

# Thermostability of the human respiratory syncytial virus fusion protein before and after activation: implications for the membrane-fusion mechanism

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Anchorless fusion (F) proteins ( $F_{TM^-}$ ) of human respiratory syncytial virus (RSV) are seen by electron microscopy as unaggregated cones when the proteolytic cleavage at two furin sites required for membrane-fusion activity is incomplete, but aggregate into rosettes of lollipop-shaped spikes following cleavage. To show that this aggregation occurred by interactions of the fusion peptide, a deletion mutant of  $F_{TM^-}$  lacking the first half of the fusion peptide was generated. This mutant remained unaggregated even after completion of cleavage, supporting the notion that aggregation of  $F_{TM^-}$  involved the fusion peptide. As exposure of the fusion peptide is a key event that occurs after activation of F proteins, the uncleaved and cleaved forms of  $F_{TM^-}$  may represent the pre- and post-active forms of RSV F protein. In an analysis of the structural differences between the two forms, their thermostability before and after proteolytic cleavage was examined. In contrast to other viral proteins involved in membrane fusion (e.g. influenza haemagglutinin), the pre-active (uncleaved) and post-active (cleaved) forms of  $F_{TM^-}$  were equally resistant to heat denaturation, assessed by spectrofluorimetry, circular dichroism or antibody binding. These results are interpreted in terms of the proposed structural changes associated with the process of membrane fusion mediated by RSV F protein.

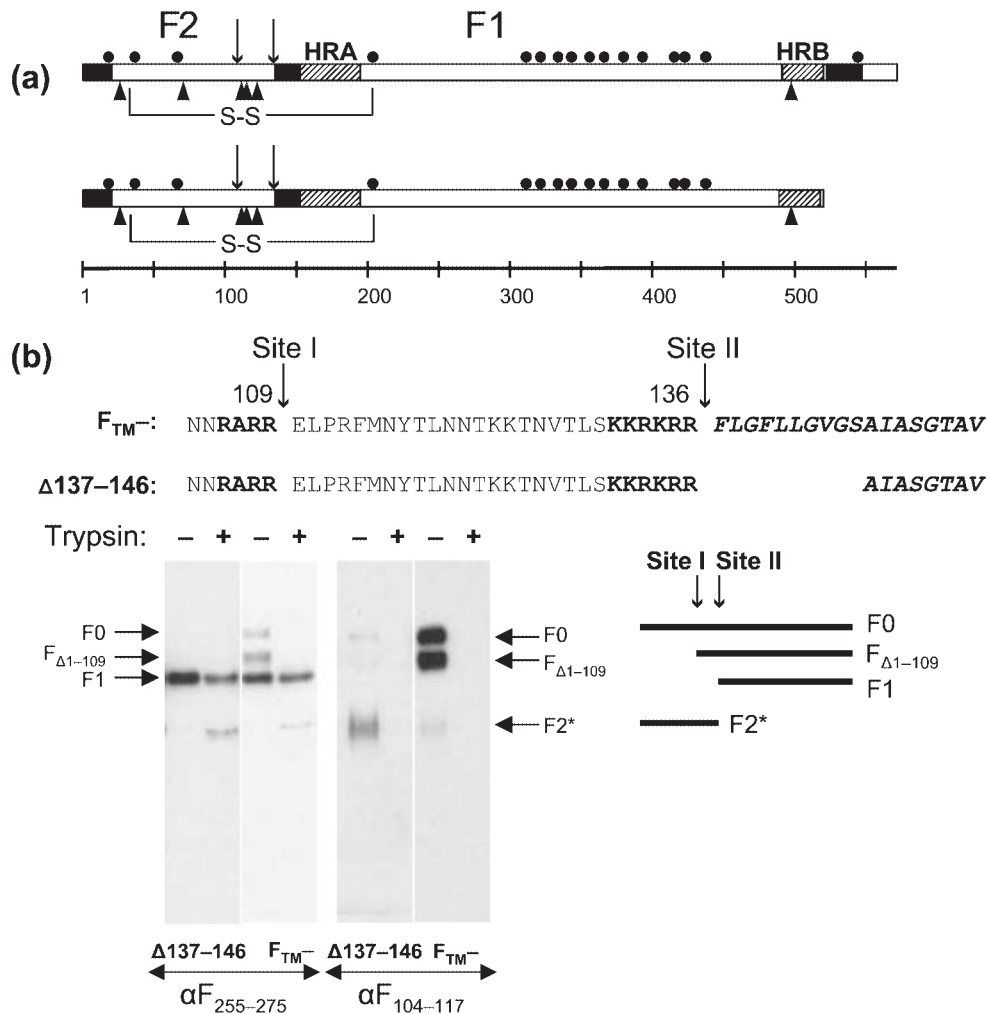
## INTRODUCTION

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 is a pathogen of considerable importance in the elderly and immunocompromised (Falsey *et al.*, 1995). The virion has two main surface glycoproteins: the attachment (G) protein (Levine *et al.*, 1987), which mediates virus binding to cells, and the fusion (F) protein (Walsh & Hruska, 1983), which is responsible for fusion of the viral and cellular membranes. The F protein also promotes fusion of the membrane of infected cells with that of adjacent cells to form characteristic syncytia. A third, small hydrophobic (SH) glycoprotein of unknown function is expressed abundantly in infected cells, but is incorporated only in small amounts into the virus particle (Collins & Mottet, 1993). The G protein of HRSV shares neither sequence nor predicted structural features with the

attachment protein of related viruses (Wertz *et al.*, 1985). In contrast, the F protein shares structural elements with its counterparts in other paramyxoviruses and all F proteins have a low, but significant level of sequence relatedness (Collins *et al.*, 1984).

The HRSV F protein is a type I glycoprotein that is synthesized as an inactive precursor (F0) of 574 aa. This precursor is cleaved by furin-like proteases during maturation to yield two disulfide-linked polypeptides, F2 from the N terminus and F1 from the C terminus. In contrast to other paramyxovirus F proteins that are cleaved only once, 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. 1a for a diagram of the primary structure). The F proteins of all paramyxoviruses have three main hydrophobic regions: one at the N terminus, which acts as the signal peptide for translocation into the ER; another region, the membrane anchor or transmembrane domain, near the C terminus; and a third region at the N terminus of the F1 chain, called the fusion peptide because it is thought, by analogy with

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**Fig. 1.** Trypsin digestion of  $F_{TM^-}$ . (a) Diagram of the F protein primary structure, denoting hydrophobic regions (filled boxes), heptad repeats (hatched boxes), cysteine residues (●), glycosylation sites (▲) and sites of proteolytic processing (↓). A diagram of the anchorless form of F ( $F_{TM^-}$ ), which lacks the C-terminal 50 aa, is shown below. (b) Partial sequences, including furin cleavage sites I and II (in bold) and the fusion peptides (italics), of wild-type  $F_{TM^-}$  and the  $\Delta 137-146$  mutant. These two proteins were purified by immunoaffinity chromatography as indicated in Methods. Aliquots ( $\sim 0.2 \mu\text{g}$ ) of each protein were either digested with  $0.2 \mu\text{g}$  trypsin/TPCK at  $4^\circ\text{C}$  for 1 h (+) or mock-digested (-). The reaction was stopped by the addition of sample buffer and boiling for 5 min. The proteins were resolved by SDS-PAGE and immunoblotted with antisera raised against synthetic peptides aa 255–275 or 104–117, as indicated. Diagrams corresponding to the bands detected in the immunoblot are shown on the right.

other fusion peptides (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 two heptad-repeat sequences, HRA and HRB, adjacent to the fusion peptide and to the transmembrane region of each monomer, respectively, are important motifs for the formation and stability of the trimers. HRA and HRB peptides form trimeric complexes in solution (Lawless-Delmedico *et al.*, 2000; Matthews *et al.*, 2000) and X-ray crystallography of these complexes reveals an internal core of three HRA  $\alpha$ -helices bounded by three antiparallel HRB  $\alpha$ -helices, packed into the grooves of the HRA coiled-coil trimer (Zhao *et al.*, 2000).

HRSV enters the cell by fusion at the plasma membrane rather than by endocytosis (Srinivasakumar *et al.*, 1991). There have been reports that the G and SH proteins enhance the formation of syncytia mediated by the F protein when the three proteins are expressed in the same cell (Heminway *et al.*, 1994; Pastey & Samal, 1997). It was thus assumed that the G and SH proteins could also enhance fusion of the viral and cellular membranes mediated by the F protein. However, spontaneous mutants (Karron *et al.*, 1997) or genetically engineered recombinants of HRSV that lack G and/or SH (Bukreyev *et al.*, 1997; Techarpornkul *et al.*, 2001) can still infect certain cell types and induce the formation of syncytia. Thus, although the G and SH proteins

may facilitate membrane fusion, it is clear that the activity for membrane fusion resides in the F protein.

We reported previously that purified HRSV F protein formed rosettes of rods with two different shapes, cones and lollipop (Calder *et al.*, 2000). The rosettes were formed by aggregation of individual rods through their transmembrane regions. A membrane-anchorless form of F that lacked the transmembrane region and the cytoplasmic tail ( $F_{TM^-}$ ) was seen mainly as unaggregated cones, although a significant proportion ( $\sim 20\%$ ) of rosetted lollipops could also be observed. Preparations of both F and  $F_{TM^-}$  contained, in addition to molecules cleaved to F1 and F2 chains, uncleaved F0 molecules and partially processed intermediates called  $F_{\Delta 1-109}$  and  $F2^*$  (Fig. 1).  $F_{\Delta 1-109}$  was generated when F0 was cleaved only at site I and  $F2^*$  when F0 was cleaved only at site II (González-Reyes *et al.*, 2001). Site II immediately precedes the fusion peptide. When cleavage of the  $F_{TM^-}$  monomers was completed, the  $F_{TM^-}$  trimers aggregated in rosettes of lollipop-shaped spikes (Ruiz-Argüello *et al.*, 2002). Aggregation of  $F_{TM^-}$  may occur through interactions of the fusion peptides, which may be exposed after completion of cleavage. As exposure of the fusion peptide is related to activation of the F protein, cones and lollipops may represent the pre- and post-activated forms of the F protein. Here, we provide evidence that the fusion peptide is involved in aggregation of  $F_{TM^-}$ , but that cleavage of the F protein trimer does not have a significant effect on the thermostability of the F protein molecule.

## METHODS

**Cells, viruses and plasmids.** Recombinant vaccinia virus expressing a soluble form of the HRSV F protein lacking the C-terminal 50 aa ( $F_{TM^-}$ ) has been described previously (Bembridge *et al.*, 1999). Plasmid pRB21 carrying a copy of the  $F_{TM^-}$  gene was mutagenized by using a QuikChange site-directed mutagenesis kit (Stratagene) and appropriate oligonucleotides (available from the authors upon request) to generate the following mutants: (i)  $\Delta 137-146$ , lacking the first 10 aa of the fusion peptide (see Fig. 1b); (ii)  $\Delta 108-130$ , lacking 23 aa between cleavage sites I and II (see Fig. 4a); and R108N/R109N/K131Q/R133Q/R135Q/R136Q (named uncleaved  $F_{TM^-}$ ), containing the indicated amino acid substitutions in cleavage sites I and II (see Fig. 4A).

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 vaccinia virus vRB12, which lacks the VP37 gene, and transfected with pRB21 plasmids that provide the VP37 gene, as well as 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 modified Eagle's medium (DMEM) 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 gene under the T7 polymerase promoter has been described previously (González-Reyes *et al.*, 2001). The  $\Delta 137-146$  deletion was introduced into this plasmid as indicated above for the  $F_{TM^-}$  gene.

HRSV (Long strain) was grown in HEp-2 cells and purified from the culture supernatant as described previously (García-Barreno *et al.*, 1988). Briefly, virus was precipitated with 6% PEG 6000, resuspended in PBS and centrifuged in a 20–60% sucrose gradient. The visible virus band was collected, diluted with PBS and spun through a 30% sucrose cushion. The virus was finally resuspended in PBS and kept at  $-80^\circ\text{C}$  until use.

**Protein purification.** HEp-2 cells grown in DMEM with 2% fetal calf serum were infected with vaccinia viruses (m.o.i. of  $\sim 0.5$ ) expressing the  $F_{TM^-}$  proteins described above. Culture supernatants were harvested 48 h post-infection, clarified of cell debris by low-speed centrifugation, concentrated 100-fold and buffer-exchanged with PBS by filtration through polyethersulfone membranes (Vivaflow; Sartorius) with 100 kDa exclusion pores. Concentrates were loaded onto immunoaffinity columns comprising an anti-F mAb (2F) bound to Sepharose [ $\sim 5.0$  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. Fractions containing the F protein, as assessed by measurement of  $A_{280}$ , were neutralized with saturated Tris, concentrated and buffer-exchanged with 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 and protein concentration was estimated by measurement of  $A_{280}$  with a calculated extinction coefficient of 0.7.

**Trypsin digestion and sucrose-gradient centrifugation.** Proteins in buffer A were incubated with the indicated amounts (see figure legends) of L-[(toluene-4-sulphonamido)-2-phenyl] ethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) for 1 h at  $4^\circ\text{C}$ . A mock-digested sample was included as a control. Proteins were loaded onto preformed 5–25% sucrose gradients in buffer A and centrifuged in a Beckman SW40 rotor at 39000 r.p.m. for 15 h at  $4^\circ\text{C}$ . Fractions (1 ml) were collected from the top of the tube. Aliquots of each fraction were analysed by SDS-PAGE and Western blotting as described previously (González-Reyes *et al.*, 2001). The antisera used for Western blotting were raised in rabbits inoculated with the synthetic peptides aa 255–275 or 104–117 of the F protein (Ruiz-Argüello *et al.*, 2002).

**Syncytium-formation assay.** BSR-T7/5 cells (Buchholz *et al.*, 1999) (a BHK-derived cell line that expresses T7 RNA polymerase constitutively) growing in microchamber culture slides were transfected with 0.5  $\mu\text{g}$  pTM1-derived plasmids as indicated in the figure legends. Transfections were done by the calcium phosphate method (MBS Mammalian Transfection kit; Stratagene). At 48 h post-transfection, cells were fixed with cold methanol for 5 min followed by cold acetone for 30 s. Fixed cells were processed for indirect immunofluorescence as described previously (García-Barreno *et al.*, 1996).

**Electron microscopy (EM).** Protein samples in buffer A were adsorbed onto carbon films and stained with 1% sodium silicotungstate (pH 7.0). A JEOL 1200 electron microscope, operated at 100 kV, was used to view the samples. Micrographs were taken under minimum-dose, accurate defocus conditions to preserve details to  $\sim 1.5$  nm (Wrigley *et al.*, 1986).

**Fluorimetry and circular dichroism.** Proteins ( $0.1-0.2$  mg  $\text{ml}^{-1}$ ) in 0.1 M sodium phosphate buffer, pH 7.0, were placed in the cell of a spectrofluorimeter (Perkin-Elmer LS3B). In some cases, 2 mM dithiothreitol (DTT) and 6.6 M urea were added to the samples. Starting at  $20^\circ\text{C}$ , the temperature was raised at a rate of  $1^\circ\text{C min}^{-1}$  to reach  $100^\circ\text{C}$  and then cooled down to  $20^\circ\text{C}$  at the same rate. At different temperatures, samples were excited at 280 nm and emission spectra were recorded between 300 and 380 nm. Spectra were corrected for values obtained with buffer alone with or without the indicated agents.

Similarly, proteins ( $0.4-0.5$  mg  $\text{ml}^{-1}$ ) in 0.1 M phosphate buffer,

pH 7.0, with or without 2 mM DTT and 6.6 M urea were heated in the cell of a spectropolarimeter (Jasco 715) at a rate of  $1\text{ }^{\circ}\text{C min}^{-1}$  from 20 to  $95\text{ }^{\circ}\text{C}$ . Spectra were recorded between 190 and 260 nm at different temperatures and corrected for buffer controls without protein. Samples were cooled down to  $20\text{ }^{\circ}\text{C}$  and final spectra were recorded.

**Antibody binding.** Proteins ( $0.1\text{--}0.2\text{ mg ml}^{-1}$ ) or purified virus ( $0.5\text{--}1.0\text{ mg ml}^{-1}$ ) were distributed in tubes and diluted 1:10 in  $0.1\text{ M}$  phosphate buffer, pH 7.0. Starting at  $30\text{ }^{\circ}\text{C}$ , the sample temperature was raised at a rate of  $1\text{ }^{\circ}\text{C min}^{-1}$  in a thermoblock. Tubes were taken at different temperatures and kept at  $4\text{ }^{\circ}\text{C}$  until the end of the incubation period. Samples were then diluted 1:100 in PBS and used to coat the wells of microtitre plates. Binding of mAbs 2F, 47F and 56F, specific for the HRSV F protein (García-Barreno *et al.*, 1989), was assessed by indirect ELISA using peroxidase-labelled anti-mouse immunoglobulin antiserum and *o*-phenylenediamine as substrate, following the manufacturer's instructions (Amersham).  $A_{492}$  was determined and expressed as a percentage of the values obtained at  $30\text{ }^{\circ}\text{C}$ .

## RESULTS

### Deletion of the N-terminal region of the fusion peptide inhibits aggregation of $F_{\text{TM}^-}$ after proteolytic cleavage and F protein-mediated fusion

We previously described a vaccinia virus recombinant expressing an anchorless form of the HRSV F protein ( $F_{\text{TM}^-}$ ) that was secreted into the culture medium of infected cells (see Fig. 1a for a diagram). Preparations of purified  $F_{\text{TM}^-}$  contained a proportion of partially cleaved intermediates. Completion of  $F_{\text{TM}^-}$  cleavage by trypsin digestion led to its aggregation in rosettes (González-Reyes *et al.*, 2001). To test whether this aggregation occurred through interactions of the hydrophobic fusion peptide of individual molecules, a deletion mutant of  $F_{\text{TM}^-}$  ( $\Delta 137\text{--}146$ ) was prepared that lacked the first 10 aa of the fusion peptide (Fig. 1b).

$F_{\text{TM}^-}$  proteins with either the wild-type sequence or the  $\Delta 137\text{--}146$  deletion were purified by immunoaffinity chromatography. Immunoblot analysis of the anchorless wild-type protein with an antiserum raised against a synthetic peptide of the F1 chain ( $\alpha\text{-F}_{255\text{--}275}$ ) revealed the presence of a major band, corresponding to the mature F1 chain, and minor bands that corresponded to the uncleaved F0 precursor and to the intermediate  $F_{\Delta 1\text{--}109}$  (cleaved only at site I) (Fig. 1b). The latter bands were particularly prominent in a Western blot with an antiserum raised against a synthetic peptide spanning residues 104–117 ( $\alpha\text{-F}_{104\text{--}117}$ ) that recognized only epitopes located in the segment between the two cleavage sites. This antibody also highlighted the F2\* intermediate. The same bands were observed in Western blots of the  $\Delta 137\text{--}146$  mutant, but their proportions relative to F1 were different from the wild-type, suggesting that the efficiency of cleavage by furin was altered after elimination of the first half of the fusion peptide. However, when the two proteins were treated with trypsin under controlled conditions, the F0,

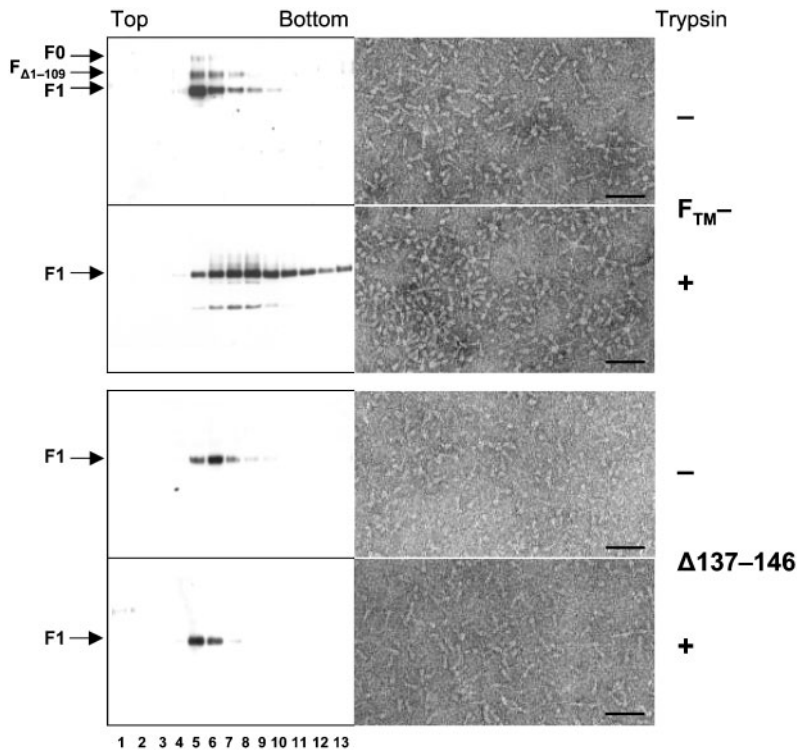
$F_{\Delta 1\text{--}109}$  and F2\* intermediates were cleaved at the remaining furin sites, whereas the F1 polypeptide remained essentially unchanged, although low levels of new bands moving faster than F1 were also observed (Fig. 1b).

To test the effect of trypsin on the aggregated state of  $F_{\text{TM}^-}$  and  $\Delta 137\text{--}146$ , the two proteins were centrifuged in 5–25% sucrose gradients or observed by EM with negative staining, before and after trypsin digestion (Fig. 2). Wild-type  $F_{\text{TM}^-}$  sedimented mainly in fractions 5–6, although trailing towards higher-density fractions was also observed. After trypsin digestion, there was a clear shift in the sedimentation profile of  $F_{\text{TM}^-}$  towards fractions of higher sucrose density. These results correlated with the observations made by EM. Whilst  $F_{\text{TM}^-}$  was seen mainly as unaggregated cone-shaped rods in mock-digested samples, this protein was seen to aggregate in rosettes of lollipop-shaped spikes after trypsin cleavage. The anchorless mutant,  $\Delta 137\text{--}146$ , sedimented in fractions 5–7 of the sucrose gradient (Fig. 2), but, in contrast to wild-type  $F_{\text{TM}^-}$ , the sedimentation profile did not change following trypsin digestion. By EM, mutant  $\Delta 137\text{--}146$  was seen to be unaggregated in both mock- and trypsin-digested samples.

The effect of the fusion-peptide deletion on membrane fusion was tested with pTM1 plasmids carrying the full-length F gene with either the wild-type sequence or the  $\Delta 137\text{--}146$  deletion. Formation of syncytia was assessed in BSR-T7/5 cells, which express the T7 polymerase constitutively (Buchholz *et al.*, 1999), transfected with the pTM1 plasmids encoding either protein under the T7 promoter. Whilst large syncytia were observed by immunofluorescence with anti-F-specific antibodies in cultures expressing wild-type F protein (Fig. 3), expression of the  $\Delta 137\text{--}146$  mutant induced high levels of F protein in individual cells, but syncytium formation was not observed.

### Thermostability of $F_{\text{TM}^-}$ before and after proteolytic cleavage

It has been suggested that viral proteins involved in membrane fusion may be in a metastable conformation that is potentially fusion-active and that a triggering event allows the transition from this conformation to a more stable and inactive post-fusion structure (Skehel & Wiley, 2000). As completion of proteolytic cleavage in the  $F_{\text{TM}^-}$  protein of HRSV leads to its aggregation, probably by exposure of the fusion peptide (see above) – a key event in the activation of any F protein for its role in membrane fusion – it was of interest to test whether cleavage of  $F_{\text{TM}^-}$  had any effect on protein thermostability that might reflect this transition. To this end, two recombinant vaccinia viruses were prepared: one expressing a mutated version of  $F_{\text{TM}^-}$  with alterations in the furin-recognition sequences that precede sites I and II (named uncleaved  $F_{\text{TM}^-}$ ), and the other expressing an anchorless  $F_{\text{TM}^-}$  protein with a deletion ( $\Delta 108\text{--}130$ ) encompassing the segment between cleavage sites I and II. The results in Fig. 4 indicated that the uncleaved  $F_{\text{TM}^-}$  sedimented mainly in fractions 4–6 of

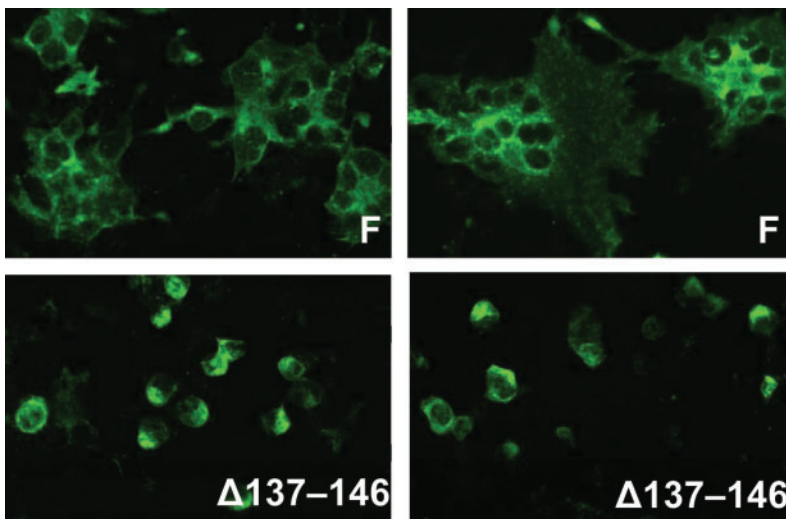


**Fig. 2.** Sucrose-gradient centrifugation and EM of the wild-type anchorless F protein and the fusion-peptide mutant. Immunoaffinity-purified wild-type ( $F_{TM^-}$ ) and mutant  $\Delta 137-146$  anchorless proteins were either undigested (-) or digested (+) with trypsin as indicated in Fig. 1. Aliquots of each protein ( $\sim 100 \mu\text{g}$ ) were loaded in linear 5–25% sucrose gradients (left panels) or negatively stained for EM (right panels). The gradients were centrifuged at 39 000 r.p.m. for 15 h at 4 °C. Thirteen 1 ml fractions (indicated below panels) were collected from the top of the tube and a 30  $\mu\text{l}$  aliquot of each fraction was analysed by Western blotting with anti- $F_{255-275}$  antiserum. Bars, 50 nm.

a 5–25% sucrose gradient and was seen as unaggregated, cone-shaped rods by EM. This mutant was seen in the form of an F0 band by Western blotting with only traces of an F1 band, generated by cleavage during the purification process. In contrast, mutant  $\Delta 108-130$  sedimented towards fractions of higher density in the sucrose gradient and was seen as rosetted, lollipop-shaped spikes by EM. Only the F1 band and traces of a faster-migrating band were observed in the Western blot of  $\Delta 108-130$ , indicating that, in this case, cleavage of the F0 precursor had been completed, probably inside the cell, by furin. Gradient fractions containing either uncleaved  $F_{TM^-}$  or  $\Delta 108-130$  were pooled and

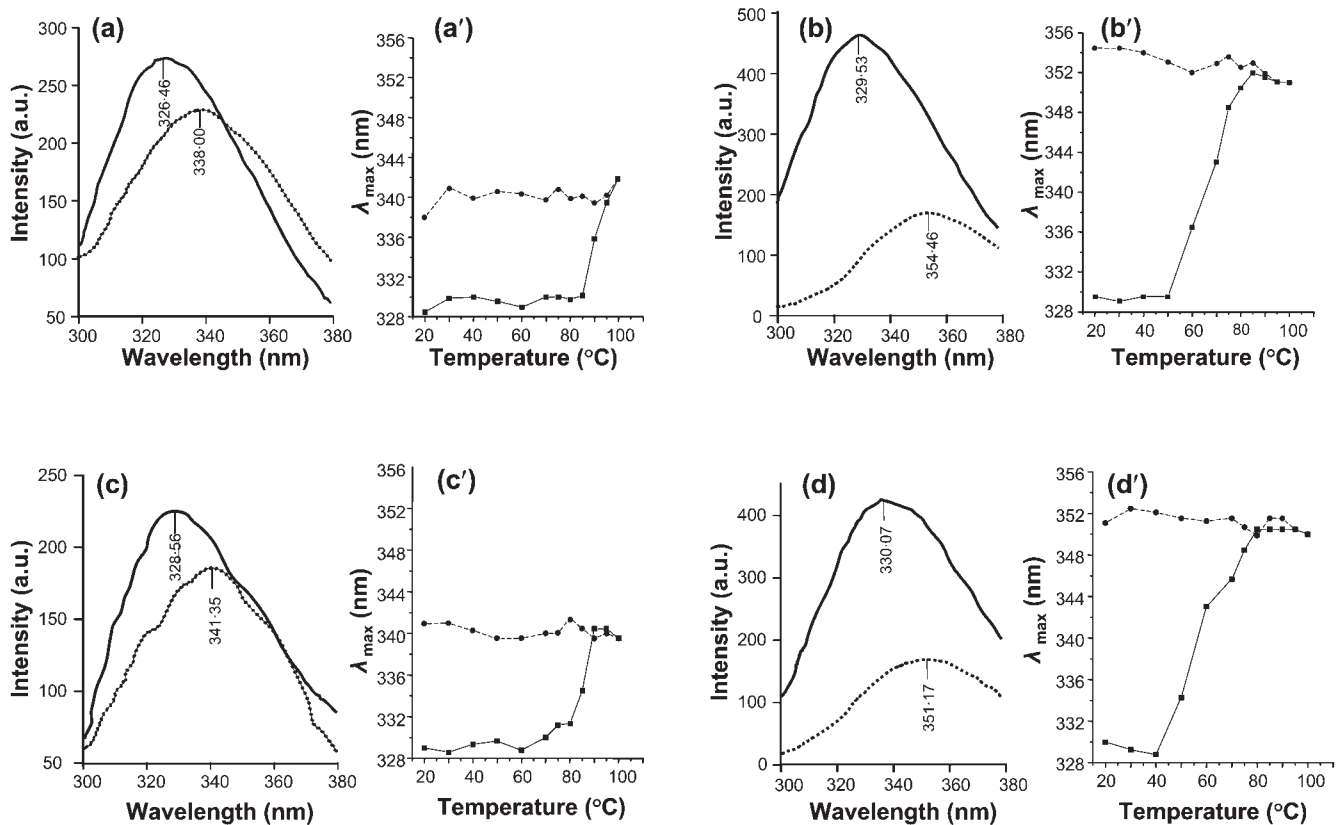
their purity assessed by SDS-PAGE and Coomassie blue staining (Fig. 4c). These gradient-purified proteins were used in thermostability tests as follows.

The thermostability of the anchorless, uncleaved  $F_{TM^-}$  and the cleaved  $\Delta 108-130$  proteins was assessed by three different types of assay: (i) spectrofluorimetry, which is responsive to changes in the environment of tryptophans in the protein structure; (ii) circular dichroism, which is sensitive to changes in secondary structure, mainly  $\alpha$ -helix content; and (iii) binding of mAbs. In some experiments, wild-type  $F_{TM^-}$  treated with trypsin (see Fig. 2) was



**Fig. 3.** Syncytium-formation assay with full-length wild-type F protein or fusion peptide mutant. BSR-T7/5 cells were transfected with pTM1 plasmids carrying inserts encoding full-length F with either the wild-type sequence (F) or the fusion-peptide deletion mutant ( $\Delta 137-146$ ). At 48 h post-transfection, cells were fixed and processed for immunofluorescence with mAb 2F as indicated in Methods. Two different fields are shown for each protein.





**Fig. 5.** Spectrofluorimetry of uncleaved  $F_{TM-}$  and mutant  $\Delta 108-130$ . (a, b) Spectra of the uncleaved  $F_{TM-}$  protein in 0.1 M phosphate buffer at 20 °C (solid line) or after heating at 100 °C (dashed line) (a) and under the same conditions, but with 2 mM DTT and 6.6 M urea added to the sample (b). The  $\lambda_{max}$  values of each spectrum are indicated. (a') and (b') represent  $\lambda_{max}$  values for uncleaved  $F_{TM-}$  spectra recorded at different temperatures whilst being heated up to 100 °C (■) or cooling down to 20 °C (●) in the same buffers as in (a) and (b), respectively. (c, d) Results obtained with  $\Delta 108-130$   $F_{TM-}$  under the same conditions as those described in (a) and (b).

fluorimetry, heating had a limited effect in denaturation of uncleaved  $F_{TM-}$  at high temperatures, but the addition of urea and DTT facilitated more extensive denaturation and significantly reduced the melting temperature ( $T_m$ ) of this protein.

Similar results were obtained with mutant  $\Delta 108-130$ . Again, heating of this protein induced only limited changes in the values of  $\lambda_{max}$  (Fig. 5c) at high temperatures (Fig. 5c'), but these changes were higher (Fig. 5d) and at lower temperatures (Fig. 5d') if 6.6 M urea and 2 mM DTT were added to the sample. The results of Fig. 5 also demonstrated that the  $T_m$  of uncleaved  $F_{TM-}$  and mutant  $\Delta 108-130$  was not significantly different.

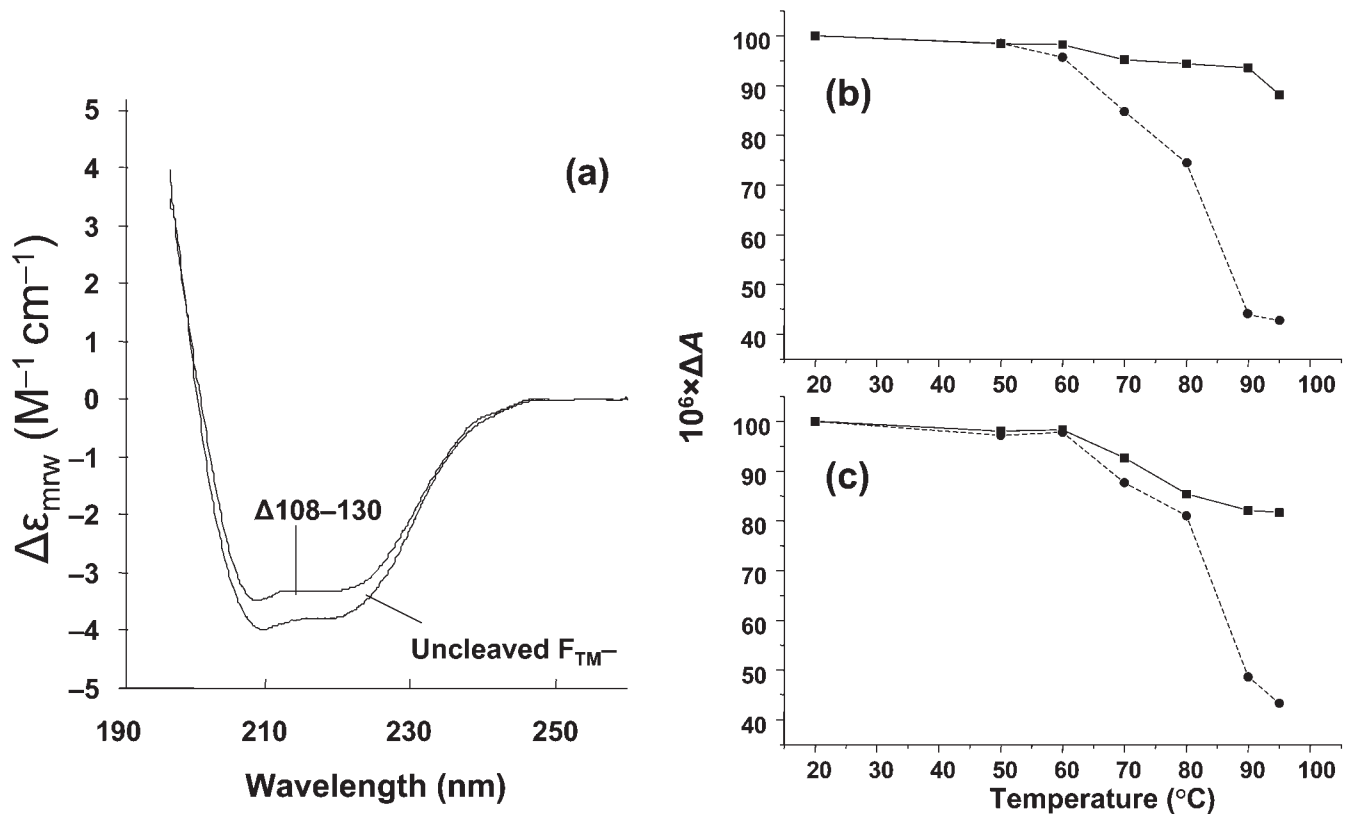
**Circular dichroism.** Fig. 6(a) shows the circular-dichroism spectra of the uncleaved  $F_{TM-}$  protein and mutant  $\Delta 108-130$ . Both spectra were very similar, indicating a relatively high  $\alpha$ -helix content for both proteins, with that for the uncleaved  $F_{TM-}$  estimated at 33 %.

Uncleaved  $F_{TM-}$  and mutant  $\Delta 108-130$  were heated

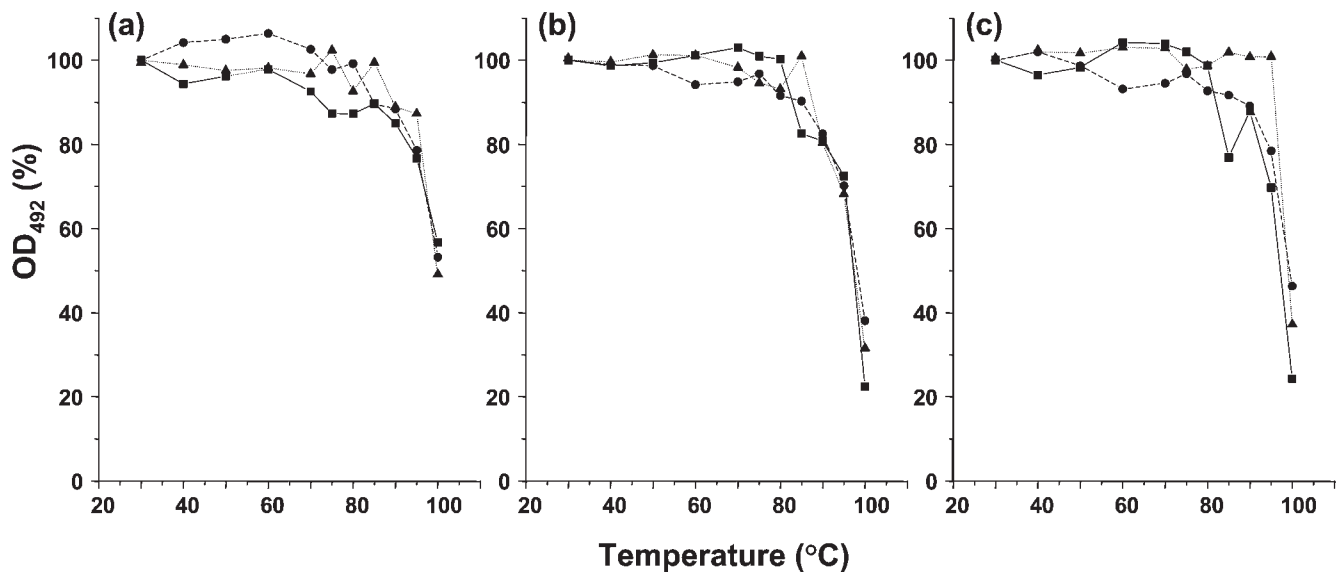
stepwise from 20 to 95 °C and circular-dichroism spectra were recorded at different temperatures. Fig. 6(b and c) show the  $\Delta A_{222}$  values for the two proteins at different temperatures. Even after reaching 95 °C, the circular-dichroism spectra remained essentially unchanged for both uncleaved  $F_{TM-}$  and  $\Delta 108-130$ , and changes in  $\Delta A_{222}$  values were very limited. Only when 6.6 M urea and 2 mM DTT were added to the buffer were significant changes in  $\Delta A_{222}$  values observed for the two proteins at temperatures between 75 and 90 °C (Fig. 6b and c). The changes observed in the circular-dichroism spectra did not revert after cooling of either protein to 20 °C (not shown).

**Antibody binding.** We have previously reported a set of mAbs (García-Barreno *et al.*, 1989) that delineated different antigenic sites in the F protein primary structure (López *et al.*, 1998) and were shown to be located in the F protein molecule by immuno-EM (Calder *et al.*, 2000).

The reactivity of three mAbs representing antigenic sites I (2F), II (47F) and IV (56F) was tested after heating both uncleaved  $F_{TM-}$  (Fig. 7a) and mutant  $\Delta 108-130$  (Fig. 7b)



**Fig. 6.** Circular dichroism of uncleaved  $F_{TM-}$  and  $\Delta 108-130$ . (a) Far-UV spectra of the two purified proteins shown in Fig. 4(c) at 20  $^{\circ}C$ . (b, c) Protein spectra were recorded at different temperatures. The values of  $\Delta A_{222}$  in each spectrum are represented on the y axis as the percentage of the values recorded at 20  $^{\circ}C$  for uncleaved  $F_{TM-}$  (b) and  $\Delta 108-130$  (c) in 0.1 M phosphate buffer, pH 7.0, without (■) or with (●) 6.6 M urea and 2 mM DTT.



**Fig. 7.** Loss of antibody binding by heating. Purified, uncleaved  $F_{TM-}$  (a), mutant  $\Delta 108-130$  (b) and HRSV (Long strain) (c) were incubated at the indicated temperatures in 0.1 M sodium phosphate, pH 7.0, as described in Methods. After heating, binding of antibodies 2F (▲), 47F (●) and 56F (■) was tested by ELISA. The results are expressed as the percentage of the values obtained at 30  $^{\circ}C$ .

at different temperatures. Binding of the three antibodies was unchanged until the two proteins were heated up to 100 °C. Then, a significant reduction in antibody binding was observed, which was not recovered by cooling the samples. This reduction in reactivity was not observed with a polyclonal rabbit antiserum raised against purified and denatured protein, excluding inhibition of antigen binding to the plates after heating (not shown).

In summary, the results of Figs 5, 6 and 7 indicated that both forms of the anchorless F protein (uncleaved and cleaved) were similarly resistant to heat denaturation. No significant differences were observed in the  $T_m$  of uncleaved  $F_{TM^-}$  and  $\Delta 108-130$  in each of the assays. The thermostability of wild-type  $F_{TM^-}$  cleaved with trypsin, as shown in Fig. 1, was not significantly different from that of  $\Delta 108-130$  as assessed by spectrofluorimetry and antibody binding (data not shown).

The antibody-binding assay allowed us to test the thermostability of full-length F protein inserted into the viral membrane. Thus, a preparation of purified HRSV was heated stepwise and binding of the three anti-F mAbs mentioned above was quantified by ELISA. The results obtained (Fig. 7c) indicated that thermostability of the F protein present in the virus preparation was essentially the same as that of the purified, anchorless F proteins.

## DISCUSSION

We reported previously that proteolytic cleavage of an anchorless F protein of HRSV led to its change from unaggregated cones to aggregated lollipops (González-Reyes *et al.*, 2001). Abrogation of cleavage at site I (after residue 109) by site-directed mutagenesis reduced the efficiency of cleavage at site II (after residue 136) and concomitantly reduced the proportion of aggregated  $F_{TM^-}$  molecules. However, abrogation of cleavage at site II inhibited aggregation of  $F_{TM^-}$ , even if cleavage at site I was still highly effective (Ruiz-Argüello *et al.*, 2002). Thus, cleavage at site II was indispensable for changes in shape and aggregation of  $F_{TM^-}$ , whereas cleavage at site I increased the efficiency of cleavage at site II.

As the fusion peptide is the main hydrophobic sequence remaining in the anchorless F protein, we assumed that the fusion peptide was exposed after proteolytic cleavage, leading to aggregation of  $F_{TM^-}$ . The results presented here support this notion. Thus, deletion of the first part of the fusion peptide (mutant  $\Delta 137-146$ ) abrogated aggregation of  $F_{TM^-}$ , even after completion of cleavage by trypsin digestion (Fig. 2). Moreover, the results of Fig. 3 indicated that deletion of aa 137–146 in the full-length F protein abrogated its capacity to induce syncytium formation. It is unclear at present whether the deletion of aa 137–146 inhibited the transition from cones to lollipops of the  $F_{TM^-}$  protein after trypsin digestion. A more detailed analysis is now required to clarify this point and to identify residues

of the fusion peptide that are indispensable for both aggregation of  $F_{TM^-}$  and membrane fusion.

We have postulated that cones and lollipops may represent the pre- and post-activated forms of the F protein. Other viral proteins involved in membrane fusion, such as influenza virus haemagglutinin (HA), adopt a metastable conformation before being activated for membrane fusion (Skehel & Wiley, 2000). Once activated, these proteins experience conformational changes that result in the formation of a more stable post-active and inactive conformation when the fusion process is completed. The best-studied example is the X-31 influenza virus HA, with a  $T_m$  of 63–65 °C at neutral pH (estimated by circular dichroism or trypsin sensitivity), but of greater than 90 °C after activation by exposure to acidic pH (Ruigrok *et al.*, 1986). Thus, it was important to compare the thermostability of  $F_{TM^-}$  molecules before and after proteolytic cleavage.  $F_{TM^-}$  was very resistant to heat denaturation before and after cleavage, as assessed by spectrofluorimetry, circular dichroism and antibody binding. This finding does not mean that the free energies for unfolding at the temperature of fusion are the same for the two forms, as enthalpy and heat-capacity changes may be affected by the proteolytic cleavage. The extreme thermostability of HRSV F protein may also be unique among paramyxoviruses. For instance, in the case of Sendai virus, heating of virus preparations induces a conformational change in the F protein at a temperature of 55 °C, as noted by changes in protease sensitivity. Fusion of Sendai virus with liposomes can be induced at the same temperature, suggesting that the conformational change is related to activation of the F protein for membrane fusion (Wharton *et al.*, 2000).

The question arises, are the uncleaved and cleaved forms of the F protein the pre- and post-active forms of this molecule? In other words, is proteolytic cleavage the triggering event for activation of the F protein of HRSV? If proteolytic cleavage is the activating event for the F protein, the pre-active conformation may not necessarily need to be in a less-stable configuration, as activation involves hydrolysis of peptide bonds. This situation is clearly different from that of influenza virus HA, where proteolysis of a precursor and subsequent exposure to an acidic environment induce the conformational changes leading to membrane fusion.

However, if the F protein is cleaved by furin inside the infected cell, it might be expected to be inactivated before reaching the cell surface, if the cleaved form of the F protein represents the post-active form of this molecule. Perhaps activation of the F protein may be modulated by the presence of partially cleaved intermediates in the same oligomer, retaining the F trimer in a pre-active state until complete cleavage at the time of virus entry, as such intermediates have been found not only in purified F protein, but also in purified virus (González-Reyes *et al.*, 2001). Alternatively, other components in the infected cell or in the virus particle may interact with the F protein to

maintain it in the pre-active state, even after proteolytic cleavage. The latter situation may apply for other paramyxoviruses, where an interaction of the attachment protein with the F protein has been reported and both proteins are needed for membrane fusion (reviewed by Lamb, 1993). In contrast, HRSV F protein alone can induce syncytium formation efficiently (Fig. 3) and viruses with F protein as their only glycoprotein infect certain cell types as efficiently as wild-type viruses (Karron *et al.*, 1997; Techaarpornkul *et al.*, 2001). Whether these differences between other paramyxoviruses and HRSV reflect the different pathways of proteolytic activation, which, in HRSV F protein, involves cleavage at two different sites compared with other paramyxoviruses, in which the F protein is cleaved only once, is unknown.

Regardless, the results presented here indicate that the changes associated with cleavage of the F protein that lead to aggregation of the anchorless form of the molecule through exposure of the fusion peptide do not significantly alter its thermostability. It is worth mentioning that the thermostability of the F protein in the virus particle, assessed by loss of antibody binding, was very similar to the thermostability of purified anchorless F protein, lending support to the relevance of the results obtained with F<sub>TM</sub> for the situation expected in the case of the infectious virus.

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