Intracellular processing of the human respiratory syncytial virus fusion glycoprotein: amino acid substitutions affecting folding, transport and cleavage

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The intracellular processing and transport of the respiratory syncytial virus (RSV) fusion (F) glycoprotein was examined by comparing the maturation and stability of wild-type F, uncleaved mutant F and chimeric F glycoproteins expressed by recombinant vaccinia viruses to that of F protein expressed by RSV. One of the recombinant viruses, vF317, expressed F protein (F317) that was processed like the RSV F glycoprotein. F317 was synthesized initially as Fo, the uncleaved glycosylated precursor of mature F protein, and formed stable oligomeric structures that were maintained following cleavage of Fo to form the disulphide bond-linked F1 and F2 subunits. Most of the newly synthesized Fo expressed by either RSV or by vF317 was sensitive to treatment with endoglycosidase H (Endo H). Following cleavage of Fo, F1 was resistant to Endo H, suggesting that conversion to complex-type sugars, which takes place in the medial Golgi apparatus, occurred simultaneously with or immediately prior to cleavage of Fo into F1 and F2. Another recombinant virus, vF313, synthesized only uncleaved F protein (F313) that comigrated with Fo. Uncleaved F313 was expressed as a stable glycosylated protein; however, unlike cleaved F317, its oligosaccharides were not modified to complex forms, as determined from its Endo H sensitivity, and uncleaved F313 did not assemble into stable oligomeric structures. Nucleotide sequence analysis of the cDNA clones encoding F313 and F317 revealed four predicted amino acid sequence differences, none of which were located at the cleavage site. Expression of chimeric F proteins obtained by restriction fragment exchange between the two cDNA clones indicated that two amino acid changes in the F1 domain, located at amino acid residues 301 (Val to Ala) and 447 (Val to Met), resulted in the expression of uncleaved F protein. A change at either of these two amino acid residues, 301 or 447, resulted in the expression of inefficiently cleaved F protein, defining an additional F protein phenotype. Pulse–chase analyses to examine the association of recombinant F glycoproteins with gradient-purified fractionated membranes or with GRP78-BiP, a protein resident in the endoplasmic reticulum (ER) which binds to nascent proteins, revealed that uncleaved F protein (F313) is associated with GRP78-BiP in the ER for a longer time than F317, and little if any F313 was transported to the cell surface. In addition, the uncleaved F protein (F313) was not recognized by a panel of F protein-specific monoclonal antibodies in ELISA or indirect immunofluorescence assays, suggesting that F313 was misfolded and, as a result, not transported properly or cleaved.

Introduction

The fusion (F) glycoprotein of paramyxoviruses is found on the surface of virions and virus-infected cells, and allows virus penetration of host cells and fusion of infected cell membranes to those of adjacent cells. F protein is synthesized initially in the form of Fo, the uncleaved precursor of the mature form of the F protein, which consists of two disulphide bond-linked subunits, F1 and F2 (Scheid & Choppin, 1977). Cleavage of F glycoprotein into F1 and F2 subunits by a cellular trypsin-like protease is essential for generating the fusion activity of the protein (Scheid & Choppin, 1977). In addition, the length and composition of amino acids at the cleavage activation site, a stretch of hydrophilic residues that precede the N-terminal hydrophobic residues of the F1 subunit, are important factors that influence the ability of paramyxovirus F proteins to be cleaved (Glickman et al., 1987; Paterson et al., 1989; Toyoda et al., 1987). A strict correlation has been demonstrated between the ability of F proteins of
different strains of Newcastle disease virus to be cleaved and the extent of virus pathogenicity (Nagai et al., 1976, 1979).

The respiratory syncytial virus (RSV) F glycoprotein open reading frame encodes 574 amino acids, as predicted from nucleotide sequence analysis (Collins et al., 1984; Elango et al., 1985). At the N terminus of the protein there is a stretch of 22 hydrophobic amino acid residues that are thought to serve as a signal peptide for the insertion of the protein into the membrane. The F2 subunit of the protein extends from the end of the signal peptide sequence to the cleavage activation site (residues 131 to 136), a hydrophilic region consisting of six consecutive basic amino acids. The N terminus of F1, which begins at residue 137 (Elango et al., 1985), contains a long stretch of hydrophobic amino acid residues and, by analogy to other paramyxovirus F proteins, is the domain responsible for the membrane fusion activity of F protein (Scheid & Choppin, 1977). A membrane anchor region consisting of 26 hydrophobic amino acid residues is found near the C terminus of F1, indicating that the F protein has a structural orientation typical of class I membrane glycoproteins. Post-translational modifications of F protein in addition to cleavage of the signal peptide and cleavage of F1 to form the F1 and F2 subunits include the formation of oligomers (Walsh et al., 1985; Arumugham et al., 1989a), the covalent attachment of palmitate to cysteine residue 550, located in the membrane anchor region (Arumugham et al., 1989b), and the addition of N-linked carbohydrates (Gruber & Levine, 1985a; Peeples & Levine, 1979). There are four potential N-linked glycosylation sites on the F2 subunit and one on the F1 subunit (Collins et al., 1984; Elango et al., 1985).

In previous work, two independently derived cDNA clones of the F protein gene were inserted into recombinant vaccinia virus (VV) vectors to express F protein (Wertz et al., 1987a, b). One of the recombinant viruses, vF317, synthesizes F protein (F317) that is cleaved to form the mature disulphide bond-linked F1 and F2 subunits and is expressed on the cell surface. Another recombinant virus, vF313, synthesizes only an F protein (F313) that comigrates with F0 and is not cleaved to form F1 and F2 subunits.

In this report, the intracellular processing and transport of the RSV F glycoprotein was examined by comparing the maturation and stability of wild-type (wt) F, uncleaved mutant F and chimeric F glycoproteins expressed by recombinant VV with those of F protein expressed by RSV. The maturation and stability of the F glycoproteins were examined by pulse–chase labelling of virus-infected cells coupled with (i) endoglycosidase H (endo H) digestion, which serves as a marker for F glycoprotein transport to the medial Golgi apparatus and the maturation of oligosaccharide chains from high mannose to complex forms, (ii) fractionation of membranes by density gradient centrifugation to monitor the intracellular transport of the recombinant F glycoproteins and (iii) immunoprecipitation with anti-BiP monoclonal antibody (MAb) to examine the association of the F proteins with GRP78-BiP, a protein resident in the endoplasmic reticulum (ER) which binds to nascent proteins, to monitor the transport of F protein from the ER to the Golgi complex. Other important objectives of this work were to determine whether differences in ability of F313 and F317 to be cleaved were a consequence of differences in intracellular transport, and to determine the genetic basis for the differences observed in F protein expression. In addition, ELISA and indirect immunofluorescence assays were used to determine whether changes in protein folding and conformation could explain the phenotypic differences observed between F313 and F317.

**Methods**

**Cells and Viruses.** HEp-2 cells were grown in modified Eagle's medium supplemented with 5% foetal bovine serum and penicillin, kanamycin and streptomycin (MEM5). Virus stocks of human RSV A2, wt VV (WR strain), temperature-sensitive (ts) VV mutant C17 (obtained from Dr. Richard C. Condit of the University of Florida, Gainesville, Fla., U.S.A.; Condit et al., 1983) and the recombinant VVs were propagated in HEp-2 cells (Stott et al., 1986). Thymidine kinase-negative (TK-) 143B cells were used for the selection of recombinant viruses and for plaque assays to determine the titre (p.f.u./ml) of VV stocks (Ball et al., 1986; Stott et al., 1986). The 143B cells were grown in MEM5 supplemented with 5-bromo-2'-deoxyuridine (20 μg/ml). The construction and isolation of recombinant VV vectors, vF313 and vF317, expressing the F protein of RSV (strain A2) have been described previously (Wertz et al., 1987a, b).

**Antibodies.** Anti-RSV serum or a panel of mouse anti-RSV MAbs specific for the F protein was used to examine the expression of RSV or recombinant F proteins by immunoprecipitation, ELISA or indirect immunofluorescence (Stott et al., 1986, 1987). Anti-RSV serum (Burroughs Wellcome Reagents) is a bovine polyclonal antiserum generated by immunization with human RSV strain A2 and bovine RSV strain 127. MAbs C1, C2, C13, C14, C18, C19, C20 and C21 were from the Compton Laboratory, AFRC Institute for Animal Health, Compton, U.K. (Stott et al., 1984). MAb 14 was provided by Dr. E.E. Walsh, University of Rochester, N.Y., U.S.A. (Walsh & Hruska, 1983), and MAB 7C2 was provided by Dr M. Trudel, Institut Armand Frappier, University of Laval, Montreal, Quebec, Canada (Trudel et al., 1986). Normal mouse serum and mouse anti-VV serum were used as negative and positive control sera, respectively, for the ELISA and the indirect immunofluorescence assay. A rat MAb (anti-BiP) specific for the cellular heavy chain-binding protein was provided by Dr Linda Hendershot of St Jude Children's Research Hospital, Memphis, Tenn., U.S.A. and Dr John Kearney of the University of Alabama at Birmingham, Ala., U.S.A.

**Nucleotide sequence analysis.** The nucleotide sequences of RSV F gene cDNA clones A25 and A26, which contain the entire coding region of the RSV F gene, were determined by dideoxynucleotide chain
termination using the Klenow fragment of *E. coli* DNA polymerase I, oligonucleotide primers and recombinant m13 phage templates (Biggin et al., 1983). The cDNA clones, which had been modified previously to contain BamHI linker ends (Ball et al., 1986; Wertz et al., 1987a, b), were excised from the VV transfer vectors and ligated into the replicative form of an m13/VV transfer vector, m13mpAB191, the construction of which is described below. The circular ssDNA genomes from recombinant phage isolates were used initially as templates for nucleotide sequencing. F gene-specific oligonucleotide primers were used initially as templates for sequencing at the 5' and 3' ends of the promoter. This primer was also used to confirm the nucleotide sequences of the cDNA clones (F343, 5' CCAATGACAGATGGGTT 3'). The orientation of inserts in the recombinant phage templates was determined by nucleotide sequencing with an oligonucleotide primer (5' TATTGCACGGTAAGGAAG 3') corresponding in sequence to bases -41 to -24 of the VV 7.5K promoter. This primer was also used to confirm the nucleotide sequences at the 5' and 3' ends of the BamHI linker-containing cDNA inserts. Nucleotide and deduced amino acid sequence manipulations were performed with a computer program (Devereux et al., 1984).

Construction of chimeric F protein genes by restriction fragment exchange. cDNA clones containing the entire coding region of the F gene were isolated previously from an RSV A2 cDNA library (Collins et al., 1984). Following modification to obtain BamHI linker ends, cDNA clone A25 extended from base 2 to base 1859 of the F gene, and included an additional 14 bases of oligo(C) adjacent to the BamHI linker at the 3' end (Wertz et al., 1987b). The cDNA clones were digested with BamHI and the resulting fragments were ligated into pGEM1 (Promega). The cDNA clones, A25 and A26, were used in the construction and isolation of recombinant virus vectors vF317 and vF313, respectively (Wertz et al., 1987a, b). Chimeric F cDNA clones, containing portions of cDNA clones A25 and A26, were obtained by exchange of restriction fragments between pGEM1/F plasmids that contained the F gene in the same orientation. The F gene restriction fragments were gel-purified and religated with the reciprocal pGEM1/F plasmids to produce chimeric F cDNA plasmids. The BamHI linker-containing inserts from the resulting pGEM1/F cDNA chimeras were gel-purified and ligated into a unique BamHI site of the m13/VV transfer vector, m13mpAB191. The m13/VV transfer vector was constructed by ligation of m13mp18 replicative form (RF) DNA (cut with HindIII and Smal) to the VV cassette (cut with HindIII and PvuII) from the VV transfer vector, pAB191, which contains the VV 7.5K promoter flanked by portions of the VV TK gene (Ball et al., 1986). Insertion of the F cDNA at the unique BamHI site placed it under the transcriptional regulation of the VV 7.5K promoter, and the orientation of the cDNA inserts in the recombinant phage DNA was confirmed by nucleotide sequencing.

Isolation of recombinant VVs expressing the F glycoprotein. Recombinant VVs were isolated by infecting HEP-2 cell monolayers (60 mm plates) with a ts VV mutant (rcS17) at a multiplicity of 0.1 followed by transfection of calcium phosphate-precipitated wt VV core DNA (3 μg) and m13/VV vector ssDNA (6 μg) (Fathi et al., 1986; Kienny et al., 1984). After incubation at the permissive temperature (33 °C) for 5 h and at the restrictive temperature (39.5 °C) for 48 to 60 h, recombinant viruses were selected by plaque purification at 37 °C on TK-143B cells in medium supplemented with 5-bromo-2'-deoxyuridine (20 μg/ml). The recombinant virus isolates were identified by blot hybridization and subjected to two additional rounds of plaque purification before preparation of virus stocks (Stott et al., 1986).

### Results

**Expression of wt and mutant F glycoproteins by recombinant VVs**

In our initial experiments designed to study the role of the F glycoprotein of human RSV in infection and immunity, two independently derived cDNA clones were used for the expression of the F glycoprotein by recombinant VVs (Wertz et al., 1987a, b). One of the recombinant viruses, vF313, expressed only an F protein that comigrated with F0 (Fig. 1, lane 3), the uncleaved glycosylated precursor of the mature form of the F protein. The other recombinant virus, vF317, expressed...
Fig. 1. RSV F glycoprotein expression by recombinant VVs. Infected and uninfected cell monolayers were metabolically labelled for 2 h with [35S]methionine. RSV-infected cells were labelled from 22 to 24 h post-infection and VV-infected cells were labelled from 2 to 4 h post-infection. Protein samples from mock-infected cells (lane M) or cells infected with RSV, VV, vF313 or vF317 (lanes 1 to 4) were immunoprecipitated with anti-RSV serum and analysed by SDS-PAGE on a 12.5% polyacrylamide gel. The M₁₅s of protein standards are shown to the left.

Fig. 2. Nucleotide and predicted amino acid sequence differences between cDNA clones A25 and A26, encoding F317 and F313, respectively, were determined by the dideoxynucleotide chain termination method (see Methods). Six nucleotide changes were observed [at residues 174 (T to C), 303 (T to C), 343 (G to A, not shown), 915 (T to C), 1352 (G to A) and 1423 (G to A, not shown)] between the cDNA clones encoding F317 and F313, respectively, leading to four predicted amino acid differences in the expressed proteins. Two amino acid differences were located in the F₁ domain of the protein (Ile to Thr at residue 54; Met to Thr at residue 97, from F317 to F313, respectively; Fig. 2) and two differences were located in the F₂ domain (Val to Ala at residue 301; Val to Met at residue 447, from F317 to F313, respectively; Fig. 2). Nucleotide variability among different F gene cDNA clones has been noted previously by sequencing several cDNA clones to obtain a consensus sequence (Collins et al., 1984). Both of the cDNA clones contained sequence differences at positions where nucleotide variability has been reported previously in the consensus sequence (Collins et al., 1984). By comparing the amino acid sequences deduced from the cDNA clones with those determined previously (for which amino acid differences were observed at residues 97, 301 and 447) the difference at residue 54 was seen to be the result of an additional change to the consensus sequence. The nucleotide sequence of both cDNA clones encoded alanine instead of proline at residue 102, which represented another nucleotide change that differed from the consensus sequence.

Although a difference in the ability of the expressed proteins to be cleaved was observed, none of the predicted differences in amino acid sequence were found at the site of cleavage (residues 136/137). Potentially, one or more of these amino acid differences could be responsible for the phenotypic differences observed between the two recombinant F glycoproteins.

Expression of chimeric F glycoproteins

To determine which of the four amino acid differences responsible for the cleavage-negative phenotype, restriction fragments were exchanged between the two clones to produce chimeric F protein cDNAs. To construct the chimeric F genes, the BamHI linker-containing F gene fragments were cloned into pGEM1 in
Fig. 3. Composition of chimeric F glycoproteins constructed by exchange of restriction fragments between F cDNA clones. The first set of chimeric F genes was created by restriction fragment exchange between pGEM1/F313 and pGEM1/F317 (a). The restriction fragments were obtained by HpaI and HindIII digestion. A second set of chimeric F genes was constructed by exchange of reciprocal restriction fragments between pGEM1/F313 and pGEM1/F317, and between pGEM1/F513 and pGEM1/F517 by digestion with HinfI and HindIII (a). The resulting chimeric F genes were excised and ligated into ml3/VV transfer vectors for the isolation of recombinant viruses expressing chimeric F proteins. The composition of the chimeric F proteins relative to the amino acid differences observed between the clones and the cleavage phenotype of the chimeric F protein [cleaved (+), uncleaved (−) or inefficiently cleaved (cl)] are shown in (b).

the same orientation to facilitate the exchange of reciprocal restriction fragments. The first set of chimeric F genes was created by restriction fragment exchange between pGEM1/F313 and pGEM1/F317. This restriction fragment exchange resulted in the construction of chimeric F cDNA clones designed to determine whether the amino acid differences in the F1 domain (residues 301 and/or 447) or those in the F2 domain (residues 54 and/or 97) affected the expression of the uncleaved F protein. The restriction fragments were obtained by HpaI and HindIII digestion (Fig. 3a). The resulting chimeric F genes were excised using BamHI and ligated into RF ml3mpAB191. After screening the phage DNA to determine the orientation of the F gene with respect to the VV 7.5K promoter, the recombinant phage DNA was used as a vector for the production of recombinant VV by homologous recombination. The resulting chimeric F proteins (Fig. 3b), synthesized by vF513 and vF517, demonstrated that the changes in the F1 domain (residues 301 and/or 447) were responsible for the expression of the uncleaved F protein (Fig. 4a, lanes 5 and 6). In addition, the chimeric F glycoprotein synthesized by vF513 expressed a faster migrating form of F2 (Fig. 4a, lane 5), suggesting that the amino acid differences observed in the F2 domain (residues 54 and/or 97) were responsible for the observed differences in the migration of F2. However, the differences observed in F2 did not affect the ability of the recombinant F proteins to be cleaved (Fig. 4a).
A second set of chimeric F genes was designed to determine whether the amino acid differences at residues 301 and/or 447 were responsible for the inability of the F₀ protein to be cleaved. The chimeric F genes were constructed by exchange of reciprocal restriction fragments between pGEM1/F313 and pGEM1/F317, and between pGEM1/F513 and pGEM1/F517 by digestion with HindIII and HindIII (Fig. 3a). The resulting chimeric F genes were excised, ligated into the m13/VV vector and screened for orientation by sequencing. The recombinant viruses synthesized chimeric F proteins (F337, F373, F533 and F577; as shown in Fig. 3b) that were cleaved inefficiently in comparison to the F protein expressed by vF317 or RSV (Fig. 4b). Further evidence for the expression of inefficiently cleaved chimeric F protein in pulse–chase labelling experiments is shown in Fig. 5. These results indicated that changes observed at both residues 301 and 447 would result in the expression of an uncleaved F protein, and that a change observed at either of these residues would result in the expression of inefficiently cleaved F protein.

Synthesis and maturation of wt, mutant and chimeric F glycoproteins

The maturation and stability of the F glycoproteins synthesized by recombinant viruses expressing wt F (F317), mutant F (F313) and chimeric F (F337 and F373) proteins were examined and compared to those of the F glycoprotein synthesized in RSV-infected cells (Fig. 5). All of the recombinant F proteins were expressed in a stable form in infected cells as shown in the lanes containing the 3 h chase period samples (Fig. 5). Like the F glycoprotein synthesized by RSV, F317 was cleaved into subunits following the 1 h chase period, as determined by the appearance of the F₁ subunit, although a small amount of F₀ remained uncleaved (and Endo H-sensitive). In all cases, the majority of F₀ expressed by either RSV or vF317 was sensitive to treatment with Endo H. However, once cleavage occurred, F₁ was resistant to Endo H, suggesting that conversion to complex-type sugars, which takes place in the medial Golgi apparatus (Kornfeld & Kornfeld,
1985), occurred simultaneously with or immediately prior to cleavage of F₀ into F₁ and F₂. F313 remained uncleaved following the 3 h chase period. Additional experiments have demonstrated that this protein remained uncleaved following a 6 h chase period (data not shown). The oligosaccharides of the uncleaved F protein were sensitive to Endo H following the 3 h chase period, which suggested that transport of F313 to the medial Golgi apparatus may be defective. F337 and F373 were cleaved, but significant amounts of F₀ remained uncleaved following the 3 h chase period. These results indicated that the chimeric F glycoproteins (F337 and F373) were cleaved inefficiently and more slowly than F317.

Previous work has indicated that the mature RSV F protein exists primarily as an oligomer, a non-covalently linked dimer consisting of two mature F protein molecules (Walsh et al., 1985; Arumugham et al., 1989a). To determine whether the wt and mutant F proteins were able to assemble into oligomers, non-reduced immunoprecipitated samples from the previous pulse–chase experiment (shown in Fig. 5) were analysed by SDS–PAGE (Fig. 6). The non-reduced F proteins synthesized by RSV or vF317 migrated on SDS–polyacrylamide gels both as F protein monomers (F), which include both F₀ and mature F that has been cleaved to form F₁ and F₂, and F protein oligomers, which confirmed the previous observation that F protein forms oligomers (Walsh et al., 1985; Arumugham et al., 1989a). In addition, F337 and F373 produced an amount of oligomeric F protein consistent with the reduced amount of cleaved product observed for these proteins in Fig. 5 (data not shown). The F317 and RSV F proteins remained in an oligomeric state following cleavage into F₁ and F₂ subunits (almost all of the RSV F protein and F317 were cleaved following the 3 h chase period as shown in Fig. 5), and were resistant to Endo H digestion. However, non-reduced uncleaved F313 migrated only in the monomeric form and was Endo H-sensitive (Fig. 6). This result suggested that uncleaved F313 does not form stable oligomers and thus may be transport-defective. Data from several laboratories have suggested oligomer formation is essential for proper transport of many glycoproteins to the cell surface (Copeland et al., 1988; Gething et al., 1986; Kreis & Lodish, 1986).

**Antibody recognition of the wt and mutant F glycoproteins**

MAbs 7C2 and L4, which define neutralization epitopes on the F protein at amino acid residues 221 to 232 and 283 to 315, respectively (Paradiso et al., 1989; Trudel et al., 1987), and MAbs C1, C2, C13, C14, C18, C19, C20 and C21, which recognize eight distinct epitopes from three overlapping antigenic sites on the RSV F protein (E. J. Stott et al., unpublished data), were used to examine the expression of the recombinant F glycoproteins synthesized in infected cells by ELISA and indirect immunofluorescence assays. These experiments were designed to determine whether the recombinant F glycoproteins maintained the epitopes recognized by the MAbs and to localize the uncleaved F protein within infected cells by using immunofluorescence assays. All of the MAbs recognized the F protein synthesized by vF317, the recombinant virus that expressed mature F protein. However, none of the MAbs recognized the uncleaved F protein (F313). Similar results were obtained from the analysis of chimeric glycoproteins F513 and F517; cleaved F513 was recognized by the panel of MAbs and uncleaved F517 was not.

In indirect immunofluorescence assays, infected cells expressing cleaved F protein (F317) were stained internally with the MAbs; however, infected cells expressing the uncleaved F protein (F313) were not (data not shown). Similar results were obtained by fluorescent antibody staining of cells expressing the recombinant F
proteins using anti-RSV serum. Since both F317 and F313 could be recognized by immunoprecipitation with the polyclonal anti-RSV serum (Fig. 1, and 4 to 8), but not by indirect immunofluorescence, we concluded that aberrant folding was responsible for the lack of intracellular recognition of F313 by the F protein-specific antibodies because only SDS-denatured proteins were immunoreactive. Therefore, immunoprecipitation of SDS-treated F glycoproteins was our only means of comparing the maturation and transport of the cleaved and uncleaved F glycoproteins in subsequent experiments.

Transport of membrane-associated wt and mutant F glycoproteins

Since cell surface or internal immunofluorescence could not be used to monitor the transport of F313 owing to the lack of antibody reactivity, biochemical methods were used. The transport of cleaved F317 and uncleaved F313 was studied further by examining the association of the F proteins with density gradient-purified membrane fractions in a pulse–chase labelling experiment (Fig. 7). During the pulse period, the F protein was found associated with the more dense membranes (fractions 2 and 3) toward the bottom of the gradient, which are enriched for rough ER membranes (Hay, 1974; Olmsted & Collins, 1989). Following the chase period, a significant quantity of the cleaved F protein (F317) was found in association with the less dense membrane fractions (fraction 1), which are enriched for smooth intracellular and plasma membranes (Hay, 1974; Olmsted & Collins, 1989). This result implied that a significant proportion of the F protein was transported to the cell surface during the chase period. However, little of the uncleaved F protein (F313) was associated with the less dense fractions following the chase period, indicating that the transport of this protein to the cell surface was defective.

Association of wt and mutant F glycoproteins with GRP78-BiP

It has been observed for several viral glycoproteins, such as the haemagglutinin protein of influenza viruses and the haemagglutinin–neuraminidase (HN) of paramyxoviruses, that unfolded or misfolded glycoproteins may associate with a resident cellular protein of the ER called BiP (Haas & Wabl, 1983) or GRP78 (Hendershot et al., 1988; Munro & Pelham, 1986). Initially, GRP78-BiP was shown to bind to immunoglobulin heavy chains blocked in transport from the ER (Bole et al., 1986). Subsequent experiments have shown that GRP78-BiP associates with a variety of other misfolded glycoproteins in the ER (Gething et al., 1986; Hurtley et al., 1989; Machamer et al., 1990; Ng et al., 1990).

To examine the association of the recombinant F glycoproteins with GRP78-BiP, proteins were immunoprecipitated with either anti-RSV sera or an anti-GRP78-BiP MAb (Fig. 8). During the pulse labelling period, the F₀ proteins synthesized by vF313 or vF317 were coprecipitated with GRP78-BiP (as observed by its
Fig. 8. Association of wt and mutant F glycoproteins with GRP78-BiP. Virus-infected cells were pulse-labelled for 15 min (odd-numbered lanes) with [35S]methionine and then incubated with unlabelled medium containing excess L-methionine for an additional 2.5 h (even-numbered lanes). Protein samples from cells infected with vF313 or vF317 were immunoprecipitated with either anti-RSV (RS) or anti-GRP78-BiP (BiP) antiserum. The Mrs of protein standards are shown to the left.

The intracellular processing and transport of the RSV F glycoprotein were examined by comparing the maturation and stability of wt F, uncleaved mutant F and chimeric F glycoproteins expressed by recombinant VVs to those of F protein expressed by RSV. Like the F glycoprotein synthesized by RSV, F317 was synthesized in the form of F0, and formed stable oligomeric structures that were maintained following cleavage into F1 and F2 subunits. Most of the newly synthesized F0 expressed by either RSV or vF317 was sensitive to treatment with Endo H. However, once cleavage had occurred, F1 was resistant to Endo H. This result suggests that conversion to complex-type sugars, which occurs in the medial Golgi apparatus (Kornfeld & Kornfeld, 1985), occurred simultaneously with or immediately prior to cleavage of F0 into F1 and F2. Evidence for the intracellular cleavage of the RSV F glycoprotein has been reported previously by Gruber & Levine (1985b), who observed the intracellular accumulation of cleaved F subunits in the presence of monensin, which causes the accumulation of glycoproteins in the Golgi apparatus. Pulse-chase analysis of the uncleaved F glycoprotein (F313) suggested that the protein was expressed as a stable, glycosylated protein that co-migrated with F0. However, F313 did not form stable oligomeric structures, its oligosaccharide chains were not modified to complex forms and are not cleaved to form F1 and F2.

Our interest in investigating the phenotypic and genetic differences between the cleaved and uncleaved F proteins was based on studies on other paramyxoviruses, which have demonstrated that cleavage of the F protein is essential for fusion (Scheid & Choppin, 1977), an important determinant of virus pathogenicity (Nagai et al., 1976, 1979). Nucleotide sequence analysis and expression of chimeric F proteins created by restriction fragment exchange revealed that two amino acid differences in the F1 domain (Val to Ala at residue 301; Val to Met at residue 447) were responsible for the lack of F protein cleavage. Expression of chimeric F proteins produced by additional restriction fragment exchanges demonstrated that a change at either residue 301 or 447 resulted in the expression of inefficiently cleaved F protein, defining an additional F protein phenotype. Although the amino acid differences observed in the F2 domain (Ile to Thr at residue 54; Met to Thr at residue 97) affected differences in the migration of F2 on polyacrylamide gels, these changes did not affect the ability of the recombinant F proteins to be cleaved. The differences in the migration of F2 may result from changes in protein folding and/or differential glycosylation, which are being investigated. None of the differences predicted for the primary amino acid sequences of the expressed proteins were found at the site of cleavage (residues 136/137), or in the N-terminal hydrophobic residues of the F1 domain.

Nucleotide variability among different F gene cDNA
clones has been noted previously by sequencing several cDNA clones to obtain a consensus sequence (Collins et al., 1984). In that study, seven nucleotide differences were observed among the four F cDNA clones examined. In this study, six nucleotide differences were observed between the two cDNA clones, all of which were nucleotide transitions and four of which resulted in amino acid differences. The nucleotide variability observed among the cDNA clones could represent the sequence heterogeneity observed for RNA virus genomes, which results from high RNA polymerase error rates (Steinhauer & Holland, 1987), and/or errors in reverse transcription during cDNA synthesis. Based on the F protein phenotypes resulting from the amino acid differences that we have described, it is possible that the amino acid differences at residues 301 and 447 could represent changes in the viral genome resulting in the production of mutant or defective viruses.

Although the length and composition of the cleavage activation site of paramyxovirus F glycoproteins are important factors governing the ability of the F protein to be cleaved and virus pathogenicity (Glickman et al., 1987; Paterson et al., 1989; Toyoda et al., 1987), our results indicate that changes in other regions of the protein may result in altered or aberrant fusion activities. Changes in amino residues 301 or 447 of the RSV F protein, which are located proximal to a cysteine-rich region of the F1 subunit, could affect protein folding mechanics by inhibiting proper intramolecular disulphide bonding and thus preventing the formation of stable F protein oligomers. Both of these amino acid residues lie proximal to cysteine residues that border the cysteine-rich region (amino acids 313 to 438), which contains 11 cysteine residues. This region of the F protein is likely to play a major role in producing and maintaining the appropriate conformation for biological function owing to the participation of cysteine residues in intramolecular disulphide linkages. The prevalence of cysteine residues in the F1 subunit is a feature conserved among all paramyxovirus F proteins described (Morisson, 1988).

We hypothesize that improper folding could explain the differences in transport observed for the cleaved and uncleaved F proteins, as indicated by the lack of recognition of the uncleaved F protein (F313) by a panel of F protein-specific MAbs, the apparent failure of F313 to form stable oligomers, and the prolonged association of F313 with GRP78-BiP in the ER. Although F313 was modified by addition of high mannose sugars, the native protein was folded in a manner which rendered the MAb-specific epitopes unrecognizable. The changes in protein folding may be due to the proximity of the amino acid substitutions (at residues 301 and 447) to the cysteine-rich region and the contribution of cysteine residues to the secondary structure of proteins. It has been shown that disulphide bonds formed on nascent F proteins are disrupted or rearranged as the protein is transported through the cell (McGinnes et al., 1985; Morrison et al., 1987). These results may be a consequence of a transient association of F protein with GRP78-BiP, which is thought to aid in the proper folding of nascent proteins in the ER (Pelham, 1986). Drastic immunoreactivity differences have been demonstrated between the immature and mature forms of the Sendai virus F protein (Mottet et al., 1986), and these differences are a result of glycosylation and intramolecular disulphide bonding, which take place sequentially during maturation (Vidal et al., 1989). Changes in intramolecular disulphide bonding would result in conformational changes during protein maturation, such as those involved in the formation of oligomeric subunits (Arunmugham et al., 1989a; Sechoy et al., 1987), which may be required for the proper transport of F protein from the ER to the Golgi complex. Studies on other viral glycoproteins have demonstrated the importance of protein folding for proper transport from the ER to the Golgi complex (Copeland et al., 1988; Gething et al., 1986; Kreis & Lodish, 1986). A variety of misfolded and/or mutant glycoproteins have been shown to associate with GRP78-BiP in the ER for longer periods than their wild-type counterparts (Gething et al., 1986; Hurtley et al., 1989; Machamer et al., 1990; Ng et al., 1990). However, the association of glycoproteins with GRP78-BiP may also be a requisite step in the normal processing of many viral or cellular glycoproteins. For example, specific and transient interactions of paramyxovirus HN proteins with GRP78-BiP in the ER have been demonstrated (Ng et al., 1989; Roux, 1990). The length and specificity of the interaction of RSV glycoproteins with GRP78-BiP in the ER is being investigated.

We thank Dr L. Andrew Ball of the University of Alabama at Birmingham for helpful discussions, the modified F gene cDNAs and VV transfer vector pAB191, Dr Richard C. Condit of the University of Florida, Gainesville for providing VV mutant tsC17, Dr Michel Trudel of the Institut Armand Frappier, University of Laval, Montreal for providing MAb 7C2, Dr Edward E. Walsh of the University of Rochester, New York for providing MAb L4, and Dr Linda Hendershot of St Jude Children's Research Hospital, Memphis, Tennessee and Dr John Kearney of University of Alabama at Birmingham for providing the anti-BiP MAb. This work was supported by Public Health Service Grants R37 AI21464 and AI20181, and by a grant from the World Health Organization Programme on Vaccine Development (G.W.W.). K.A was supported in part by Public Health Service Grant T32-HL 07553. Support for maintenance of the Wisconsin-GCG computer programs utilized here was provided by the Center for AIDS Research P30 AI27767.

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(Received 22 October 1991; Accepted 7 January 1992)