Post-translational processing and oligomerization of the fusion glycoprotein of human respiratory syncytial virus

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The post-translational maturation of the fusion protein (F) of human respiratory syncytial virus was investigated. Chemical cross-linking experiments indicated that F forms homotetramers and provided evidence that the intermonomer contacts involve primarily the F1 subunit. Homooligomerization as measured by sedimentation in sucrose gradients was insensitive to carbonyl cyanide m-chlorophenylhydrazone, indicating that it occurs in the endoplasmic reticulum. Cleavage of the F0 precursor to yield the F1 and F2 subunits was blocked by monensin or brefeldin A, indicating that it takes place in distal cisternae of the trans Golgi compartment or in the more distal trans Golgi network. The F0 precursor was not detected at the cell surface in surface immunoprecipitation experiments, indicating that cleavage is intracellular. The appearance of the cleaved F1 protein at the cell surface was concurrent with that of the attachment glycoprotein (G); this and other information indicated that the type 2 membrane orientation of G is not obligatorily associated with a reduced transit rate. Examination of F maturation in the presence of tunicamycin provided evidence that its expression at the cell surface depends upon cleavage and not directly upon glycosylation.

Human respiratory syncytial virus (RSV), a member of the genus Pneumovirus of the family Paramyxoviridae (for reviews see Collins, 1991; McIntosh & Chanock, 1990), encodes three transmembrane surface proteins, the fusion protein (F), responsible for virus penetration (Walsh et al., 1985), the heavily glycosylated attachment protein (G), and the small hydrophobic protein (SH) of unknown function.

RSV F is synthesized as a precursor F0 (69K) which is cotranslationally N-glycosylated and post-translationally processed by endoproteolysis into two subunits, F1 (49K) and F2 (20K), which are linked by disulphide bonds (Fernie et al., 1985; Gruber & Levine, 1985a, b; and references therein). The cellular location of the cleavage of RSV F0 has not been clearly identified; one study has shown that the cleavage is blocked by monensin (Fernie et al., 1985), an ionophore reported to block transport between the medial and trans Golgi compartments (Griffiths et al., 1983), but in another study monensin did not inhibit cleavage (Gruber & Levine, 1985a, b). Based on indirect evidence, it also has been reported that the F0 precursor is expressed at the cell surface, consistent with extracellular cleavage (Gruber & Levine, 1985a). F has been shown to be sulphated (Cash et al., 1977) and palmitylated (Arumugham et al., 1989c). (For reviews of post-translational processing and the exocytic pathway see Dunphy & Rothman, 1985; Kornfeld & Kornfeld, 1985; Griffiths & Simons, 1986; Rose & Doms, 1988; Klausner, 1989.)

RSV-infected cells were labelled for 5 min with [35S]methionine followed by chase incubations of between 5 and 60 min (Fig. 1). Duplicate monolayers from each incubation were incubated intact on ice with an anti-RSV serum for surface immunoprecipitation (Fig. 1c), or lysed and analysed by immunoprecipitation with the same antiserum to detect total cell-associated protein (Fig. 1b). Consistent with previous observations (Fernie et al., 1985; Gruber & Levine, 1985b), the cleavage product F1 appeared after approximately 20 min of chase. Some of the F0 precursor was still detectable after 60 min of chase, indicating that processing was somewhat inefficient.

Surface immunoprecipitation showed that the transit times for appearance of F and G at the cell surface were approximately the same, 20 to 30 min (Fig. 1c). It has been suggested previously (Blumberg et al., 1985) that the type 2 membrane orientation is associated with a reduced rate of transit, based on the observed slow processing of the attachment haemagglutinin–neuraminidase (HN) glycoprotein of other paramyxoviruses (Morrison & Ward, 1984; Blumberg et al., 1985). The
Fig. 1. Kinetics of intracellular maturation and cell surface expression of RSV F and G. Replicate cultures of RSV-infected HEp-2 cells were labelled for 5 min with [35S]methionine, washed and incubated in non-radioactive chase medium for 0, 5, 10, 15, 20, 30, 40 or 60 min (lanes 1 to 8) and cell lysates were prepared. Replicate aliquots were analysed by immunoprecipitation with (a) preimmune serum or (b) rabbit antiserum specific to RSV which had been purified by two cycles of banding in sucrose gradients. In addition, a duplicate set of cultures was analysed by (c) surface immunoprecipitation with the RSV-specific serum. A plate of uninfected labelled cells (lane 10) was analysed by surface immunoprecipitation in parallel. As a control for the surface immunoprecipitation to monitor possible association of antibody with intracellular labelled protein during the subsequent lysis and processing, a plate of unlabelled RSV-infected cells prepared in parallel was reacted with anti-RSV antibodies, washed well and lysed (lane 11). The lysate was transferred to an additional plate of labelled RSV-infected cells (a duplicate of the 60 min chase) and the combined lysate was processed to collect antibody–antigen complexes in parallel with the experimental samples above. Any radiolabelled protein immunoprecipitated from this sample would represent antibody exchange or non-specific precipitation, and the actual amount of non-surface-specific background would be twofold greater than apparent because the specific activity of the RSV proteins in the mixed lysate would be half that of the experimental samples. In this particular experiment, a background of non-surface immunoprecipitation was evident. Immunoprecipitated proteins were analysed by SDS–PAGE on 10% gels. The positions are indicated for the precursor F0, subunit F1 (F2 was not retained on these gels), the 48000 (G48) and 50000 (G50) Mr N-glycosylated precursors of G (Wertz et al., 1989; our unpublished data), and mature G.

fact that RSV F and G have opposite orientations (type 1 and type 2, respectively) but similar transit times indicates that this structural feature is not the only determinant of and is not obligatorily associated with a reduced transit rate. Furthermore, the third integral RSV membrane protein, SH, has an extremely rapid transit rate and a type 2 orientation (Olmsted & Collins, 1989; our unpublished data). F0 was not detected at the surface (Fig. 1c), even at times when it was abundant intracellularly (Fig. 1b), showing that cleavage occurs intracellularly.

RSV-infected cells were then labelled for 5 min with [35S]methionine followed by a 1 h chase in the presence of one of three inhibitors of exocytosis, carbonyl cyanide m-chlorophenylhydrazone (CCCP), monensin or brefeldin A (BFA), which block protein processing at progressively later stages: CCCP uncouples oxidative phosphorylation and blocks transport from the endoplasmic reticulum (ER) (Copeland et al., 1988); monensin appears to block transport between the medial and trans Golgi cisternae, as mentioned above; BFA blocks anterograde (outward) transport from the ER whereas retrograde transport continues, resulting in a backflow of Golgi cisternae and their resident enzymes into the ER or an intermediate ER–Golgi compartment (Doms et al., 1988; Lippincott-Schwartz et al., 1990; and references therein). Marker enzymes from the cis, medial and at least part of the trans Golgi compartments have been reported to be returned to the ER, in which they processed ER-retained protein efficiently, whereas enzyme markers are not redistributed from the more distal trans Golgi network (TGN) (Chege & Pfeffer, 1990). Therefore, in operational terms, BFA interrupts post-translational processing at a later stage than does monensin. In the pulse–chase experiments, CCCP was added immediately after the 5 min labelling period and was maintained in the medium thereafter, whereas monensin or BFA were added 45 min prior to the pulse and maintained thereafter. In addition, duplicate plates received tunicamycin, an inhibitor of N-glycosylation, which was added 2 h prior to labelling and maintained thereafter. Following the labelling and chase, cells were analysed intact by surface immunoprecipitation with anti-RSV antiserum, or were lysed and analysed by immunoprecipitation of intracellular proteins with an F-specific antipeptide antiserum.
Surface expression of F was blocked completely by CCCP (not shown), monensin or BFA (Fig. 2a), demonstrating the efficiency of each in inhibiting exocytosis. The cleavage of F₀ into F₁ and F₂ was completely blocked by each inhibitor (Fig. 2a), indicating that cleavage occurs in or after the protein has left the distal cisternae of the trans Golgi compartment. The apparent size of F₀ was slightly larger with CCCP treatment and successively smaller with the monensin and BFA treatments, consistent with successive trimming of the N-linked sugars (Kornfeld & Kornfeld, 1985). The acquisition of resistance to endoglycosidase H was sensitive to CCCP, monensin or BFA, indicating that the enzymes responsible for this were not redistributed by BFA treatment and presumably were located in distal cisternae of the trans Golgi compartment or in the TGN (not shown).

In the presence of tunicamycin, F accumulated as the unglycosylated, uncleaved precursor (Fig. 2b) which, like the glycosylated form, was not transported to the cell surface (Fig. 2a). Although glycosylation was required for efficient proteolytic processing of F₀, some cleavage of the unglycosylated precursor was detected intracellularly (Fig. 2b, species labelled ‘F₁, ungly’), as has also been observed previously (Fernie et al., 1985). Although the amount of the unglycosylated F₁ cleavage product produced during the 1 h chase incubation was small, it was detected readily at the cell surface (Fig. 2a), suggesting that it was transported efficiently following cleavage. The surface expression of unglycosylated F₁ was blocked by monensin (not shown) and BFA (Fig. 2a), confirming that its cleavage and transport followed the exocytic pathway. The identification of this species as unglycosylated F₁ was confirmed by its immunoprecipitation with F-specific monoclonal antibodies (Fig. 2c).

Also, when analysed by SDS-PAGE in the absence of reducing agent, all of the surface-associated unglycosylated F₁ migrated with the Mr of unglycosylated F₀, migrated with the Mr of unglycosylated F₀, suggesting that it was transported efficiently following cleavage and was sensitive to CCCP, monensin or BFA, indicating that the enzymes responsible for this were not redistributed by BFA treatment and presumably were located in distal cisternae of the trans Golgi compartment or in the TGN (not shown).

were immunoprecipitated with anti-RSV serum. The immunoprecipitates were analysed by electrophoresis on 10% gels under (R) reducing or (N) non-reducing conditions. F₁, ungly is the unglycosylated form of the disulphide-linked F₁ and F₂ subunits. The reduced recovery of F₁, ungly under non-reducing conditions probably is due to trapping in disulphide-linked aggregates (Vidal et al., 1989).
It was interesting that $F_0$ in glycosylated or unglycosylated form was not detected at the cell surface even though substantial amounts remained intracellularly long after the labelling pulse. In contrast, $F_1$ in either glycosylated or unglycosylated form was detected readily at the surface. This suggested that cleavage might be a requirement for transport of $F$ to the cell surface in mammalian cells, and $F_0$ might be specifically retained intracellularly. Glycosylation appeared to be involved only in that it facilitates cleavage, presumably by helping to form or stabilize a native, easily cleaved structure of $F_0$. Studies with Sendai virus and Newcastle disease virus have shown that cleavage of $F$ is accompanied by a change in conformation and, in the latter case, a change in charge attributable to elimination of the basic cleavage peptide (Hsu et al., 1981; Kohama et al., 1981). Perhaps these changes are a prerequisite for transport of $F$ from the TGN. We have attempted to compare further the transport of cleaved and uncleaved $F$ using site-directed mutagenesis of cDNA to alter the cleavability and charge of the cleavage site (R. A. Olmsted & P. L. Collins, unpublished data). However, the alterations appeared to interfere with additional earlier steps in processing, complicating the use of these mutants in addressing this question.

To investigate oligomerization of $F$, replicate cultures were labelled for 5 min and chased for 1 h using CCCP, monensin or BFA as described above, and intracellular proteins were analysed by sedimentation in sucrose gradients at pH 5.8 in the presence of Triton X-100 (Fig. 3). Control experiments in which lysis and analysis were at pH 6.5, 7.0 or 7.5 yielded similar results (not shown). When the total protein profile across the fractionated gradients was analysed by SDS–PAGE directly without immunoprecipitation (not shown), most intracellular proteins were present as monomeric species in fractions 9 to 11 (fraction 1 represents the bottom of the gradient and fraction 12 the top). Under these conditions, the influenza A virus H trimer (Copeland et al., 1988) and the human parainfluenza virus type 3 HN tetramer (Collins & Mottet, 1991) migrated with peaks in fraction 3 to 5 (not shown).

The position of $F$ in the gradient was determined by immunoprecipitation of gradient fractions with antipeptide antiserum. The peak of mature $F$ was in fraction 4.

(Copeland et al., 1988; Doms et al., 1989; Collins & Mottet, 1991) in an SW41 rotor at 38000 r.p.m. for 20 h at 40 °C. The gradients were harvested into 12 1 ml fractions which were adjusted to pH 7.5, immunoprecipitated with $F$-specific antipeptide antiserum and analysed by SDS–PAGE on 10% gels. The gradient fractions are numbered, with 1 being the bottom and 12 the top, and the fractions containing the peak of the $F$ oligomer (4) and the peak of non-oligomerized proteins (9 and 10) are shown. The positions of $M$, markers (M) are shown.
was from cells treated with CCCP, monensin or BFA. The observation that oligomerization was unaffected by the addition of CCCP (or any of the other inhibitors) would be consistent with this step occurring in the ER, as has been generally observed for oligomeric proteins (Rose & Doms, 1988). The finding that the cleaved and uncleaved forms cosedimented (as can be seen readily in the mock-treated control which contained both forms) showed that the number of monomers in the F spike was unchanged by cleavage.

The number of monomers in the F oligomer was investigated by reaction with a series of dimethyl diimidate chemical cross-linkers which differed in the number of CH₂ units separating the two functional groups, as described previously (Collins & Mottet, 1991). RSV-infected cells were labelled for 4 h in the presence of [³⁵S]methionine followed by a 1 h chase to allow cleavage of most of the material to occur. The cells were lysed and reacted with the chemical cross-linking agents, the cross-linkers were inactivated, and proteins were collected by immunoprecipitation with antipeptide antiserum and analysed by SDS-PAGE.

Under non-reducing conditions (Fig. 4a), monomeric F₁,₂ (labelled 1X) migrated with an Mr of 63000, which is somewhat less than the value of 69K for reduced F₀ and might reflect intramolecular disulphide bonding. This has been noticed previously, for example, for the Newcastle disease virus F (McGinnes et al., 1985). Also, the non-cross-linked, non-reduced sample included a minor band of 130K (labelled 2X), which would correspond to a dimeric form (predicted Mr, 126000) observed previously following analysis by SDS–PAGE without heat denaturation and reduction (Arumugham et al., 1989b; Walsh et al., 1985). The major cross-linked species included, at the lower cross-linker concentration, primarily the dimeric form as well as a 190K species (labelled 3X) which was of the appropriate size to be a trimer of F₁,₂ (predicted Mr, 189000) and, at the higher cross-linker concentration, a 245K species (labelled 4X) which was of the appropriate size to be a tetramer of the F₁,₂ monomer (predicted Mr, 252000). These oligomeric forms of F were not detectably immunoprecipitated by SH-specific or G-specific antisera (not shown); together with the Mr estimates, this showed that the cross-linked species did not significantly involve G or SH.

When analysed under reducing conditions (Fig. 4b), the non-cross-linked sample contained primarily the 49K F₁ subunit (labelled 1X; the 20K F₂ subunit was not retained on these gels) as well as a small amount of residual uncleaved F₀ precursor which, for unknown reasons, typically resolved into three or more closely spaced bands. The major new species obtained upon cross-linking were of 93K and 136K (labelled 2X and 3X, respectively, in Fig. 4b), which were approximately of the appropriate sizes to be dimers and trimers of the F₁ subunit (predicted Mr, 98000 and 147000, respectively). The 136K reduced trimer (species 3X in Fig. 4b) was similar in electrophoretic mobility to the 130K non-reduced dimer (species 2X in Fig. 4a), but was clearly distinct because, for example, the non-reduced dimer was not abundant in some lanes of material treated with the higher concentration of cross-linker (a, lanes 3 and 4), which was of the appropriate size to represent trimers of the F₁,₂ subunit (predicted Mr, 252000). These oligomeric forms of F were not detectably immunoprecipitated by SH-specific or G-specific antisera (not shown); together with the Mr estimates, this showed that the cross-linked species did not significantly involve G or SH.

It has been shown previously that F can be isolated as a homodimer [2X (F₁,₂)] when analysed by SDS-PAGE without heating or reduction, or following chemical cross-linking (Walsh et al., 1985; Arumugham et al., 1989a). The tetramers [4X (F₁,₂)] described in the present paper presumably represent a less stable association between two such SDS-stable dimers. Arumugham et al. (1989b) have shown that a cross-linked form of the SDS-stable dimer was converted into an F₁ dimer by reduction. Here, that observation was confirmed and
extended to show that the cross-linked tetramer was reduced in size by reduction to yield species of the appropriate size to be dimers and trimers (and possibly tetratomers) of the $F_1$ subunit. Thus, the $F$ spike appears to involve the association of two homodimers into a homotetramer, and the intermonomer associations both within and between dimers appear to involve the $F_1$ subunit. The $F_2$ subunits might form the core of the tetramer, and in such a configuration the hydrophobic N termini of the $F_1$ subunits might associate to form a fusogenic tetrapeptide. The $F_2$ subunits might be oriented externally around this core. In this context, it is interesting to note that the $F_2$ subunit is the most divergent region of the mature protein with regard to amino acid sequence (Johnson & Collins, 1988), and also contains most of the carbohydrate side chains [one of which is variably present among different strains (Collins, 1991)]. Sequence diversity and glycosylation might be mechanisms by which some antigenic diversity in this important neutralization antigen is produced.

Sendai virus $F$ also appears to be a homotetramer which has been suggested to be organized around the $F_1$ subunit (Sechoy et al., 1987). RSV and Sendai virus Fs have been shown previously to have a low but significant level of amino acid sequence relatedness and to be similar with regard to general linear structural features such as size and approximate locations of the cleavage site, hydrophobic domains and cysteine residues (Spriggs et al., 1986). Here, this similarity has been extended by the finding of evidence of shared elements of quaternary structure. It has been noted that the Fs of nine other paramyxoviruses including Sendai virus have a leucine zipper motif that is located between four and eleven amino acids N-terminal of the transmembrane anchor region and involves four leucine (occasionally isoleucine) residues located at intervals of seven positions (Buckland & Wild, 1989). It has been suggested that these participate in oligomerization. RSV $F$ has a similar motif that begins 12 residues from the anchor; this motif is unusual in having isoleucine at three of the four positions but nonetheless might be a functional counterpart.

Arumugham et al. (1989a) detected small quantities of disulphide-linked $F$ and $G$ in infected cells, raising the possibility that these two proteins form heterooligomers. Here, the two proteins were found to appear at the cell surface concurrently, but coprecipitation or chemical cross-linking of $F$ and $G$ were not detected. The lack of detection of $G$ could reflect its poor labelling with methionine, and therefore these observations do not completely rule out the possibility of coassembly of these two proteins.

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