Sequence elements of the fusion peptide of human respiratory syncytial virus fusion protein required for activity

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We have reported previously the expression and purification of an anchorless form of the human respiratory syncytial virus (HRSV) F protein (FTM) representing the ectodomain of the full-length F. FTM molecules are seen as unaggregated cones by electron microscopy but completion of proteolytic cleavage of the F0 monomers in the FTM-trimer leads to a change in shape from cones to lollipops that aggregate into rosettes. This aggregation apparently occurs by interaction of the fusion peptides of FTM molecules that are exposed after cleavage. Since exposure of the fusion peptide is a key event in the process of membrane fusion, changes associated with FTM cleavage may reflect those occurring in full-length F during membrane fusion. Deletions or substitutions that changed either the length, charge or hydrophobicity of the fusion peptide inhibited aggregation of FTM, and these mutants remained as unaggregated cones after cleavage. In contrast, more conservative changes did not inhibit the change of shape and aggregation of FTM. When the same changes were introduced in the fusion peptide of full-length F, only the mutations that inhibited aggregation of FTM prevented membrane fusion. Thus, the conformational changes that follow completion of cleavage of the FTM protein require a functional fusion peptide. These sequence constraints may restrict accumulation of sequence changes in the fusion peptide of HRSV F when compared with other hydrophobic regions of the molecule.

INTRODUCTION

Human respiratory syncytial virus (HRSV) is an enveloped, non-segmented negative-stranded RNA virus, classified within the genus Pneumovirus of the family Paramyxoviridae (Collins et al., 2001). It is the main cause of severe lower respiratory tract infections in very young children (Glezen et al., 1986) and a pathogen of considerable importance in the elderly and high-risk adults (Falsey et al., 2005). It is thought that HRSV enters the cell by attachment of the G glycoprotein (Levine et al., 1987) to the cell membrane, followed by fusion of the viral and cell membranes at neutral pH promoted by the fusion (F) glycoprotein (Walsh & Hruska, 1983) at the cell surface (Srinivasakumar et al., 1991). The F protein also mediates fusion of the membrane of infected cells with that of adjacent cells to form characteristic syncytia. HRSV encodes a third, small hydrophobic (SH) glycoprotein, of unknown function, that is expressed to high levels at the surface of infected cells but is incorporated inefficiently in the virus particle (Collins & Mottet, 1993).

Many authors have reported that the attachment (HN or H) glycoprotein and the F glycoprotein of paramyxoviruses are both needed for membrane fusion (review by Lamb, 1993). For instance, co-expression of HN (or H) and F in the same transfected cell is required for syncytium formation. It has been proposed that HN, on binding to the cell receptor, undergoes conformational changes that in turn could induce conformational changes in F, leading to its activation for membrane fusion.

There have been reports that G and SH enhance the formation of syncytia mediated by HRSV F when the three proteins are expressed in the same cell (Heminway et al., 1994; Pastey & Samal, 1997). It was suggested that G and SH were involved in activation of F in a manner similar to that proposed for the activation of paramyxovirus F by HN. However, spontaneous mutants (Karron et al., 1997) or genetically engineered recombinants of HRSV that lack G and/or SH (Bukreyev et al., 1997; Techavrapornkul et al., 2001) infect certain cell types in culture and induce formation of syncytia. In addition, expression of F alone in transfected cells promotes syncytium formation (González-Reyes et al., 2001). Thus, it is clear that, in the case of HRSV, the activity for membrane fusion resides in the F protein, and its activation for membrane fusion is not dependent on...
the activity of other viral glycoproteins, at least in viruses with F as the only surface glycoprotein or in transfected cells expressing only F.

The HRSV F protein is a type I glycoprotein that is synthesized as an inactive precursor (F0) of 574 amino acids. This precursor is cleaved by furin-like proteases during maturation to yield two disulfide-linked poly peptides, F2 from the N terminus and F1 from the C terminus. The F0 precursor of HRSV and the related bovine RSV are cleaved twice, after residues 109 (site I) and 136 (site II), which are preceded by furin recognition motifs (González-Reyes et al., 2001; Zimmer et al., 2001) (see Fig. 1 for a diagram of the primary structure). In contrast, the F0 precursor of other paramyxoviruses is cleaved only once, at a position equivalent to that of site II of HRSV F (Lamb & Kolakofsky, 2001).

HRSV F shares structural features with the F protein of other paramyxoviruses, despite limited sequence identity. Thus, all F proteins have three main hydrophobic regions: one, at the N terminus, which acts as the signal peptide for translocation into the ER; another region, which is the membrane anchor or transmembrane domain near the C terminus; and a third region at the N terminus of the F1 chain that is called the fusion peptide because it is thought, by analogy with other fusion peptides (Harter et al., 1989; Durrer et al., 1996), to be inserted into the target membrane during the process of membrane fusion. The mature F protein is a homotrimer in which heptad repeat sequences, HRA and HRB, are adjacent to the fusion peptide and to the transmembrane region of each monomer, respectively.

HRA and HRB peptides of Simian virus 5 (SV5) (Joshi et al., 1998) or HRSV F (Lawless-Delmedico et al., 2000; Matthews et al., 2000) form trimeric complexes in solution. X-ray crystallography of these complexes revealed an internal core of three HRA α-helices bounded by three antiparallel HRB α-helices packed into the grooves of the HRA coiled-coil trimer (Baker et al., 1999; Zhao et al., 2000).

We have reported that purified HRSV F protein forms rosettes of rods with two different shapes: cones and lollipops (Calder et al., 2000). The rosettes are formed by aggregation of individual rods through their transmembrane regions. A membrane-anchorless form of F that lacks the transmembrane region and the cytoplasmic tail (FTM−) is seen mainly as unaggregated cones, although a significant proportion (approx. 20 %) of rosetted lollipops can also be observed. Preparations of both F and FTM− contain, in addition to molecules cleaved to F1 and F2 chains, uncleaved F0 molecules and partially processed intermediates called F0−1−109 and F2*. F0−1−109 is generated when F0 is cleaved only at site I and F2* when F0 is cleaved only at site II (González-Reyes et al., 2001). Site II immediately precedes the fusion peptide (Fig. 1).

When cleavage of the monomers is completed in vitro by controlled trypsin digestion of purified FTM−, the trimer cones aggregate in rosettes of lollipop-shaped spikes (Ruiz-Argüello et al., 2002). Deletion of the first 10 amino acids of the fusion peptide eliminates aggregation of FTM−, lending support to the hypothesis that, after completion of cleavage in all the monomers, FTM− undergoes a conformational change (reflected in a change of shape) that exposes the fusion peptide and leads to aggregation. We now provide evidence that the conformational changes that follow activation of HRSV F by proteolytic cleavage require a functional fusion peptide.

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**Fig. 1.** F protein primary structure and mutants used in this study. The HRSV F protein primary structure is represented by a rectangle (top), denoting the hydrophobic regions (filled boxes), heptad repeats A and B (HRA and HRB; hatched boxes), cysteine residues (●) and sites of proteolytic processing (sites I and II). The anchorless form of F (FTM−), which lacks the C-terminal 50 amino acids, is shown below. SP, Signal peptide; FP, fusion peptide; TM, transmembrane region. F1 and F2 are the polypeptide chains generated after proteolytic cleavage of the F0 precursor and that remain covalently linked by a disulfide bond (S–S), as indicated. A partial sequence of the F protein (residues 106–155), including furin cleavage sites I and II (in bold) and the fusion peptide (bold and italic), is shown below the protein diagram, as well as the point mutations and amino acid deletions made for this study.
METHODS
Cells, viruses and plasmids. The recombinant vaccinia virus that expresses a soluble form of the HRSV F protein (Long strain) lacking the C-terminal 50 amino acids (F_{TM}−) was described by Bembridge et al. (1999). Plasmid pRB21 carrying a copy of the F_{TM}− gene (pRB21/F_{TM}−) was mutagenized using the Quick-Change site-directed mutagenesis kit (Stratagene) and the oligonucleotides shown in Table 1 to generate the mutants depicted in Fig. 1. Integrity of the F_{TM}− insert and presence of the desired mutations was confirmed by automatic sequencing.

The different pRB21 plasmids were used to rescue vaccinia viruses expressing the corresponding F proteins by the method of Blasco & Moss (1995). This is based on the recovery of recombinant viruses able to form plaques from cells infected with the vaccinia virus vRB12, which lacks the VP37 gene, and transfected with pRB21 plasmids that provide the VP37 gene, in addition to the desired gene. Recombinant vaccinia viruses were plaque-purified three times to ensure homogeneity of the virus stocks. These were prepared in CV-1 cells grown in Dulbecco’s medium supplemented with 10% fetal calf serum, as described previously (Calder et al., 2000).

A pTM1-derived plasmid carrying a full-length cDNA insert of the HRSV F protein (pTM1/F) under a T7 polymerase promoter has also been described previously (González-Reyes et al., 2001). The same mutations introduced in pRB21/F_{TM}− were introduced in pTM1/F using the same kit and oligonucleotides.

Protein purification. HEp-2 cells grown in Dulbecco’s medium with 4% fetal calf serum were infected with vaccinia viruses (m.o.i. ~0.5 p.f.u. per cell) expressing the F_{TM}− proteins described in the previous section. Culture supernatants were harvested 48 h post-infection, cleared of cell debris and concentrated 100-fold and buffer-exchanged to PBS by filtration through polyethersulfone membranes (Vivashflo; Sartorius) of 100 kDa exclusion pore size. The concentrates were loaded onto immunoaffinity columns made with an anti-F mAb 2F (Garcia-Barreno et al., 1989) bound to Sepharose ~3-0 mg antibody (g resin)−1. Columns were washed with 20 vols PBS and eluted with 20 vols 0.1 M glycine/Tris, pH 2.5. Elution of F protein was followed by absorbance at 280 nm. Column fractions were neutralized with saturated Tris and concentrated and buffer-exchanged to buffer A (10 mM Tris/HCl pH 7.5, 150 mM NaCl) with Vivaspin (Sartorius). Purity was assessed by SDS-PAGE and Coomassie blue staining. Specific bands were visualized by Western blots with antisera raised against either an F1-derived peptide (2F355–275) or a peptide encompassing residues 104–117 (2F104–117) (González-Reyes et al., 2001). The latter antiserum recognizes exclusively epitopes located in the segment between the two cleavage sites (I and II) of the F0 precursor. Protein concentration was estimated by absorbance at 280 nm with a calculated absorbance coefficient of 0.7 for a 1 mg ml−1 solution.

Trypsin digestion and sucrose-gradient centrifugation. Purified F_{TM}− proteins in buffer A were incubated with TPCK-trypsin (Sigma) for 1 h at 4 °C, at a ratio of 2.5 μg trypsin (mg protein)−1. Mock-digested samples were included as controls. The proteins were then

Table 1. Oligonucleotides used for site-directed mutagenesis

Two complementary oligonucleotides were used for each PCR-based mutagenic reaction, but only those of positive polarity are shown in the table for simplicity. Each oligonucleotide name denotes the F nucleotides included in its sequence, followed by the amino acid substitution(s) introduced after PCR-based mutagenesis. The altered residues are underlined in the oligonucleotide sequences. The last two oligonucleotides indicate the nucleotides deleted (Δ) and the corresponding amino acids (in parentheses).

<table>
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<tr>
<th>Oligonucleotide</th>
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<td>OF411-444/G139A</td>
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<td>GAAAGAAGAAGATTTTCTGTTTTTGCGACTGTATCATCTAAGGCTC</td>
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Effect of mutations in the fusion peptide on aggregation of F\textsubscript{TM}\textsuperscript{−} after trypsin cleavage

The mutations described in the previous section were introduced into recombinant vaccinia viruses to express the corresponding F\textsubscript{TM}\textsuperscript{−} proteins. Wild-type and mutant proteins were purified from culture supernatants of cells infected with the corresponding vaccinia virus recombinants by immunoaffinity chromatography. Purity was checked by SDS-PAGE followed by Coomassie blue staining of the purified proteins. Fig. 2(a) shows results obtained with representative mutants. In all cases, a major band with the mobility of the fully processed F1 chain and variable amounts of a contaminating ∼20 kDa band were observed. However, minor bands of lower mobility than F1 were also present in all preparations, as reported before (González-Reyes et al., 2001). These bands were identified as the uncleaved F0 precursor and the intermediate F\textsubscript{A1−109} (cleaved only at site I), based on reactivity with two antisera: one raised against a peptide of the F1 chain (xF\textsubscript{255−275}) (Fig. 2b) and the other against a peptide spanning residues 104–117 (xF\textsubscript{104−117}) (Fig. 2c), which recognizes only epitopes located between cleavage sites I and II of the F polypeptide (González-Reyes et al., 2001). The latter antiserum also recognizes an F2\textsuperscript{−} band, corresponding to an intermediate of the F2 chain cleaved only at site II (Fig. 2c). In some mutants, the relative proportions of F0, F\textsubscript{A1−109} and F2\textsuperscript{−} to F1 were essentially the same as in the wild-type, but in others there were significant quantitative differences. For instance, wild-type proportions were maintained in the L141V mutant but the relative intensities of F0, F\textsubscript{A1−109} and F2\textsuperscript{−} to F1 were reduced in the G151V mutant. This might reflect changes in the accessibility to furin of cleavage sites I or II as a consequence of local changes introduced by certain mutations in the F\textsubscript{TM}\textsuperscript{−} structure.

To test the effect of the different mutations on aggregation of F\textsubscript{TM}\textsuperscript{−}, the purified proteins were treated with limited amounts of trypsin to complete cleavage at sites I and II, as described previously (Ruiz-Argüello et al., 2002). The proteins were then analysed either before or after trypsin digestion by sedimentation in 10–25 % sucrose gradients and by EM. The results obtained are shown in Figs 3 and 4 for the wild-type F\textsubscript{TM}\textsuperscript{−} protein and for five representative mutants.

Wild-type F\textsubscript{TM}\textsuperscript{−} sediments mainly in fractions 5 and 6 of the gradient, although trailing towards higher-density fractions was also observed. After trypsin treatment, there was a clear shift in the sedimentation profile of F\textsubscript{TM}\textsuperscript{−} towards fractions of higher sucrose concentration (Fig. 3). These results correlated with observations made by EM. While F\textsubscript{TM}\textsuperscript{−} was seen mainly as unaggregated cone-shaped rods in mock-digested samples, this protein was aggregated in rosettes of lollipop-shaped spikes after trypsin cleavage (Fig. 4). It should be noted that, while F0 and F\textsubscript{A1−109} were observed in the gradient fractions before trypsin treatment of F\textsubscript{TM}\textsuperscript{−}, they were not present after trypsin digestion. This indicated completion of cleavage of those intermediates at sites I and II to generate mature F1 chain (Fig. 3), although
**Fig. 2.** Electrophoretic analysis of purified F<sub>TM</sub> proteins. The wild-type F<sub>TM</sub> protein and the different anchorless mutant proteins indicated above each lane were purified by immunoaffinity chromatography as indicated in Methods. Purified proteins were resolved by SDS-PAGE under reducing conditions and revealed by either Coomassie blue staining (a) or Western blotting with antisera α<sub>F</sub><sub>255–275</sub> (b) or α<sub>F</sub><sub>104–117</sub> (c), as indicated. Lane M, molecular mass markers. The positions of the F1 band and the proteolytic intermediates F0, F<sub>Δ1–109</sub> and F2* are indicated. The asterisk (*) denotes a contaminating band observed by Coomassie blue staining.

**Fig. 3.** Sucrose-gradient centrifugation of the wild-type anchorless F protein and fusion peptide mutants. Immunoaffinity-purified wild-type F<sub>TM</sub> and mutant anchorless proteins (shown in Fig. 1) were either left untreated (−) or treated with TPCK-trypsin (+) as indicated in Methods. Aliquots of each protein (~100 μg) were loaded on linear 10–25% sucrose gradients and centrifuged at 39 000 r.p.m. for 15 h at 4°C. Thirteen 1 ml fractions were collected from the top of the tube and 30 μl of each fraction was analysed by Western blot with α<sub>F</sub><sub>255–275</sub>. A partial hydrophobicity plot (residues 120–180), including the predicted fusion peptide (FP), obtained with the algorithm of Kyte & Doolittle (1982) is shown to the right of each blot. The hydrophobicity plot of the wild-type sequence (dotted line) is included in each panel for comparison.
other, minor, faster-migrating bands were also observed. The efficiency of cleavage by trypsin of the P0 and F_{D1-109} intermediates in F_{TM^{-}} preparations of the mutants was similar to that of the wild-type (see other panels of Fig. 3).

The F_{TM^{-}} mutant Δ141–150 also sedimented in fractions 5–7 of the sucrose gradient (Fig. 3) but, in contrast to the wild-type anchorless protein, the sedimentation profile did not change after trypsin digestion. In the EM, the mutant Δ141–150 was seen unaggregated after both mock and trypsin treatment (Fig. 4).

L141 was replaced with either V or N in two different mutants (Fig. 1). The L141V substitution shortened the side chain of the residue by a methyl group and did not change the hydrophobicity profile of the fusion peptide significantly. In contrast, the L141N substitution led to a moderate decrease in the hydrophobicity of the first half of the fusion peptide (Fig. 3). The purified L141V F_{TM^{-}} mutant protein sedimented mainly in fractions 5–8 of the sucrose gradient (Fig. 3) and it was seen as unaggregated cones by electron microscopy (Fig. 4). However, after trypsin treatment, this mutant sedimented in fractions of higher density towards the bottom of the tube (Fig. 3) and it was seen aggregated in rosettes of lollipop-shaped spikes under the EM (Fig. 4). The L141N F_{TM^{-}} protein also sedimented in fractions 5–8 of the sucrose gradient but, in contrast to the L141V mutant, trypsin digestion did not alter its sedimentation profile (Fig. 3). In addition, the L141N F_{TM^{-}} molecules remained as unaggregated cone-shaped spikes after trypsin treatment (Fig. 4).

Fig. 4 also shows the sedimentation behaviour of another pair of F_{TM^{-}} mutants, G151A and G151V, in which the hydrophobicity of the fusion peptides was marginally increased. The sedimentation properties of the G151A F_{TM^{-}} mutant resemble those of the wild-type protein both before and after trypsin digestion (Fig. 3). Thus, completion of cleavage by trypsin led to a shift of its sedimentation profile towards the bottom of the tube. This behaviour correlated with a change in shape from unaggregated cones before trypsin treatment to aggregated lollipops after trypsin digestion (Fig. 4). In contrast, neither the sedimentation profile nor the shape of the G151V F_{TM^{-}} mutant was changed after completion of cleavage by trypsin (Figs 3 and 4). In this case, the mutant molecules sedimented in fractions 5–8 of the gradient and were seen to be unaggregated irrespective of trypsin treatment.
In all cases in which \( \text{F}_{\text{TM}}^- \) was observed to aggregate after trypsin treatment, the subsequent change of shape, from cones to lollipops, was also noticed. In these mutants, a small proportion of molecules were already cleaved and aggregated in small rosettes before trypsin treatment. However, mutations that inhibited aggregation of \( \text{F}_{\text{TM}}^- \) after trypsin treatment also inhibited the change of shape.

**Effect of mutations in the fusion peptide on syncytium formation**

The mutations shown in Fig. 1 were also introduced in the full-length F gene, inserted in the pTM1 vector under a T7 promoter. As reported previously (González-Reyes et al., 2001), transfection of BSR-T7/5 cells (which express the T7 RNA polymerase constitutively) with the wild-type pTM1/F plasmid led to expression of F and formation of large syncytia that were visualized by immunofluorescence 48 h after transfection (Fig. 5). The mutants L141V and G151A also induced large syncytia after transfection of BSR-T7/5 cells (Fig. 5). These two mutations did not inhibit aggregation of \( \text{F}_{\text{TM}}^- \) after trypsin treatment. In contrast, BRS-T7/5 cells transfected with pTM1 plasmids carrying the F gene with the deletion Δ141-150 or the amino acid substitutions L141N or G151V expressed high levels of the corresponding F proteins, detected by immunofluorescence, but formation of syncytia was not observed. Thus, the same mutations that inhibited \( \text{F}_{\text{TM}}^- \) aggregation after trypsin treatment (Figs 3 and 4) eliminated the formation of syncytia by the full-length F protein (Fig. 5).

**Location of mutations in a putative α-helical fusion peptide**

The mutations introduced in the full-length F and tested for syncytium formation are shown in the α-helical wheel of Fig. 6. Irrespective of their position on the helical wheel, mutations that drastically altered the chemical properties of the amino acid side chain (shown in dark grey) inhibited syncytium formation. In contrast, those changes that maintained the chemical properties of the amino acid side chain (shown in light grey) induced syncytium formation in transfected cells. In addition to the single mutants shown in Fig. 6, double and triple mutants in which S146, S150 and T152 were replaced by A were also made. This was done to eliminate partially or completely all the polar amino acids of the fusion peptide. All these double and triple mutants also induced syncytium formation in transfected cells (Fig. 7), indicating that a peptide without polar residues is still capable of mediating membrane fusion. Thus, at least for this activity, as assessed in the syncytium formation assay, the polar character of those residues is dispensable.

Only a subset of fusion peptide mutants were tested for \( \text{F}_{\text{TM}}^- \) aggregation after trypsin treatment (enclosed in squares in Fig. 6). In all cases, mutations that drastically altered the properties of the particular amino acid side chain inhibited the conformational change and the aggregation of \( \text{F}_{\text{TM}}^- \) and correlated with inhibition of syncytium formation by full-length F. Mutants that maintained the chemical character of the substituted amino acid allowed the conformational change and aggregation of \( \text{F}_{\text{TM}}^- \) and syncytium formation by full-length F.

**DISCUSSION**

Previous studies have addressed the sequence requirements of the F protein fusion peptides of other paramyxoviruses, such as *Newcastle disease virus* (Sergel et al., 2001) or SV5 (Russell et al., 2004), for membrane fusion. However, while sharing structural features with other paramyxovirus F proteins, HRSV F also shows important differences. For instance, HRSV F is cleaved twice during the maturation process, while other paramyxovirus F proteins are cleaved only once. Furthermore, HRSV F is capable of inducing syncytium formation in the absence of other viral proteins (González-Reyes et al., 2001), while most paramyxovirus F proteins require co-expression of the attachment (HN or H) protein for membrane fusion (Lamb, 1993). Thus, findings...
for other paramyxovirus F proteins may not be directly applicable to HRSV F.

In addition, this study addresses the sequence requirements of the fusion peptide for the conformational changes that follow completion of cleavage of an anchorless form of HRSV F (F_{TM-}). Since these changes apparently involved exposure of the fusion peptide (Ruiz-Argüello et al., 2004), a key step during the process of membrane fusion, they may reflect changes in the full-length F during the process of membrane fusion. This hypothesis is strengthened by the correlation reported here between the sequence requirements for aggregation and change of shape of F_{TM-} and induction of syncytium formation by full-length F. An explanation of the inhibition of F_{TM-} aggregation by mutations in the fusion peptide is not available, but it may be that the mutations cause local structural disturbances which prevent exposure of the fusion peptide. Whether fusion peptide mutations that inhibit syncytium formation mediated by other paramyxovirus F proteins also inhibit the conformational changes that follow activation of those molecules has not been reported.

Our findings differ substantially from those reported for other viral glycoproteins involved in membrane fusion. For instance, exposure of the purified ectodomain of influenza virus haemagglutinin (HA) to acidic pH, a key event in triggering HA-mediated membrane fusion (Skehel et al., 1982), induces conformational changes in HA that expose its fusion peptide and result in HA aggregation. However, acidification of HA fusion peptide mutants that are inactive for membrane fusion also induces conformational changes

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**Fig. 6.** Representation of the F protein fusion peptide in an \( \alpha \)-helical wheel. The residues of the HRSV F protein fusion peptide (a) are represented in an \( \alpha \)-helical wheel (b). The amino acid substitutions tested in the full-length F for syncytium formation are shown next to the corresponding wild-type residues. Mutations enclosed in squares were also tested for aggregation of F_{TM-} after cleavage by trypsin. Mutations that inhibited either of those activities are shaded dark grey while those that displayed wild-type phenotypes are shaded light grey.

**Fig. 7.** Syncytium formation by F protein mutants with changes in polar residues of the fusion peptide. Plasmids encoding either wild-type F or single, double or triple substitutions of S146, S150 and T152 by A were used to transfect BSR-T7/5 cells, as indicated in each panel. Forty-eight hours later, the cells were fixed and processed for immunofluorescence with mAb 2F.
and aggregation of the HA ectodomain (Cross et al., 2001). Thus, while aggregation of the HA ectodomain after exposure to low pH does not require an active fusion peptide, the structural changes that follow completion of cleavage of HRSV F\textsuperscript{TM} and lead to its aggregation are inhibited in mutants with an inactive fusion peptide.

It has been postulated that the fusion peptides of viral type I fusion proteins adopt an \(z\)-helical conformation, at least after their insertion into the target membrane (Durrer et al., 1996). However, the effects of the mutations analysed in this study were independent of their location in an \(z\)-helical wheel representing the putative \(z\)-helix structure of the HRSV F fusion peptide. Furthermore, those effects were closely related to changes in the chemical properties of specific residues, irrespective of changes in hydrophobicity or polar character. Thus, although the fusion peptide of HRSV F may need a certain hydrophobic character for insertion into the target membrane, there are other sequence requirements for its activity. These requirements may restrict the accumulation of changes in this part of the molecule, consistent with its sequence conservation among related pneumoviruses and among paramyxoviruses in general (reviewed by Collins et al., 2001).

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