Cleavage at the Furin Consensus Sequence RAR/KR\textsubscript{109} and Presence of the Intervening Peptide of the Respiratory Syncytial Virus Fusion Protein Are Dispensable for Virus Replication in Cell Culture

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Received 22 April 2002/Accepted 17 June 2002

Proteolytic processing of the respiratory syncytial virus F (fusion) protein results in the generation of the disulfide-linked subunits F1 and F2 and in the release of pep27, a glycopeptide originally located between the two furin cleavage sites FCS-1 (KKR\textsubscript{136}) and FCS-2 (RAR/KR\textsubscript{109}). We made use of reverse genetics to study the importance of FCS-2 and of pep27 for BRSV replication in cell culture. Replacement of FCS-2 in the F protein of recombinant viruses by either of the sequences NANR\textsubscript{109}, RANN\textsubscript{109} or SANN\textsubscript{109}, respectively, abolished proteolytic processing at this position, whereas the cleavage of FCS-1 was not affected. All mutants replicated in calf kidney and Vero cells in the absence of exogenous trypsin, although somewhat higher titers of BRSV containing the NAN\textsubscript{109} or the RAN\textsubscript{109} motif were achieved in the presence of trypsin. The virus mutants showed a reduced cytopathic effect which was lowest in the case of the SANN\textsubscript{109} mutant. These findings demonstrate that cleavage at FCS-2 is dispensable for replication of respiratory syncytial virus in cell culture. A deletion mutant containing FCS-1 but lacking FCS-2 and most of pep27 replicated in cell culture as efficiently as the parental virus, indicating that this domain of the F protein is not essential for virus maturation and infectivity.

Human respiratory syncytial virus (HRSV) 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 (7). A very similar disease is caused by BRSV in calves (1, 2, 20, 33).

The envelope of the respiratory syncytial viruses (RSV) contains three glycoproteins: the attachment protein G, the small hydrophobic protein SH, and the fusion protein F. Several studies indicate that both the G and the SH proteins are dispensable for virus replication in cell culture but may have some accessory function in the host (4, 18, 19, 35, 39). The F protein mediates fusion between the viral and cellular membrane and is therefore essential for virus replication. Since fusion does not require low pH, cells infected with RSV can fuse with adjacent cells resulting in multinucleated syncytia. Syncytium 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 (16, 29). Recent studies suggest that certain glycosaminoglycans of the cell surface are required for HRSV infection (13, 14, 23, 27). The G protein, as well as the F protein, have been demonstrated to bind to these carbohydrate structures (10, 11, 18, 23).

The primary sequence of the F protein from different serotypes of HRSV and BRSV is highly conserved but shows only little homology with other paramyxovirus fusion proteins. However, with respect to size, locations of hydrophobic domains, heptad repeats, and cysteine residues the RSV F protein shares many structural features with other paramyxovirus fusion proteins. A property that is even more common and also found with other virus families is the synthesis of the fusion protein as an inactive precursor F\textsubscript{0} that has to be proteolytically cleaved to become fusion active (21, 22). This posttranslational modification results in the exposition of a hydrophobic fusion peptide at the N terminus of the membrane-anchored fragment. The fusion peptide is supposed to play a crucial role in the fusion process. The majority of viral fusion proteins, including the RSV F proteins, contain a multibasic cleavage motif of the consensus sequence RX(K/R)R immediately upstream of the fusion peptide. This sequence is recognized by the ubiquitous subtilisin-like endoprotease furin of the trans-Golgi network (21, 22). A few viruses are not activated by furin. Their fusion proteins usually contain a monobasic cleavage site that is cleaved by trypsin-like proteases. The type of the cleavage motif has been shown to be an important determinant for virus pathogenicity (21). A unique feature of the RSV F proteins is the cleavage of F\textsubscript{2} at two conserved furin consensus sequences, RAR/KR\textsubscript{109} (FCS-2) and KKKR\textsubscript{136} (FCS-1), resulting in the generation of three proteolytic fragments, the large membrane-anchored subunit F\textsubscript{1} with the hydrophobic fusion peptide at its N terminus, the small subunit F\textsubscript{2} which is linked to F\textsubscript{1} via a disulfide bridge, and a small peptide composed of 27 amino acids (pep27) originally located between the two cleavage sites (12, 41). All three products have been shown to contain N-linked oligosaccharide side...
The two cloning steps were performed using Expand reverse transcriptase (Roche Diagnostics) and random hexamers for priming. Nucleotides 1 to 744 of the F gene were amplified from the cDNA by PCR with oligonucleotides bF-S(1-27) and bF-AS(724-744) and according to the following protocol: initial denaturation at 94°C for 1.5 min, followed by 35 two-step cycles (each composed of denaturation at 94°C for 30 s and annealing-extension at 60°C for 30 s), with a final elongation step at 72°C for 7 min. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and analyzed on a UV illuminator.

Radioimmunoprecipitation. Confluent monolayers of PT-11 cells grown in 25-cm² flasks were inoculated in duplicate with the indicated recombinant BRSVs (MOI of 0.1) for 3 h at 37°C. After removal of the inoculum, the cells were washed three times with 2% fetal calf serum and overlaid with medium containing 2% fetal calf serum and 0.9% methylcellulose (Sigma). After incubation for 3 days, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS) and then fixed with 3% formaldehyde in PBS for 15 min. The fixed cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS). The cells were washed three times with PBS and incubated for 1 h at room temperature with a horseradish peroxidase-linked goat anti-serum directed to the RSV matrix protein (monoclonal antibody 18G9D1; diluted 1:40 in PBS).
FIG. 1. Amino acid changes introduced into the F protein of recombinant BRSVs. A section of the BRSV fusion protein comprising amino acids 102 to 139 is shown. The furin consensus sequences are underlined, and the positions of the amino acids located N terminally of the furin cleavage site are indicated. Identical amino acids are represented by dots, and dashes indicate deleted amino acids. The positions of the amino acids located N terminally of amino acids 102 to 139 is shown. The furin consensus sequences are (RARR) or the BRSV (RAKR) fusion protein to either NANR or NANR abolished cleavage by furin, whereas cleavage at FCS-1 was not affected (41). In the modified FCS-2 motifs, single arginine residues either at position 106 or position 109 were preserved making these sites susceptible to trypsin-like proteases. To evaluate the role of FCS-2 in the proteolytic activation of RSV, we generated two recombinant BRSV mutants, rBRSV-F(R106N/K108N) and rBRSV-F(K108N/R109N), differing from the parental virus in the same amino acid exchanges described above (Fig. 1). The mutants were rescued after transfection of BSR-T7/5 cells with the modified antigenic plasmid together with four plasmids directing the expression of the polymerase complex (3). Since we first hypothesized that cleavage at FCS-2 might be required to activate fusion activity of the virus mutants, we added trypsin to the cell culture supernatant. However, the recovery of the mutants was also successful in the absence of exogenous trypsin. To exclude the possibility that endogenous trypsin-like proteases could help at the preserved arginine residues, we generated another recombinant BRSV, rBRSV-F(R106N/K108N/R109N), in which FCS-2 was replaced by the amino acid sequence SANN (Fig. 1). Like the other two mutants, this virus was efficiently recovered from transfected BSR-T7/5 cells. In addition to mutants containing amino acid exchanges, we constructed and rescued a recombinant virus, rBRSV-F(A106-130), with a deletion of 25 amino acids in the F protein. The deletion comprised FCS-2 and most of pep27, retaining only FCS-1 and the two basic amino acids at positions 131 and 132 (Fig. 1). Virus stocks were prepared by two passages on PT-11 (bovine calf kidney) cells. To verify the identity of the recombinant virus mutants, total RNA was extracted from infected cells and the region between nucleotides 5570 and 6313 of the RNA genome was amplified by RT-PCR. Although the PCR product derived from the parental virus genome was ca. 750 bp long, the deletion mutant was characterized by a PCR product of 670 bp, thus confirming the deletion within the F gene (data not shown). All changes introduced into the F gene were also verified by sequencing of the RT-PCR products.

RESULTS

The RSV fusion protein contains two conserved furin consensus sequences: FCS-1 is located immediately upstream of the fusion peptide, whereas FCS-2 is separated from the fusion peptide by a stretch of 27 amino acids, designated pep27 (Fig. 1). Previously, we reported that changing FCS-2 of the HRSV (RARR) or the BRSV (RAKR) fusion protein to either NANR or NANR abolished cleavage by furin, whereas cleavage at FCS-1 was not affected (41). In the modified FCS-2 motifs, single arginine residues either at position 106 or position 109 were preserved making these sites susceptible to trypsin-like proteases. To evaluate the role of FCS-2 in the proteolytic activation of RSV, we generated two recombinant BRSV mutants, F(R106N/K108N) and F(K108N/R109N), differing from the parental virus in the same amino acid exchanges described above (Fig. 1). The mutants were rescued after transfection of BSR-T7/5 cells with the modified antigenic plasmid together with four plasmids directing the expression of the polymerase complex (3).

We have previously shown that FCS-2 cleavage mutants are characterized by a large size F2 subunit designated F2+ (41). The difference in molecular weight between F2 and F2+ is due to the glycosylated pep27 that remains attached to F2. This phenotypical marker allowed us to distinguish between parental and mutant rBRSVs. The F proteins were immunoprecipitated from metabolically labeled PT-11 cells 2 days after infection and analyzed by Tricine-SDS-PAGE under reducing conditions (Fig. 2). The parental F protein (lane a) appeared as three distinct bands: the precursor F0 (72 kDa), the large subunit F1 (50 kDa), and the small subunit F2 (17 kDa). In the absence of the glycosylated pep27, F2 differed from the parental F protein in the smaller size of its precursor F0 (lane e). Since the FCS-2 mutants might be activated by cellular proteases secreted into the medium, we also analyzed the F proteins incorporated into mature virus particles. At 4 days postinfection, the virions were pelleted from the clarified supernatants through a 25% sucrose cushion, separated by...
SDS-polyacrylamide gel electrophoresis under nonreducing conditions, and analyzed by the Western blot technique (Fig. 2B). The F protein of the parental rBRSV appeared as a 72-kDa disulfide-linked complex (F₁,₂) composed of F₁ and F₂ (lane a). Addition of trypsin to the cell culture supernatant did not change this pattern (lane b). The presence of F₂⁺ in the FCS-2 cleavage mutants caused a molecular weight shift of the disulfide-linked complex to ca. 82 kDa (F₁,₂⁺) (lanes c, e, and g). A 72-kDa band that would indicate cleavage by endogenous proteases was not detected with any of the mutants. However, the addition of trypsin to the cell culture medium led to a partial cleavage of rBRSV-F(R106N/K108N) (lane d) and rBRSV-F(K108N/R109N) (lane f) and also of rBRSV-F(R106S/K108N/R109N) (lane h). However, cleavage of the latter mutant did not result in the F₁,₂ complex of 72 kDa, indicating that cleavage has occurred at another basic amino acid within pep27. The deletion mutant showed the same pattern as the parental virus (compare lanes a and b with lanes i and j). After proteolytic release of pep27 from the parental F protein, there is no major difference between the two F proteins that could be detected by the Western blot.

The growth characteristics of the parental and mutant rBRSVs were analyzed by using the bovine kidney PT-11 cell line, as well as African green monkey kidney (Vero) cells. The cells were infected in duplicate with the viruses at an MOI of 0.1, and supernatants were collected over a 6-day period at 24-h intervals. The virus titers were quantitated in duplicate by a plaque assay facilitated by immunological staining of the matrix protein. In the absence of trypsin (Fig. 3A), the FCS-2 cleavage mutants showed a somewhat reduced virus release in the beginning, but at day 6 postinfection they reached the titers of the parental virus. In the case of the mutants rBRSV-F(R106N/K108N) and rBRSV-F(K108N/R109N), this growth retardation was compensated for by the addition of acetylated trypsin (0.5 μg/ml) to the cell culture supernatant (Fig. 3B). In contrast, trypsin did not affect replication of either the parental virus or the mutant rBRSV-F(R106S/K108N/R109N) (Fig. 3C). The deletion mutant rBRSV-F(Δ106-130) replicated in PT-11 cells with a kinetics comparable to that of the parental virus. Likewise, the presence of trypsin had no effect on replication of this virus. Very similar growth kinetics were observed with Vero cells, although BRSV generally grew to lower titers in this cell line (not shown). On the other hand, BRSV caused a much more pronounced cytopathic effect in Vero cells than in PT-11 cells. At day 3 postinfection, we observed giant multinucleated cells in the Vero cell monolayer infected with the parental rBRSV (Fig. 4). Syncytium formation was also induced by the FCS-2 cleavage mutants; however, the syncytia were of smaller size and contained much fewer nuclei, indicating that the mutations introduced into the FCS-2 cleavage site affect cell-to-cell fusion. The syncytia formed by the mutants rBRSV-F(R106N/K108N) and rBRSV-F(K108N/R109N) grew to almost the parental virus level during the following 24 h. In striking contrast, the size of syncytia formed by the mutant rBRSV-F(R106S/K108N/R109N) did not change with time (data not shown). The deletion mutant showed a phenotype similar to that of the triple mutant, e.g., formation of very small syncytia that did not increase in size after longer incubation. Taken together, these results indicate that both FCS-2 and the intervening peptide pep27 are dispensable for virus replication in cell culture.

**DISCUSSION**

The fusion protein of RSV resembles many other viral fusion proteins in the location of a furin recognition site immediately upstream of the fusion peptide. Cleavage at this site by furin or a related cellular protease results in the location of the fusion peptide at the N terminus of the membrane-anchored subunit and is associated with a conformational change as shown for influenza virus hemagglutinin and the fusion proteins of simian virus 5 and RSV (6, 9, 12). Many viral fusion proteins require this posttranslational modification in order to become fusion active (21, 22). For example, blocking this step by specific furin inhibitors has been shown to reduce the infectivity of human immunodeficiency virus type 1 (15). In addition, recombinant measles was demonstrated to require on exogenous trypsin for activation of infectivity if the furin motif of the viral fusion protein was changed into a trypsin-like motif (26). However, reverse genetics showed that the conserved
furin motifs found in the Ebola virus glycoprotein and in the human cytomegalovirus glycoprotein B are dispensable for virus growth in cell culture (28, 34). A major difference between these and the former viruses is the location of the furin cleavage site distantly from the postulated hydrophobic fusion domains.

A unique feature of RSV is the additional cleavage of the F protein at a second furin consensus sequence, FCS-2, separated from the fusion peptide by 27 amino acids. Previous studies by using a plasmid-driven or vaccinia virus-based expression system revealed that F-mediated syncytium formation was significantly affected when either FCS-1 or FCS-2 were changed into a furin-resistant motif by site-directed mutagenesis, indicating that cleavage at both sites might be important for activation of the RSV fusion protein (12, 41). In the present study, we learned from reverse genetics that cleavage at FCS-2 is not essential for virus infectivity though the FCS-2 cleavage mutants did not grow as efficiently as the parental virus during the first replication cycles. If a single arginine was left with the modified motif (NANR109 or RANN109), the addition of trypsin to the cell culture supernatant compensated for this growth retardation, whereas it had no supporting effect on rBRSV-F(R106S/K108N/R109N) that did not contain any arginine or lysine residues in the modified FCS-2. Accordingly, trypsin treatment caused a partial cleavage of the mutants rBRSV-F(R106N/K108N) and rBRSV-F(K108N/R109N), whereas there was no evidence for cleavage by endogenous trypsin-like proteases. However, the mutant F protein of rBRSV-F(R106S/K108N/R109N) was also cleaved by trypsin but probably at a different site. Potential cleavage sites within pep27 are Arg119 and Lys-Lys124. The amino acid changes made with the FCS-2 also led to a reduced syncytium formation by the recombinant BRSV mutants. These findings suggest that the fusion activity of the FCS-2 mutants is not abolished but impaired. Probably, the glycosylated pep27 that remains attached to the F2 subunit of the FCS-2 cleavage mutants interferes with conformational rearrangements necessary for optimal fusion activity (12). In accordance with this view, the deletion mutant lacking pep27 did not show any growth retardation and addition of trypsin had no supporting effect on this virus. Nevertheless, the deletion mutant also showed a drastically reduced syncytium formation activity in Vero cells. It should be noted that the mature parental F protein differs from the deletion mutant with respect to the C terminus of its F2 subunit. Although the former ends with the sequence RAKR109, the latter has two additional amino acids and terminates with the sequence KKRKRR111. This C terminus did not impair the infectivity (virus-to-cell fusion) of rBRSV-F(Δ106-130) as indicated by the growth kinetics. However, it might interfere with cell-to-cell fusion, suggesting that the structural requirements for these two processes differ from each other. Differences between virus-to-cell fusion and syncytium formation have also been reported for the fusion protein of other enveloped viruses (8, 32, 37).

The second furin consensus sequence RAK/R222 ZIMMER ET AL. J. VIROL. R in the RSV fusion protein is highly conserved in all HRSV and BRSV strains isolated so far, suggesting that this cleavage site has a role in the viral life cycle. Our results indicate that cleavage at FCS-2 is not critical for virus replication in cell culture. However, it might be advantageous for RSV replication in vivo.
One possible function of pep27 and the dual cleavage might be related to the host immune response. The RSV fusion protein has recently been shown to inhibit proliferation of T cells by cell-to-cell contact (31). In analogy to measles virus (38), proteolytic activation of RSV F might be necessary for this function. It will be interesting to determine, by using the mutants described here, how the proteolytic processing of the fusion protein may influence its inhibitory activity. Another possible function might be related to pep27 itself, the intervening peptide released upon furin cleavage at FCS-2 and FCS-1. Like FCS-2, this peptide is dispensable for virus replication in cell culture. However, the motif FYGLM<sub>129</sub> in pep27 of BRSV FCS-2, this peptide is dispensable for virus replication in cell function might be related to pep27 itself, the intervening peptide released upon furin cleavage at FCS-2 and FCS-1. Like FCS-2, this peptide is dispensable for virus replication in cell culture. However, the motif FYGLM<sub>129</sub> in pep27 of BRSV suggests a possible role in the host. FYGLM<sub>120</sub> matches the signature sequence FXGLM characteristic for the tachykinin family of bioactive peptides (36). Substance P and other members of this family exhibit multiple activities, including the induction of bronchoconstriction, mucus secretion, histamine release, vasodilation, and others (5, 17, 25, 30). It remains to be experimentally shown whether pep27 of BRSV or a further processed form of it exhibits a similar tachykinin-like activity. Although the pep27 peptides of all known BRSV isolates are highly homologous to one another, they show only little similarity with the pep27 of HRSV. In particular, the latter lacks the tachykinin motif, indicating that the pep27 peptides of BRSV and HRSV might exhibit different activities. Whatever the function of the two different peptides exactly is, we should take into account that both peptides might contribute to the pathogenicity of RSV. This idea is especially important for the development of live attenuated RSV vaccines or other virus vectors expressing the RSV F protein, as well as for DNA vaccines which are based on the F gene. In this regard, the deletion mutant rBRSV-F(Δ106-130) is of particular interest since it is expected to lack the proposed activity of pep27. Another feature of this mutant that makes it an interesting vaccine candidate is the reduced cytopathic effect in infected cells. Finally, this virus will help us to study the role of pep27 in infection of the host.

ACKNOWLEDGMENTS

We thank R. Riebe for providing the PT-11 cell line. We acknowledge the help of Jose Antonio Melero and Claes Örvel, who made monoclonal antibodies available to us. This work was supported by grants from the European Community (QLK2-CT-1999-00443) and the Deutsche Forschungsgemeinschaft (HE 1168/11-1/2) to G.H.

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