accordance with this result, no syncytia were observed in the untreated and in the trypsin-treated sample (row 4). The mutant hF:R135S (row 5) was capable of inducing syncytia that were smaller than those formed by the parental F protein (row 1). Consistent with this phenotype, the mutation introduced into FCS-1 did not completely prevent furin action resulting in a substantial amount of F1 in addition to F1+ (Fig. 4A, lane m). Trypsin treatment did not enhance syncytia formation (Fig. 6, row 5). This finding can also be explained by the result shown in Fig. 4 (lane n), where this mutant protein was only partially degraded by trypsin retaining an amount of F1 comparable to the amount of F1 in the untreated sample (compare lanes m and n of Fig. 4A). Taken, together, mutant analysis indicates that cleavage at FCS-2 is required for the F protein to induce syncytia formation. Trypsin activation mutants could be generated with mutations introduced into FCS-2 but not into FCS-1.

**DISCUSSION**

Membrane fusion plays an important role in the vesicular transport between cellular compartments (37), in the fertilization of the mammalian egg (38, 39), and in cell entry of enveloped viruses (40). Best understood is this process in the case of enveloped viruses that initiate infection by a fusion event be-
tween the viral lipid envelope and the membrane of the target cell. Viral fusion activity is mediated by a viral surface protein and often involves a stretch of hydrophobic amino acids. This so-called fusion peptide is believed to interact with the lipids of the target membrane resulting in a destabilization of the cell membrane (reviewed in Ref. 41). In this way, a fusion process is initiated that is believed to proceed via hemifusion (fusion of the outer leaflets of the two lipid bilayers), formation of small pores (fusion of both leaflets of the two partner membranes), and extension of the pores (42, 43). Virus-mediated fusion must be regulated to ensure that the viral fusion protein only reacts with the target but not with other cellular or viral membranes.

Studies with the hemagglutinin of influenza viruses suggest that after synthesis the viral fusion proteins adopt a metastable conformation, in which the fusion peptide is not exposed to the hydrophilic environment. In order to trigger the fusion of membranes, the fusion protein has to undergo a conformational change. In the course of this intramolecular rearrangement, the fusion peptide becomes exposed and thus is able to interact with the target membrane (44–47). With viruses that enter cells by endocytotic uptake, e.g., influenza viruses and vesicular stomatitis virus, the conformational change may be induced by the low pH encountered within endosomes. In the case of viruses that fuse with the plasma membrane, the conformational change may result from the binding to a specific cell surface receptor as has been shown for the interaction between HIV and chemokine receptors (48). Whatever cellular factor, low pH or specific surface receptor, is used to trigger the fusion activity, with most enveloped viruses the exposure of the fusion peptide will only occur after proteolytic cleavage of the fusion protein into two subunits. In most cases, cleavage is achieved by furin-like enzymes in the trans-Golgi network, a late compartment of the secretory pathway. In this way, the fusion protein may be prevented from aberrant fusion reactions within vesicular compartments such as endoplasmic reticulum or Golgi. As shown for the influenza hemagglutinin (49), proteolytic cleavage may also induce a first conformational change in the viral fusion protein.

The fusion protein of RSV resembles many other viral fusion proteins in the location of the fusion peptide immediately downstream of a furin recognition motif (2, 3). Proteolysis at this site by furin-like enzymes results in two cleavage products with the fusion peptide at the N terminus of the membrane-anchored subunit F1. A unique feature of the RSV F proteins is that there are two furin recognition motifs and that cleavage has to occur at both FCS-1 and FCS-2 in order to render the protein fusion-active. Other viral fusion proteins only contain a single recognition site for furin. This is also true for the fusion proteins of the related pneumonia virus of mice and the avian pneumoviruses (50, 51). In the glycoprotein of HIV, gp160, there is a stretch of basic amino acids (KAKRR$^{504}$) that is separated from the actual furin cleavage site (REKR$^{511}$) by only three amino acids. However, despite the basic character, the sequence of the former peptide is not consistent with the furin recognition motif RX/R/K/R. Analysis of mutants has revealed that only the furin motif REKR$^{511}$, but not the KAKRR$^{504}$ sequence is required for proteolytic activation of the HIV glycoprotein (52).

Modification of FCS-1 in the RSV fusion protein by site-directed mutagenesis resulted in the appearance of an F1$^{+}$ band that is expected if cleavage occurs only at FCS-2 and pep27 remains attached to the F2 subunit. In addition to F1$^{+}$, some F1 was observed with all FCS-1 mutants. The F1 band was most prominent in the mutant R135S. This is consistent with other reports that the basic amino acid at the −2 position within the furin recognition motif is less important for cleavage by furin than are the R(−1) and R(−4) residues (53). Syncytia formation indicated that the fusion activity was reduced but not abolished in this mutant. In contrast, no syncytia were observed with the mutants R136K and R133K/R136K indicating that the amount of F1 protein observed in these mutants was not sufficient for a detectable fusion activity. The RSV fusion proteins contain a lysine at position −6 and basic residues at this position have been reported to compensate for less favorable amino acids at the −1 and −4 position (54). Therefore, the low level of F1 in the R136K and R133K/R136K mutants may result from cleavage by furin. The amino acid exchanges in the FCS-1 mutants were chosen such that an arginine or lysine was retained at the −1 position. In this way it was expected that trypsin treatment should result in proteolytic activation as has been shown for the fusion proteins of other paramyxoviruses that have a single arginine at the cleavage site (2, 3). However, the F proteins of the R136K and R133K/R136K mutants were almost completely degraded and consequently no fusion activity was induced by trypsin. With the R135S mutant, some F1 was retained but still more than half of the protein was degraded and the low fusion activity was not enhanced by trypsin treatment. From this result we conclude that mutations in the FCS-1 motif affect the conformation of the fusion protein by exposing trypsin-sensitive domains. This is in contrast to the mutant fusion protein of a recombinant measles virus that becomes fusion-active after trypsin treatment (28).

Modification of FCS-2 by site-directed mutagenesis resulted in the appearance of an F2$^{+}$ band that is expected if cleavage occurs only at FCS-1 and pep27 remains attached to F2. With mutants R106N/R108N and R108N/R109N cleavage at FCS-2 was almost completely abolished, whereas some F2 was detectable with mutants R106N and R106K/R109K. The former mutants retained only a single basic amino acid of the furin recognition site, whereas the two latter mutants contain two or three basic amino acids. However, these basic residues do not form a genuine furin cleavage site and there are no nearby basic residues to compensate for less favorable amino acids at the −1 and/or −4 positions. Therefore, we cannot exclude that the small amount of F2 observed with mutants R106N and R106K/R109K is due to cleavage by a protease other than furin, e.g. a trypsin-like enzyme. It should be noted that in the same cells the influenza C glycoprotein HEF that contains a single arginine residue at the cleavage site was partially cleaved. Therefore, a trypsin-like enzyme is expected to be present in BSR-T7/5 cells and may be responsible for the partial cleavage of the mutants mentioned above. In contrast to FCS-1, FCS-2 mutants were not degraded by trypsin. Treatment with this protease rather converted F1$^{+}$ into F1$^{−}$. Concomitantly the fusion activity was enhanced as indicated by the formation of syncytia. This result suggests that, in contrast to FCS-1, mutations at FCS-2 have no detrimental effect on the conformation of the RSV F protein. Interestingly, proteolytic activation was possible also with mutant R108N/R109N. In this mutant trypsin can cleave at Arg$^{106}$, but not at amino acids 108 or 109. Conversely, cleavage of the parental protein by furin and cleavage of mutant R106N/R108N by trypsin is possible at Arg$^{109}$ but not at Arg$^{106}$. This result indicates that proteolytic activation of the RSV fusion protein does not require a site-specific cleavage at FCS-2.

The data presented here demonstrate that cleavage at both FCS-1 and FCS-2 is required for the F protein to induce syncytia formation. Our pulse-chase experiment indicates that both F1$^{+}$ and F2$^{+}$ are present in cells expressing F protein.

---

3 G. Zimmer, unpublished data.
suggesting that furin or a furin-like enzyme that acts on F protein does not have a strict temporal preference for FCS-1 or FCS-2. The data obtained with our mutants also show that cleavage at either site does not depend on prior cleavage of the other site. As a consequence of the two proteolytic events, pep27 is released from the polypeptide chain of the fusion protein and there is no cysteine residue to form disulfide bonds with either F1 or F2. Consistent with this view, mutations in either FCS-1 or FCS-2 resulted in a band with reduced electrophoretic mobility (F1$_{-2}$ or F$_{1,2,+}$ respectively). The release of pep27 may facilitate the structural rearrangement required to convert the RSV fusion protein into its fusogenic form. This may explain why in the case of RSV cleavage at FCS-1 is not sufficient to make the F protein fusion-competent.

Pep27 in the F protein of both HRSV and BRSV is glycosylated. The oligosaccharide(s) do not prevent the cellular pro- teases from activating the fusion protein. This is different from the hemagglutinin of influenza viruses. In a strain of the H5 subtype, an oligosaccharide in the vicinity of the furin recognition motif prevented cleavage by furin and rendered the virus apathogenic. A variant derived from this virus that had lost this carbohydrate side chain because of a mutation in the glycosylation motif prevented cleavage by furin and rendered the virus apathogenic. A variant derived from this virus that had lost this carbohydrate side chain because of a mutation in the glycosylation motif was highly pathogenic (55).

Mutations in FCS-2 like R106N/R108N and R108N/R109N are potential candidates for the generation of a live attenuated RSV vaccine by reverse genetics (31, 56). Recombinant RSV containing a fusion protein that depends on activation by trypsin-like proteases rather than by furin are expected to be less virulent than the parental virus. In contrast to furin that is required for the release of H5N1 virus, pep27 in the F protein of both HRSV and BRSV is glycosylated. The release of pep27 may facilitate the structural rearrangement required to convert the RSV fusion protein into its fusogenic form. This may explain why in the case of RSV cleavage at FCS-1 is not sufficient to make the F protein fusion-competent.

Pep27 in the F protein of both HRSV and BRSV is glycosylated. The oligosaccharide(s) do not prevent the cellular pro- teases from activating the fusion protein. This is different from the hemagglutinin of influenza viruses. In a strain of the H5 subtype, an oligosaccharide in the vicinity of the furin recognition motif prevented cleavage by furin and rendered the virus apathogenic. A variant derived from this virus that had lost this carbohydrate side chain because of a mutation in the glycosylation motif prevented cleavage by furin and rendered the virus apathogenic. A variant derived from this virus that had lost this carbohydrate side chain because of a mutation in the glycosylation motif was highly pathogenic (55).

Mutations in FCS-2 like R106N/R108N and R108N/R109N are potential candidates for the generation of a live attenuated RSV vaccine by reverse genetics (31, 56). Recombinant RSV containing a fusion protein that depends on activation by trypsin-like proteases rather than by furin are expected to be less virulent than the parental virus. In contrast to furin that is required for the release of H5N1 virus, pep27 in the F protein of both HRSV and BRSV is glycosylated. The release of pep27 may facilitate the structural rearrangement required to convert the RSV fusion protein into its fusogenic form. This may explain why in the case of RSV cleavage at FCS-1 is not sufficient to make the F protein fusion-competent.

Pep27 in the F protein of both HRSV and BRSV is glycosylated. The oligosaccharide(s) do not prevent the cellular pro- teases from activating the fusion protein. This is different from the hemagglutinin of influenza viruses. In a strain of the H5 subtype, an oligosaccharide in the vicinity of the furin recognition motif prevented cleavage by furin and rendered the virus apathogenic. A variant derived from this virus that had lost this carbohydrate side chain because of a mutation in the glycosylation motif prevented cleavage by furin and rendered the virus apathogenic. A variant derived from this virus that had lost this carbohydrate side chain because of a mutation in the glycosylation motif was highly pathogenic (55).

Mutations in FCS-2 like R106N/R108N and R108N/R109N are potential candidates for the generation of a live attenuated RSV vaccine by reverse genetics (31, 56). Recombinant RSV containing a fusion protein that depends on activation by trypsin-like proteases rather than by furin are expected to be less virulent than the parental virus. In contrast to furin that is required for the release of H5N1 virus, pep27 in the F protein of both HRSV and BRSV is glycosylated. The release of pep27 may facilitate the structural rearrangement required to convert the RSV fusion protein into its fusogenic form. This may explain why in the case of RSV cleavage at FCS-1 is not sufficient to make the F protein fusion-competent.
