Multiple glycosylated forms of the respiratory syncytial virus fusion protein are expressed in virus-infected cells

Helen W. McL. Rixon, Craig Brown, Gaie Brown and Richard J. Sugrue

MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, UK

Analysis of the respiratory syncytial virus (RSV) fusion (F) protein in RSV-infected Vero cells showed the presence of a single F1 subunit and at least two different forms of the F2 subunit, designated F2a (21 kDa) and F2b (16 kDa), which were collectively referred to as [F2]a/b. Enzymatic deglycosylation of [F2]a/b produced a single 10 kDa product suggesting that [F2]a/b arises from differences in the glycosylation pattern of F2a and F2b. The detection of [F2]a/b was dependent upon the post-translational cleavage of the F protein by furin, since its appearance was prevented in RSV-infected Vero cells treated with the furin inhibitor dec-RVKR-cmk. Analysis by protein cross-linking revealed that the F1 subunit interacted with [F2]a/b via disulphide bonding, to produce equivalent F protein trimers, which were expressed on the surface of infected cells. Collectively, these data show that multiple F protein species are expressed in RSV-infected cells.

The human respiratory syncytial virus (RSV) fusion (F) protein is initially synthesized as an inactive precursor (F0), which undergoes proteolytic activation in the trans-Golgi compartment (Collins & Mottet, 1991; Anderson et al., 1992; Bolt et al., 2000; Sugrue et al., 2001) resulting in disulphide-linked F1 and F2 subunits (F2s–sF1). We have previously examined processing of the RSV (A2 strain) F protein in Vero C1008 cells (Sugrue et al., 2001) where we noted that the F2 domain was difficult to detect, compared with the F1 subunit, following metabolic labelling of the F protein with [35S]methionine. Thus, to overcome this problem, RSV-infected cells were labelled with [3H]glucosamine and the F protein isolated by radioimmunoprecipitation (RIP) using mAb19 (Taylor et al., 1992) and analysed by SDS–PAGE (Fig. 1A). Although mAb19 recognizes the F1 subunit (Taylor et al., 1992), the F2 subunit is co-precipitated since both subunits are covalently linked in F2s–sF1 by a disulphide bridge (Scheid & Choppin, 1977; Gruber & Levine, 1985). Only F2s–sF1 was detected and specific labelled protein bands corresponding to the F1 and F2 proteins were visualized. The F1 subunit migrated as a single 50 kDa species, while at least three different F2 protein species could be identified whose sizes were estimated as 22, 16 and 10 kDa, and which were designated F2a, F2b and F2c respectively. The intensity of these bands, as visualized by autoradiography, decreased in the order F2a F2b F2c. It is interesting to note the similarities in size of F2a and F2b in RSV-infected cells with the mono- and di-glycosylated mutant forms of the F2 subunit transiently expressed in mammalian cells (Zimmer et al., 2001). The origin of the F2c species is less certain since it is a minor species that is only readily seen following longer labelling pulses with [3H]glucosamine (6–8 h pulse). Whereas we detected F2a and F2b in several other different cell lines (CV-1, HELa and HEp2), F2c was only detected in Vero cells. Its size is similar to that of the deglycosylated F2, and it may represent an F2 species that is generated by specific proteolytic activity within Vero cells. In this report F2a and F2b will be collectively referred to as [F2]a/b. The F protein present in RSV-infected cells was analysed by Western blotting using mAb 19 (Fig. 1B), which showed that the F1 subunit migrated as a single 50 kDa protein in SDS–PAGE. No smaller F1 protein-related products were detected thus showing that the 22–10 kDa proteins detected in our RIP assays are not degradation products of the F1 subunit. We also noted that similar levels of the [3H]glucosamine-labelled F1 and [F2]a/b protein bands were detected in RSV-infected cells, either in the absence or presence of 6 µg/ml actinomycin D, an inhibitor of host cell RNA polymerase activity (unpublished data). No labelled proteins were detected in mock-infected cells, either in the presence or absence of the drug, thus confirming the viral nature of these proteins.

The [3H]glucosamine-labelled F protein was examined in RSV-infected Vero cells treated with the furin inhibitor dec-RVKR-cmk (Fig. 1C). Addition of dec-RVKR-cmk resulted in the formation of the endo H-resistant form of the non-cleaved F protein (F0EH1), described previously (Sugrue et al., 2001). This coincided with a substantial reduction in the levels of detectable F1 and [F2]a/b. In addition, we detected the F protein cleavage intermediate (gp60), described previously (Sugrue et al., 2002, Journal of General Virology, 83, 1290–1297).
shown) were detected by autoradiography. Apparent molecular masses were isolated by RIP and analysed using 15% SDS–PAGE as described previously (Pagler, 1995; Sugrue et al., 2001). Briefly, cell monolayers were treated with RSV at a multiplicity of 2 and following adsorption at 33 °C for 2 h, the cells were incubated at 33 °C for a further 18 h. At 20 h post-infection, the medium was removed and the cells incubated in DMEM minus glucose for 1 h prior to radiolabelling after which they were pulse-labelled with 100 μCi/ml d-[^3H]glucosamine hydrochloride for 6 h. The F protein was isolated by RIP and analysed using 15% SDS–PAGE as described previously (Sugrue et al., 2001). The labelled protein bands (as shown) were detected by autoradiography. Apparent molecular masses were estimated using Rainbow high- (14–3–220 kDa) and low- (2.5–46 kDa) range protein markers (Amersham). (B) Western blot analysis of the F protein from cell lysates using mAb19. Mock-infected (M) or RSV-infected (I) monolayers were extracted with lysis buffer (1% NP-40, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 2 mM PMSF, 20 mM Tris–HCl, pH 7.5) at 4 °C for 10 min. The clarified lysate was resuspended in boiling mix, separated by SDS–PAGE and transferred onto a PVDF membrane. After transfer, the membrane was washed with PBSA and blocked for 18 h at 4 °C in PBSA containing 1% MarveI and 0.05% Tween 20. It was then washed twice in PBSA prior to incubation with mAb19 for 60 min. The membrane was washed four times in PBSA containing 0.05% Tween 20 and probed using goat anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma). The protein bands were visualized using the ECL protein detection system (Amersham). (C) The detection of [F2]ab is dependent upon F protein cleavage. RSV-infected Vero cells were either mock-treated (−) or treated (+) with 80 μM dec-RVKR-cmk 1 h before pulse-labelling with [3H]glucosamine for 4 h. The F protein was isolated by RIP and analysed using 15% SDS–PAGE. * Indicates the position of gp60. The positions of F0EHr and the F1, [F2]a/b are indicated. (D) Cell surface biotinylation of the F protein. The positions of mock-infected (M) and RSV-infected (I) Vero cells were biotin-labelled as described previously (Altin & Pagler, 1995). The results of our analysis clearly showed that both the F1 and [F2]a/b subunits were efficiently labelled with biotin (Fig. 1D). This suggests that the different F protein species are expressed on the surface of RSV-infected cells.

N-linked glycosylation is initiated in the endoplasmic reticulum (ER) where a mannose core structure is initially attached to the protein. This is subsequently trimmed and complex carbohydrates (e.g. fucose, glucosamine and galactosamine) are then added to the trimmed mannose core, yielding the final carbohydrate structure. RSV-infected Vero cells were labelled with either d-[^3H]glucosamine, d-[2-[^3H]mannose or L-[6-[^3H]fucose, and the F protein analysed by RIP and SDS–PAGE (Fig. 2A). These [3H]sugars label specific residues within the carbohydrate moiety that is attached to the F protein: [3H]mannose labels both mannose and fucose residues, [3H]glucosamine labels the sugars glucosamine and galactosamine, while only fucose residues are labelled using [3H]fucose (Cummings, 1993). In each case, the different F protein subunits were readily detected following radiolabelling with these sugars (Fig. 2A). The relative intensity of each labelled subunit may be determined both by the number and structure of individual oligosaccharide moieties which are present. However, the ability to directly label the F2 protein subunits with terminal sugars shows that they are modified by complex carbohydrates and are mature forms of the F2 subunit.

The glycosylation status of the F protein subunits was investigated using endoglycosidase H (endo H) and PNGase F (Fig. 2B, C). PNGase F is able to remove the entire carbohydrate moiety from proteins modified by N-linked glycosylation while endo H specifically removes hybrid and high-mannose sugar chains. In this analysis, both the [35S]methionine- and [3H]glucosamine-labelled F protein were assayed for their sensitivity to deglycosylation by these enzymes. Analysis of the [35S]methionine-labelled F protein subunits by SDS–PAGE (Fig. 2B) showed, as expected, that the electrophoretic mobility of F1 and [F2]a/b remained unchanged following endo H treatment. The electrophoretic mobility of the F1 and F2 subunits increased following PNGase F treatment, yielding streptavidin–HRP (1/1000 dilution, Amersham). The membrane was washed with PBSA and the protein bands were visualized using the ECL protein detection system (Amersham). The biotinylated F protein species are indicated.
single protein bands of 48 and 10 kDa respectively, which is consistent with their deglycosylation. This confirms that [F2]_ab is modified by complex N-linked carbohydrate chains. In a similar analysis, endo H treatment of [3H]glucosamine-labelled F protein had no effect either on the intensity or the electrophoretic migration of the F1 and [F2]_ab protein bands (Fig. 2C). However, treatment with PNGase F resulted in the efficient removal of [3H]glucosamine label from each of the F protein subunits. In addition, examination of the F protein in RSV-infected cells treated with tunicamycin, a nucleoside antibiotic that inhibits N-linked glycosylation, showed the presence of 48 and 10 kDa proteins, the expected sizes for the deglycosylated F1 and F2 subunits (data not shown). The formation of a single 10 kDa F protein following enzymatic deglycosylation using PNGase F (or by tunicamycin), suggests that the different forms of the F2 protein may arise from differences in N-linked glycosylation.

The [3H]glucosamine-labelled F protein was analysed by non-reducing SDS–PAGE (Fig. 3A), under which conditions the F protein migrated with a molecular mass of approximately 70 kDa, the expected size for the non-reduced monomeric form of F2–sF1. A single 70 kDa F protein species was detected following short labelling periods (e.g. 1 h) whereas the non-reduced F2–sF1 appeared as a closely spaced doublet band following longer labelling. The absence of the protein bands corresponding to F1 and [F2]_ab in non-reducing SDS–PAGE clearly shows they are disulphide bonded within F2–sF1. This doublet arises from the disulphide bonding of the F1 subunit with [F2]_ab, the latter accumulating during longer pulse-labelling times (data not shown). It is interesting to note that the size difference of the proteins within the doublet is similar to the differences in the size between F2a and F2b. A similar F protein 70 kDa doublet band was also observed under non-reducing conditions by Western blotting using mAb19 (Fig. 3B). In addition, in both assays, a larger F protein species (approx 140 kDa) was detected, which is similar in size to the F protein dimer that has been reported (Arumugham et al., 1989; Collins & Mottet, 1991; Anderson et al., 1992). This suggests that the non-reduced F protein has an increased resistance to heat denaturation during sample preparation compared with the reduced form of the F protein.

The ability of F1 and [F2]_ab to form equivalent oligomeric structures was examined using bi-functional protein cross-linking reagents, namely diithiobis(succinimidyl propionate) (DSP) and disuccinimidyl suberate (DSS). These reagents contain two reactive groups that are separated by a spacer arm spanning 11–12 Å (Wong, 1991). In this way, components of protein complexes, formed through non-covalent interactions (e.g. hydrogen bonding), can be stabilized during extraction and subsequent processing by SDS–PAGE. We anticipated that the potential size of cross-linked F protein multimers would be beyond the resolution of the Tris–glycine electrophoresis system and instead used 3–8 % Tris–acetate SDS–PAGE, which allows accurate resolution of protein species in the range 40–400 kDa (Hames, 1990). Separation of the non-reduced F protein by 3–8 % Tris–acetate polyacrylamide gels, followed by Western blotting, showed that the 70 kDa doublet band that was detected by Tris–glycine SDS–PAGE was resolved into two clearly distinguishable protein species, whose sizes were approximately 42 and 55 kDa (Fig. 3C). The observed difference in electrophoretic migration of F2–sF1 between the Tris–acetate and Tris–glycine systems presum-
H. W. McL. Rixon and others

Fig. 3. [F2]a/b interacts with the F1 subunit in the mature F protein. (A) RSV-infected Vero cells were labelled for either 1 or 4 h with [3H]glucosamine and the F protein was isolated by RIP and analysed by 15% non-reducing SDS–PAGE. (B) Western blot analysis of non-reduced F protein using mAb19 was performed as described in Fig. 1. The mature monomeric (F2s–sF1) and dimeric (F2s–sF1)2 F protein species are indicated. (C) Western blot analysis of F protein in RSV-infected Vero cells by non-reducing NuPAGE 3–8% Tris–acetate SDS–PAGE gel electrophoresis (Invitrogen) using mAb19. (D) Analysis of in situ cross-linked F protein oligomers. RSV-infected Vero cells were pulse–chase labelled using [35S]methionine as described in Fig. 2. Increasing concentrations of DSP and DSS, prepared in PBSA, pH 8.0, were added to the infected monolayers, which were incubated at 4 °C for 1 h. The cross-linking reactions were quenched with PBSA+20 mM glycine and lysates prepared using lysis buffer +20 mM glycine. The F protein complexes were isolated by RIP and analysed by 3–8% non-reducing Tris–acetate SDS–PAGE. The [35S]methionine-labelled protein bands were detected by phosphorimaging as described in Fig. 2. The cross-linker concentrations are shown, nt, Non-treated. The F protein complexes are indicated together with their molecular masses. We have assigned the monomeric F2s–sF1 a molecular mass of 50 kDa which is the average mass of the two F protein species present in the doublet band. The 420 kDa position was estimated by extrapolation of the calibration curve and is within the fractionation range of this electrophoresis system.

ably is a consequence of the different buffer systems used that influence binding of SDS to the F protein (Hames, 1990).

RSV-infected cell monolayers were pulse–chase labelled using [35S]methionine and treated with DSP and DSS which allowed the F protein to be cross-linked in situ. The F protein was isolated using RIP and analysed by 3–8% Tris–acetate SDS–PAGE under non-reducing conditions (Fig. 3D). In the absence of cross-linker, the monomeric form of F2s–sF1 was clearly visible, and an additional very faint 100 kDa doublet protein band (dimeric F protein) was also observed. The addition of cross-linking reagents to the monolayers resulted in the formation of two doublet bands, whose sizes were approximately 100 and 150 kDa, consistent with an F protein dimer and trimer respectively.

Our findings suggest that in RSV-infected cells the mature F protein, like several other viral fusion proteins, exists as a homotrimeric complex. This is in agreement both with the homotrimeric F protein complexes identified in other paramyxoviruses (Russell et al., 1994) and structural information obtained from recombinant F protein and model peptides (Calder et al., 2000; Matthews et al., 2000). The formation of these cross-linked F protein oligomers coincided with a reduction in the amount of immunoprecipitated monomeric F protein. Similar results were obtained using a variety of other protein cross-linkers (data not shown). Analysis of the cross-linked F protein oligomers by Western blotting failed to detect the presence of the G protein (unpublished observations), indicating that the G protein is not present in the cross-linked
F protein complexes, confirming previous observations that have failed to detect stable interactions between the F and G glycoproteins (Collins & Mottet, 1991). These data allow us to conclude that the different glycosylated F protein species are able to form equivalent homotrimeric complexes, a prerequisite for transfer through the secretory pathway (Gething et al., 1986; Kreis & Lodish, 1986), which are subsequently transported to the surface of RSV-infected cells. These conclusions were supported by density-gradient centrifugation analysis which showed co-migration of F1 and [F2]a/b (data not shown).

We have identified multiple forms of F2s–sF1, which differ in their pattern of F2 glycosylation. Differential processing of the RSV F protein is not unique within the paramyxoviruses and similar observations have been reported in measles virus fusion protein (Alkhatib et al., 1990; Bolt & Pedersen, 1998). The biological consequences of these different F protein species are unclear, but they may result in subtle differences that could affect some aspects of F protein function, e.g. interaction of the F protein with heparin sulphate, the putative virus receptor (Karron et al., 1997; Karger et al., 2001) or by influencing fusion activity (Alkhatib et al., 1994; Zimmer et al., 2001). The importance of glycosylation in receptor binding for other viral fusion proteins has been documented (Fenouillet et al., 1989, 1990).

We thank Duncan McGeoch for critical review of the manuscript. We are grateful to Geraldine Taylor at the Institute of Animal Health, Compton, UK for providing mAb19. C.B. was funded by a Wellcome Trust Vacation Scholarship.

References


Received 18 June 2001; Accepted 24 September 2001