A point mutation in the F\textsubscript{1} subunit of human respiratory syncytial virus fusion glycoprotein blocks its cell surface transport at an early stage of the exocytic pathway

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Vaccinia virus recombinants expressing either wild-type or mutant forms of human respiratory syncytial (RS) virus (Long strain) fusion (F) glycoprotein were obtained. Proteolytic processing of the precursor, F\textsubscript{0}, and cell surface transport of the F glycoprotein were unaffected in the recombinants, except in those that contained the replacement Phe \to Ser at position 237 of the F\textsubscript{1} subunit. In recombinants containing this mutation, either alone or in combination with others, the traffic of the F molecule was arrested at some intermediate step of its transport to the cell surface and, consequently, the endoproteolytic cleavage of the F\textsubscript{0} precursor was inhibited. Immunofluorescence staining of infected cells and endoglycosidase H (Endo-H) sensitivity assays indicated that the arrest occurred before the mid-Golgi compartment. Dimerization and folding of the F protein were also affected by the Phe\textsuperscript{237} \to Ser substitution. Other amino acid replacements at positions 236 or 237 of the F\textsubscript{1} subunit had various effects upon F\textsubscript{0} maturation. These results are discussed in terms of the maturation requirements for the RS virus F molecule.

Introduction

The fusion (F) glycoprotein of human respiratory syncytial (RS) virus mediates fusion of the viral membrane with that of the host cell to initiate a new infective cycle (Walsh & Hruska, 1983). The F protein also mediates fusion between an infected cell and adjacent cells, leading to syncytium formation (reviewed by Collins, 1991). Although there is limited sequence identity of the RS virus F protein with those of other paramyxoviruses (< 20%), they all share structural features such as the locations of the hydrophobic domains, the carbohydrate side chains and the cysteine residues (reviewed by Morrison & Portner, 1991). In contrast, the second major external RS virus glycoprotein, the attachment (G) glycoprotein, shares neither sequence nor structural features with the attachment proteins (HN or H) of other paramyxoviruses (Satake et al., 1985; Wertz et al., 1985).

The RS virus F glycoprotein is synthesized as an inactive precursor, F\textsubscript{0}, with a molecular mass of 68 kDa, which is cleaved by trypsin-like proteases to generate two smaller polypeptides, F\textsubscript{1} (49 kDa) and F\textsubscript{2} (20 kDa), which remain linked by disulphide bridges (Gruber & Levine, 1985a). The cleavage site is preceded by six basic residues located at the C-terminal end of the F\textsubscript{2} subunit and is followed by a hydrophobic peptide located at the N-terminal end of the F\textsubscript{1} subunit. The mature F protein contains 15 cysteines, 11 of which are closely spaced around the middle of the F\textsubscript{1} subunit and are probably important in folding of the F monomer. The F\textsubscript{1} subunit has a single potential site for N-glycosylation whereas the F\textsubscript{2} subunit has 4–5 potential sites. The F molecule has been shown to be sulphated (Cash et al., 1977) and palmitoylated (Arumugham et al., 1989a).

Shortly after its synthesis, the F\textsubscript{0} precursor is oligomerized in the endoplasmic reticulum (ER) to form SDS-stable dimers and less stable homotetramers (Collins & Mottet, 1991). The transport time, before the F protein appears at the cell surface, is 20–30 min (Fernie et al., 1985; Gruber & Levine, 1985b; Collins & Mottet, 1991). The endoproteolytic cleavage of the F\textsubscript{0} precursor occurs at late times of its transport through the exocytic pathway, most likely in the trans-Golgi compartment or in the trans-Golgi network (Collins & Mottet, 1991).

Inoculation of laboratory animals with either purified antigen (Walsh et al., 1987) or vaccinia virus recombinants which express the F protein (Olmsted et al., 1986; Stott et al., 1987) induces a protective immune response against challenge by RS virus. This protective immunity is broadly cross-reactive for strains of the two antigenic groups (A and B) into which RS virus isolates have been subdivided. In addition, monoclonal anti-
bodies (MAbs) directed against the F protein confer passive protection in mice to an RS virus challenge (Taylor et al., 1984, 1992). These results, which highlight the relevance of the F protein for a protective immune response, have led to studies on the antigenic organization of the F molecule. Several neutralizing epitopes have been mapped onto the F protein primary structure (Arbiza et al., 1992; Bourgeois et al., 1991; López et al., 1990; Martín-Gallardo et al., 1991; Trudel et al., 1987). We have recently characterized two antigenic areas (II and IV) of the F protein involved in neutralization and fusion inhibition (Arbiza et al., 1992; Taylor et al., 1992). The first area (II) was located in a trypsin-resistant fragment corresponding to the N-terminal third of the F1 subunit (positions 258–272). The second area (IV) included residue 429, located in a trypsin-sensitive region towards the C-terminal end of the cysteine-rich domain of the F1 subunit. Structural studies with synthetic peptides from antigenic area II illustrated the conformational requirements of most epitopes included in this area (López et al., 1993). We now report on the generation and characterization of vaccinia virus recombinants expressing mutant forms of the RS virus F protein in which proteolytic processing of the F0 precursor and its expression at the cell surface are both inhibited.

**Methods**

**Viruses.** The Long strain of human RS virus (group A) and the escape mutant R47F/4, selected with MAb 47F (García-Barreno et al., 1989), were grown in HEp-2 cells and purified from culture supernatants as described previously (García-Barreno et al., 1988).

Wild-type and recombinant vaccinia viruses (see later) were grown in CV-1 cells. Intracellular virus was released by three cycles of freezing and thawing followed by centrifugation through a 45% sucrose cushion at 70,000 g for 60 min.

**Antibodies.** MAbs 47F, 2F, 55F, 56F, 70F, AK13A2 and 19, which recognize the F glycoprotein of the Long strain, have been described (Arbiza et al., 1992; López et al., 1990; Taylor et al., 1992; Mathelse et al., 1995). Antiserum against the F protein was raised in rabbits inoculated with immunoaffinity-purified Long F protein (García-Barreno et al., 1989).

**Preparation of plasmids and vaccinia virus recombinants.** Four basic plasmids (pSLF88, LF1, pSCF and pGFR47) were employed to construct the vaccinia virus recombinants used in this study (Table 1). The origins of plasmids pSLF88, LF1 and pSCF have been described previously (Cristina et al., 1990; López et al., 1988; Portela et al., 1989). They all contain full-length cDNA copies of the Long F gene inserted into vectors pBSV9, pGEM-4 or pSC11, respectively. Plasmid pGFR47 was produced by inserting an F cDNA copy, obtained from the RS virus mutant R47F/4, into the Small site of pGEM-4.

From the above plasmids, F gene inserts were isolated and cloned into the pSC11 vector (kindly donated by B. Moss; NIH, Bethesda, USA) following the procedure of Chakrabarti et al. (1985), to obtain the vaccinia virus recombinants listed in Table 1. A recombinant control (VA-5C) was made with plasmid pSC11 without an insert.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Nucleotide changes*</th>
<th>Amino acid changes*</th>
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<tbody>
<tr>
<td>VA-5C†</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>VA-F</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VA-FT</td>
<td>797 (A → T)</td>
<td>262 (Asn → Tyr)</td>
</tr>
<tr>
<td>VA-FR47</td>
<td>76 (T → C)</td>
<td>–</td>
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<tr>
<td></td>
<td>212 (A → T)</td>
<td>67 (Asn → Tyr)</td>
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<td>371 (A → T)</td>
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<td>680 (T → C)</td>
<td>223 (Asn → Tyr)</td>
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<td>723 (T → C)</td>
<td>237 (Phe → Ser)</td>
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<tr>
<td></td>
<td>797 (A → T)</td>
<td>262 (Asn → Tyr)</td>
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<tr>
<td></td>
<td>1338 (C → T)</td>
<td>442 (Ala → Val)</td>
</tr>
<tr>
<td></td>
<td>1354 (A → G)</td>
<td>–</td>
</tr>
<tr>
<td>VA-FS1</td>
<td>680 (T → C)</td>
<td>223 (Phe → Leu)</td>
</tr>
<tr>
<td>VA-FS2</td>
<td>723 (T → C)</td>
<td>237 (Phe → Ser)</td>
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<td>680 (T → C)</td>
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<td>237 (Phe → Ser)</td>
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<td>VA-FS4</td>
<td>680 (T → C)</td>
<td>223 (Phe → Leu)</td>
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<td>VA-FS6</td>
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</tr>
<tr>
<td></td>
<td>797 (A → T)</td>
<td>262 (Asn → Tyr)</td>
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</tbody>
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* Nucleotide and amino acid changes in the F protein encoded by the different vaccinia virus recombinants, relative to the F protein of RS virus Long strain. NA, Not applicable; –, no change.
† VA-5C is a recombinant control obtained with pSC11 without an F insert.

Plasmids used for preparation of the vaccinia virus recombinant VA-FT and the series VA-FS1 to VA-FS6 (Table 1) were obtained by site-directed mutagenesis, using the PCR procedure of Higuchi et al. (1988), as described previously (Arbiza et al., 1992). The plasmids in Fig. 5 that contained mutations at positions 236 or 237 of the F protein gene were also obtained by site-directed mutagenesis. The presence of the desired mutation in each plasmid was confirmed by sequencing. The PCR primers as well as details of the protocols for the generation of plasmids and recombinants are available from the authors upon request.

**Radiolabelling of cells and preparation of extracts.** HEp-2 or CV-1 cells were infected with either RS virus or vaccinia virus recombinants, as described previously (García-Barreno et al., 1988). At the times indicated in the figure legends, the medium was replaced by methionine-depleted Dulbecco's modified Eagle's medium containing 2.5% dialysed fetal calf serum and [35S]methionine (250 μCi/ml). Extracts were made by resuspending the cultures in lysis buffer (10 mM-Tris–HCl, pH 7.6, 140 mM-NaCl, 1 mM-EDTA and 1% octyl-glucoside). The extracts were clarified by centrifugation at 10,000 g for 10 min.

Transient expression of the F protein was done in HeLa-T4 cells (Maddon et al., 1986) by using the vaccinia virus-T7 recombinant vTF7-3, as described by Fuerst et al. (1986). Cell cultures were infected with vTF7-3 (kindly donated by B. Moss, NIH, Bethesda, USA) at 10 p.f.u./cell and subsequently transfected for 5 h with the plasmids listed in Fig. 5 using lipofectin (1 ng/106 cells) (Gibco BRL). Radiolabelling and preparation of extracts were done as indicated above.

**Immunochromatographic techniques**

(i) Western immunoblotting. The procedure of Towbin et al. (1979) was followed using biotinylated anti-rabbit or anti-mouse immunoglobulins, streptavidin-peroxidase and 4-chloro-l-naphthol, as recommended by the manufacturer (Amersham).
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(a) kDa

(b) F0 F1

Fig. 1. Western blot analysis of F proteins expressed by vaccinia virus recombinants. The following protein extracts were separated by SDS-PAGE: lane 1, purified Long virus; lane 2, uninfected HEP-2 cells; lane 3, Long-infected HEP-2 cells; lane 4, R47F/4-infected HEP-2 cells; lane 5, VA-5C-infected CV-1 cells; lane 6, VA-F-infected CV-1 cells; lane 7, VA-FT-infected CV-1 cells; lane 8, VA-FR47-infected CV-1 cells. After electrophoresis the proteins were electrotransferred to Immobilon membranes and these were developed with anti-F antiserum (a) or MAb 47F (b). The positions of molecular mass markers (left) and F0 and F1 polypeptides (right) are indicated.

(ii) Immunoprecipitation. The F protein-related products from radiolabelled extracts were selectively bound to anti-F MAbs adsorbed to protein A-Agarose beads (Boehringer Mannheim). The bound material was separated by centrifugation and, after washing, eluted in electrophoresis sample buffer and analysed by SDS-PAGE and autoradiography. For endoglycosidase H (Endo-H) treatment the immunoprecipitated F protein was eluted in 0.5% SDS and 1% 2-mercaptoethanol, boiled for 2 min and was made up to a final concentration of 50 mM with respect to sodium citrate, pH 5.5. Endo-H digestions were done with 1000 U (New England Biolabs) for 60 min at 37 °C.

To test dimerization of the F glycoprotein we followed the procedure described by Arumugham et al. (1989b) for purified F protein. MAb 47F was purified by protein A-Sepharose chromatography (García-Barreno et al., 1989) and covalently bound to CNBr-activated Sepharose following the manufacturer’s instructions (Pharmacia). The 47F-Sepharose beads were added to cell extracts (16 h at 4 °C). The bound material was separated by centrifugation and eluted in electrophoresis sample buffer without 2-mercaptoethanol and without heating. The eluted material was analysed by SDS-PAGE and autoradiography.

(iii) Immunofluorescence. Cells growing in tissue culture chamber slides (Nunc) were infected with vaccinia virus recombinants at 5–10 p.f.u./cell. After 20–30 h, cells were fixed and processed for indirect immunofluorescence (Rueda et al., 1994) using mouse MAbs and FITC-conjugated sheep anti-mouse immunoglobulin.

Alternatively, infected cells were detached from the plates with 0.03% EDTA in Mg2+- and Ca2+-free PBS and incubated with the MAbs indicated in the figure legends for 30 min at 4 °C. After washing with PBS, the cells were stained with FITC-labelled anti-mouse immunoglobulin and fixed with 1% paraformaldehyde.

Results

Cloning and expression of F cDNAs from wild-type and mutant R47F/4 viruses

We described previously the isolation and characterization of RS virus mutants (Long strain) which escaped neutralization by MAb 47F (López et al., 1990). One of them (R47F/4) had a transversion A→U at nucleotide 797 of the F mRNA that changed the amino acid at position 262 from Asn to Tyr. This amino acid substitution eliminated several epitopes included in antigenic area II of the F glycoprotein (Arbiza et al., 1992).

We sought to confirm that the substitution Asn262→Tyr was sufficient to reproduce the antigenic changes observed in the virus R47F/4 by cloning and expressing the mutant F mRNA. Poly(A)+ RNA obtained from HEP-2 cells infected with R47F/4 was used as template to obtain a cDNA copy which was cloned in pGEM-4 (Cristina et al., 1990). One of the recombinant plasmids (pGFR47) contained a copy of F cDNA with the following changes, relative to the wild-type F (Long): (i) extra sequences (about 1000 nucleotides) at the 5' end whose origins have not been investigated further and (ii) a full-length cDNA of the F gene with eight nucleotide changes which encoded the six amino acid substitutions...
Fig. 2. For legend see facing page.
were either fixed, permeabilized and stained by immuno-
with the vaccinia virus recombinants. After 24h, the cells
processing in VA-FR47-infected cells was the result of a
was apparently associated with the extra mutations of
contained an F 0 band recognized by anti-F antiserum
inefficient proteolytic processing of the F molecule when
expressed from vaccinia virus recombinants (Wertz
Tyr was sufficient to eliminate the epitope 47F. Other
Western blot for the presence of wild-type and mutant F
products (L6pez et al., 1989), which contained a
cDNA copy of the Long F gene, were used to infect CV-
recombinants, together with the previously
described VA-F (Portela et al., 1989), which contained a
cDNA copy of the Long F gene, were used to infect CV-
extracts of CV-1 cells infected with VA-F or VA-FT (Fig.
1 d). In contrast, MAb 47F reacted with the F1 subunit of
Long virus and VA-F but with neither R47F/4 nor VA-
FT (Fig. 1 b), indicating that the replacement Asn ~62
intraacellular and membrane staining. Surface
fluorescence was detected unambiguously in these
cultures when staining was done in unixed cells (Fig.
2 e, f). In contrast, cells infected with VA-FR47 showed
only intracellular staining (Fig. 2g) but no membrane
fluorescence was observed in either permeabilized or
unxed cells (Fig. 2h). The same results were obtained
with polyclonal rabbit anti-F serum (not shown).

Characterization of the genetic lesion responsible for F
glycoprotein maturation arrest in VA-FR47
To relate the phenotypic changes observed in VA-FR47
to any of the mutations present in the original cDNA, we
prepared a second set of vaccinia virus recombinants
containing single or multiple amino acid substitutions.
Two of the mutations present in pSCFR47 (amino acids
67 and 120) were included in the Fsubunit, which shows
the highest degree of amino acid differences between the
RS virus strains (López et al., 1988). Changes in the
amino acid at position 442 of the F subunit have been
found among RS virus isolates (López et al., 1988). None
of these three mutations significantly altered the pre-
dicted secondary structure of the F protein (not shown).
Thus, we decided to explore the effects of amino acid
changes at positions 223, 237 and 262, either individually
or in combination, upon F protein maturation. To this
end, the series of vaccinia virus recombinants VA-FS1
to VA-FS6 (Table 1) was obtained (see Methods). These
recombinants, together with VA-FT, represented single,
double and triple mutants at the positions indicated
above.

Cells infected with the different vaccinia virus recom-
binants were tested by immunofluorescence for intra-
cellular and cell surface expression of the F protein. Fig.
3 shows the fluorescence pattern of CV-1 cells infected
with the recombinants listed in Table 1 that are not
presented in Fig. 2. The surface of cells infected with the
single mutants VA-FS1 (Fig. 3a, b) or VA-FT (Fig.
2e, f) or with the double mutant VA-FS4 (Fig. 3g, h)
were clearly stained with MAb 19. In contrast, this MAb
failed to stain the surface of cells infected with the
mutants that included the amino acid substitution Phe237
Ser, either alone (VA-FS2; Fig. 3c, d) or in com-
bination with other changes (VA-FS3, Fig. 3e,f). VA-

Fig. 2. Immunofluorescence staining of CV-1 cells infected with the vaccinia virus recombinants VA-5C (a, b), VA-F (c, d), VA-FT
(e, f) or VA-FR47 (g, h). After infection (24 h later) the cells were either fixed and stained (a, c, e, g) or detached from the plates and
stained with MAb 19(b, d, f, h).
Fig. 3. Immunofluorescence of CV-1 cells infected with the VA-FS1 to VA-FS6 vaccinia virus recombinant series. CV-1 cells were infected with VA-FS1 (a, b), VA-FS2 (c, d), VA-FS3 (e, f), VA-FS4 (g, h), VA-FS5 (i, j) or VA-FS6 (k, l). Cells were processed as described in the legend to Fig. 2.

FS5, Fig. 3i, j; VA-FS6, Fig. 3k, l). Thus, the replacement Phe237 → Ser was sufficient to block F protein transport to the cell surface.

To correlate the absence of surface expression with inhibition of F₀ proteolytic processing, cells infected with the different vaccinia virus recombinants were labelled for 18 h with [35S]methionine and chased for 2 h with cold methionine. The F protein-related products were analysed after binding to anti-F MAb. The results shown in Fig. 4 indicate that extracts of cells infected with any of the mutants that included the Phe237 → Ser substitution contained only the unprocessed precursor (Fig. 4, lanes 5, 7, 9, 10 and 11). However, cells infected with the mutants that included the substitutions Phe237 → Leu (Fig. 4, lane 4), Asn262 → Tyr (lane 6) or both (lane 8) contained the F₀ precursor and the F₁ subunit as well as some of the F₁ degradation products (p20). The unprocessed (F₀) and processed (F₁ and F₂) F protein-related products of the different vaccinia virus recombinants could be readily labelled with [3H]mannose (not shown), indicating that addition of N-linked sugar chains to F₀ was unaffected by the Phe237 → Ser replacement. The amount of F protein-related products in Phe237 → Ser mutants was lower than in wild-type virus as a consequence of a lower rate of synthesis, noted in pulse-chase experiments (not shown). It is possible that
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The substitution Phe\textsuperscript{237} → Ser involved an unsafe amino acid change, as deduced by Bordo & Argos (1991) from amino acid replacements that are found in protein families. We then tested whether or not other amino acid replacements at position 237 of the F molecule would inhibit protein maturation. New plasmids were generated by site-directed mutagenesis using PCR. One of the plasmids had a fortuitous mutation at position 236 (Glu → Lys) and the others had the replacements Phe\textsuperscript{237} to Leu, Val, Tyr, Lys or Asp. The different plasmids were used in the transient expression system of Fuerst et al. (1986). Processing of the F\textsubscript{0} precursor and Endo-H sensitivity were assessed after radiolabelling cells and immunoprecipitation of extracts with anti-F MAbs (Fig. 5b). The results obtained indicated that certain amino acid changes at position 237 (Phe → Leu, Phe → Tyr or Phe → Asp) had no effect upon F\textsubscript{0} processing. In contrast, the changes Phe → Val or Phe → Lys at position 237, and to lesser extent the change Glu → Lys at position 236, inhibited F\textsubscript{0} processing. In all cases the remaining F\textsubscript{0} precursor was sensitive to Endo-H digestion whereas the F\textsubscript{1} subunit was Endo-H-resistant.

When cells transfected with the plasmids of Fig. 5(b) were tested by immunofluorescence, surface staining was observed only in those cells that processed the F\textsubscript{0} precursor to significant levels (not shown). Thus, transport of the F glycoprotein to the cell surface was tightly associated with processing of the F\textsubscript{0} precursor, independent of the amino acid present at position 237.

Failure in folding and oligomerization have been associated with ER protein arrest in other systems.
Fig. 5. Endo-H treatment of F glycoproteins. HeLa-T4 cells were infected with vTF7-3 and transfected with the plasmids indicated beneath panels; the control plasmid pSC11 is indicated. Two separate experiments are represented (a, b). Cultures were labelled for 1 h with [35S]methionine starting 2 h after transfection and chased for 15 h in medium with cold methionine. Extracts were made and immunoprecipitated with a pool of anti-F MAb's. The precipitates were treated (+) or untreated (−) with Endo-H and analysed by SDS-PAGE and autoradiography. The Endo-H-digested form of F is indicated (FoH).

Fig. 6. Detection of F dimers. HeLa-T4 cells were either mock-infected or infected with Long virus, or infected with vFT7-3 and transfected with either LF1 or LFS2. The cells were then labelled for 2 h with [35S]methionine. Extracts were eluted with SDS-PAGE sample buffer without heating and without 2-mercaptoethanol (2-ME)(left panel). Other extracts were treated as indicated above panels.
Fig. 7. Immunofluorescence of HEp-2 cells infected with the vaccinia virus recombinants VA-F (a, c, e, g, i, k) or VA-FS2 (b, d, f, h, j, l). Cells were fixed 24 h after infection and stained with the following MAb's: 2F (a, b), 55F (c, d), 47F (e, f), AK13A2 (g, h), 56F (i, j) or 19 (k, l).
(Gething & Sambrook, 1992). To explore the dimerization properties of wild-type and mutant forms of the F protein, extracts of either Long-infected cells or cells transiently expressing F proteins encoded by LF1 or LFS2, plasmids were immunoprecipitated with MAb 47F-Sepharose beads and eluted in sample buffer. The unheated precipitate of extracts expressing wild-type F protein (either Long-infected or LF1-transfected cells) contained a band of high molecular mass, corresponding to F dimers (Fig. 6). This material dissociated to form F monomers upon heating and $F_1 + F_2$ chains (although $F_2$ was not detected under the conditions shown in Fig. 6) upon heating and reduction. In contrast, the material immunoprecipitated from LFS2-transfected cells was unable to penetrate the separating gel without heating and reduction, suggesting the formation of aggregates that remained at the top. When the precipitate was boiled and treated with 2-mercaptoethanol, the LFS2-encoded polypeptide generated the unprocessed F monomer band. Other bands shown in Fig. 6 correspond to cell protein contaminants that were not reproducible in other experiments.

To test whether alterations in the oligomerization properties of the LFS2-encoded polypeptide were associated with misfolding of the F molecule, cells infected with either VA-F or VA-FS2 were fixed and stained with the MAbs indicated in Fig. 7. These MAbs recognize epitopes representative of three antigenic sites identified in the F molecule (Garcia-Barreno et al., 1989). MAbs AK13A2 and 47F, which recognize epitopes from antigenic site II and react with the $F_1$ subunit in Western blots (Arbizu et al., 1992; López et al., 1993), stained the cells infected with VA-F (Fig. 7e, g) or VA-FS2 (Fig. 7f, h). Similar results were obtained with MAb 19, which reacted in Western blots with the $F_1$ subunit epitope belonging to antigenic site IV (Fig. 7k, l). In contrast, MAbs 2F and 55F from antigenic site I and MAAb 56F from antigenic site IV, which recognize epitopes sensitive to the harsh treatment of Western blot assays, did not stain the cells infected with VA-FS2 (Fig. 7b, d, j) although normal staining was observed in cells infected with VA-F (Fig. 7a, c, i). These results indicate that the mutant F protein with the replacement $\text{Phe}^{237} \rightarrow \text{Ser}$ does not express conformational epitopes which are destroyed by Western blot treatment and suggests misfolding of the molecule.

**Discussion**

This study began by cloning an F gene that contained six point mutations (pGFR47) and was defective in both processing the $F_0$ precursor and its transport to the cell surface. Vaccinia virus recombinants that contained amino acid replacements at positions 223, 237 or 262 of the F protein, either alone or in combination, were used to identify the mutation $\text{Phe}^{237} \rightarrow \text{Ser}$ as responsible for this phenotypic trait.

There are numerous examples of mutations in virus and non-virus glycoproteins that inhibit their traffic to the cell surface (Einfeld & Hunter, 1991). In most studies, site-directed mutagenesis has been used to test the significance of certain protein domains (membrane anchor, cytoplasmic tail, etc) that were predicted to be important for cell surface transport (Gething et al., 1989; Parks & Lamb, 1990; Qadri et al., 1991). In other studies, temperature-sensitive mutants identified unpredicted residues which were essential for the same process (Garten et al., 1991). The main concept arising from these studies is that proper folding and oligomerization of glycoproteins is necessary for their transport through the exocytic pathway.

The fluorescence staining pattern of cells infected with vaccinia virus recombinants indicated that the $\text{Phe}^{237} \rightarrow \text{Ser}$ F mutant was blocked at some early stage of the transport pathway, most likely in the ER or cis-Golgi. This was corroborated by the sensitivity of the unprocessed $F_0$ precursor to Endo-H treatment. Misfolding of the mutant protein was suggested by the lack of reactivity with MAbs that recognize conformational epitopes and improper oligomerization was suggested by the absence of $F$ dimers in immunoprecipitates that were neither heated nor reduced. Anderson et al. (1992) also reported a vaccinia virus recombinant (F313) that expressed a double mutant (Val$^{201} \rightarrow \text{Ala}$ and Val$^{247} \rightarrow \text{Met}$) of the RS virus F glycoprotein. In this case, each of the individual changes was insufficient to block $F_0$ maturation, but the double mutation inhibited both $F_0$ processing and cell surface transport. Using different methodology to that of our work, evidence was presented which suggested that the F protein encoded by the recombinant F313 was misfolded and did not oligomerize (Anderson et al., 1992).

The inhibitory effect of mutations $\text{Phe}^{237} \rightarrow \text{Ser}$, Val$^{201} \rightarrow \text{Ala}$ or Val$^{247} \rightarrow \text{Met}$ upon $F_0$ cleavage is most likely related to its impaired surface transport. In agreement with this statement Collins & Mottet (1991) reported that cleavage of the $F_0$ precursor occurs at late stages of its cell surface transport (distal cisternae or trans-Golgi network) in RS virus-infected cells.

Other amino acid replacements at positions 236 or 237 had different effects upon F protein maturation that did not follow strictly the rules of ‘safe’ substitutions deduced from the comparison of protein families (Bordo & Argos, 1991). For instance, the replacements $\text{Phe}^{237} \rightarrow \text{Lys}$ and $\text{Phe}^{237} \rightarrow \text{Asp}$, which are equally infrequent among protein families, had opposite effects upon $F_0$ processing (Fig. 5). Residue 237 is conserved among the F proteins of human (Johnson & Collins, 1988) and...
bovine RS viruses (Lerch et al., 1991; Pastei et al., 1993; Walravens et al., 1990) and in the equivalent position of other pneumoviruses (Chambers et al., 1992). Since the amino acid replacements Phe\textsuperscript{237} \rightarrow Leu, \rightarrow Tyr or \rightarrow Asp did not impair F\sb{1} processing and surface transport, there may be restrictions to changes in residue 237 of the RS virus F glycoprotein which are not reflected at the individual protein level.

The mutation Phe\textsuperscript{237} \rightarrow Ser is located within the N-terminal third of the F\sb{1} subunit. This region is highly resistant to trypsin digestion and contains several neutralizing epitopes clustered around positions 262, 268 and 272 (Arbiza et al., 1992). We previously reported that the reactivity of MAbs with synthetic peptides that spanned positions 215–275 was dependent upon peptide length and conformation (López et al., 1993). Thus, residue 237 seems to be located in a region of the mature F molecule that is important for its antigenicity and has a strong tendency to retain its folded conformation.

Chemical cross-linking studies have suggested that F oligomerization involves the association of two SDS-stable homodimers into a homotrimer. Intermonomer associations both within and between dimers appear to involve the F\sb{1} subunit (Collins & Mottet, 1991). Accordingly, the substitution Phe\textsuperscript{237} \rightarrow Ser, reported here, or the substitutions Val\textsuperscript{301} \rightarrow Ala and Val\textsuperscript{747} \rightarrow Met, reported by Anderson et al. (1992), may disturb F protein oligomerization by altering intermonomer interactions. Further studies should clarify the precise role of these residues and other regions of the F molecule in assembly of the mature three-dimensional structure.

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