Respiratory Syncytial Virus Glycoprotein Expression in Human and Simian Cell Lines

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SUMMARY

Glycoproteins synthesized in both human (HeLa and HEp-2) and simian (Vero and BS-C-1) cell lines following infection with two different strains of respiratory syncytial virus (A2 and Long) were analysed by SDS–PAGE following immunoprecipitation with monoclonal antibodies. Minor virus strain-dependent differences in the large glycoprotein, G, and the fusion protein polypeptides F1 and F2 were observed together with minor cell line-dependent differences in the size of the F2 polypeptide. Major quantities of two glycoproteins, termed Ga (50K) and Gb (45K), were detected in A2 strain-, and to a lesser extent in Long strain-, infected simian cells. These proteins were also present in infected human cells, but in much reduced amounts. Immunoprecipitation with anti-G monoclonal antibodies demonstrated that Ga and Gb shared different epitopes with G.

Two glycoproteins have been resolved by SDS–PAGE of respiratory syncytial (RS) virions (Lambert & Pons, 1983). These are the large glycoprotein (G, approx. 90000 mol. wt., 90K), which may be responsible for viral attachment to cells (Walsh et al., 1984a; Gruber & Levine, 1985a), and the putative RS virus fusion protein (F), the two disulphide-linked portions of which are resolved as F1 (approx. 50K) and F2 (approx. 20K) under reducing conditions (Fernie & Gerin, 1982). A 45K precursor of the G glycoprotein and an uncleaved 70K precursor of the F glycoprotein have also been identified in infected cell lysates (Gruber & Levine, 1985b; Fernie et al., 1985).

The virion glycoproteins are immunogenic in animals (Walsh et al., 1984b, 1985) and antibodies to both have been demonstrated in naturally infected human infants (Burns et al., 1984; Ward et al., 1983; Vainionpaa et al., 1985). Studies in mice (Taylor et al., 1983) and cotton rats (Walsh et al., 1984b) indicate that monoclonal antibodies (MAbs) may be protective. Consequently, a purified glycoprotein preparation may offer advantages over conventional killed or live virus vaccines which have so far proved unsuccessful in the prevention of RS virus bronchiolitis. In seeking a source of purified glycoproteins for pilot studies we have investigated the production of glycoproteins by two laboratory strains of virus (Long and A2) in a variety of cell lines. We report minor cell line- and virus strain-dependent differences in the mobility of the two glycoproteins in polyacrylamide gels and a major difference in the production of G protein-related low molecular weight molecules between the simian (Vero, BS-C-1) and human (HeLa, HEp-2) cell lines tested.

SDS–PAGE under reducing conditions (Fig. 1) demonstrated that the major glycoprotein labelled with [3H]glucosamine in both Long and A2 strain-infected HeLa and Vero cell lysates was G. In both cases Long strain G (86K and 85K in HeLa and Vero cells respectively) was marginally larger than that of the A2 strain (84K in both cell lines). In A2-infected and to a lesser
extent in Long-infected Vero cells a broad band of similar intensity to G, which we initially thought was F₁, was observed in the 50K region of the gel. A less intense but more discrete band was found at a similar position in samples from infected HeLa cells. A third set of bands representing the F₂ fraction of F was resolved in the 20K region of the gel. As with G, minor variations in size with both virus strain and cell line were detected. Long strain F₂ was smaller than that of the A2 strain in both cell lines, and F₂ molecules synthesized in Vero cells were smaller than those in HeLa cells for both strains of virus. In contrast to the 50K band, all the F₂ bands were of similar intensity.

Lysates of HeLa and Vero cells infected with the Long or A2 strains of RS virus were immunoprecipitated with an excess of the anti-F MAb 1A12 (Routledge et al., 1985) (Fig. 2) and analysed by SDS-PAGE under reducing conditions. A slight virus strain-dependent difference in the size of the F₁ molecule was observed, the Long strain F₁ (50K) being smaller than that of the A2 strain (52K). Several high mol. wt. bands were also resolved in the anti-F immunoprecipitates, mainly in those from the heavily labelled HeLa cells. One of these migrated to the same position as the G glycoprotein, which has frequently been observed to co-precipitate in small quantities with F. The identity of the others is unknown.

In the SDS–PAGE of infected cell lysates described earlier (Fig. 1), the intensity of the 50K band, initially identified as F₁, implied that the proportion of F₁ to F₂ synthesized was greater in Vero cells than in HeLa. However, in the anti-F MAb immunoprecipitates (Fig. 2), HeLa cells rather than Vero cells yielded the greater quantity of F₁ and for both cell types the ratio of F₁ to F₂ was approximately equal. In non-reducing SDS-PAGE (see Fig. 4) the F₁ and F₂ in the immunoprecipitates migrated as the F₁₂ disulphide-linked 70K molecule. In Vero cell lysates run under similar conditions the 50K band did not shift position. These observations are inconsistent with the Vero cell lysate 50K band being F₁.

The virus strain- and cell-line-dependent differences in glycoprotein expression described above for HeLa and Vero cells were paralleled in HEp-2 and BS-C-1 cells respectively (data not shown).

Two anti-G MAbs, 4G4 and 3F4 (Routledge et al., 1985), which recognized distinct epitopes on the G protein as determined by additive binding in an ELISA system (Fig. 3), were used to precipitate HeLa, HEp-2, Vero and BS-C-1 cell lysates infected with either the Long or A2 strain of RS virus and labelled with [³H]glucosamine (Fig. 4). As a control, lysates were also precipitated with the anti-F MAb 1A12. From the Vero/A2 lysate, MAb 4G4 precipitated G at 84K and a second broad band of with an average mol. wt. of 50K. From the same lysate MAb 3F4 precipitated G together with a broad band with an average mol. wt. of 45K. The difference in size of the low mol. wt. bands precipitated by 4G4 and 3F4 suggested that they were different molecules. The 4G4 50K band was therefore termed Ga, and the 3F4 45K band Gb. The absence of these bands from the anti-F immunoprecipitate precluded their non-specific precipitation by murine monoclonal antibody.

In both anti-G MAb precipitates of infected HeLa cells the major band resolved was G. Low mol. wt. bands were also seen, but compared to the quantities yielded by Vero cells the amounts were minor. The size difference between Ga and Gb of Vero cells was also apparent in HeLa cells.

The results with HEp-2 and BS-C-1 cells again paralleled those of HeLa and Vero cells respectively. For all four cell lines the Long strain Gb (47K) was slightly larger than the A2 strain Gb (45K) molecule. No obvious variation in the size of Ga was detected, therefore in each infected cell line Ga was larger than Gb.

To confirm the specificity of Ga precipitation by the anti-G 4G4 MAb a Vero/A2 lysate was first exhaustively absorbed with the anti-G 3F4 MAb to remove G and Gb (Fig. 5). In the control precipitation of unabsorbed lysate with 4G4, G and Ga were precipitated as before. 4G4 precipitated only a trace quantity of G from the absorbed lysate, but still a considerable amount of Ga. Anti-G 3F4 MAb precipitation of the unabsorbed and absorbed lysate demonstrated the efficient removal of G and Gb (data not shown). Thus, Ga is not recognized by MAb 3F4, supporting the supposition that Ga and Gb are different G-related glycoproteins. Furthermore, precipitation of Ga by 4G4 from the absorbed lysate indicates that Ga is specifically co-
Fig. 1. SDS-PAGE of RS virus-infected cell lysates. Confluent monolayers of (a) HeLa or (b) Vero cells in Costar cluster trays were cultured, mock-infected (lanes 1) or infected with RS virus Long (lanes 2) or A2 (lanes 3) strain at an m.o.i. of 5. Twenty-four post-infection when 50% of cells were showing c.p.e. the monolayers were labelled for 5 h with 55 µCi/ml [3H]glucosamine hydrochloride (Amersham) in the presence of high salt (255 mM NaCl). Monolayers were then solubilized in 250 µl volumes of reducing sample buffer, boiled and electrophoresed on 10% polyacrylamide gels (Laemmli, 1970) and fluorographed as described (Routledge et al., 1985). Non-radioactive phosphorylase a, bovine serum albumin, alcohol dehydrogenase and chymotrypsinogen a (mol. wt. 91K, 67.5K, 41K and 25.1K respectively) were used as mol. wt. markers (M). Their positions on Coomassie Brilliant Blue-stained fluorographed gels was marked with radioactive ink. When running 12.5% polyacrylamide gels to determine the size of the F2 molecules (not shown) cytochrome c (12-4K) was included as fifth marker. Fluorographs have been cut to remove lanes not referred to in the text. Strips adjacent to each other have been run on the same gel and are of the same exposure except in Fig. 6 where multiple exposures were used. DF, dye front.

Fig. 2. SDS-PAGE of RS virus anti-F protein immunoprecipitates. Monolayers of (a) HeLa or (b) Vero cells mock-infected (lanes 3) or infected with Long (lanes 1) or A2 (lanes 2) strain RS virus were labelled for 5 with 100 µCi/ml [3H]glucosamine hydrochloride in isotonic maintenance medium as described in Fig. 1. Monolayers were solubilized in 0.5 ml volumes of RIPA buffer pH 9.5 (0.15 M NaCl, 0.1% w/v SDS, 1% v/v Triton X-100, 1% w/v sodium deoxycholate, 0.01 M Tris) and stored at -70 °C. When required, lysates were rapidly thawed, homogenized, incubated at 37 °C for 30 min, clarified at 10000 g for 45 min at room temperature and then incubated at 37 °C for a further 2 h to complete solubilization. Four-hundred µl volumes of lysate were then immunoprecipitated with anti-F MAb 1A12 coupled to Sepharose beads; details of these procedures have been previously described (Routledge et al., 1985). The immunoprecipitates were boiled in 200 µl volumes of reducing sample buffer and electrophoresed on 10% polyacrylamide gels. M, mol. wt. markers.

precipitated with the G protein, a point confirmed by immunoperoxidase staining of Western blots in which 4G4 detected both G and Ga (data not shown).

To investigate the possibility of precursor–product relationships among Ga, Gb and G, Vero/A2 lysates pulse- and pulse-chase-labelled with [3H]glucosamine were immunoprecipi-
Fig. 3. Additive binding ELISA. Anti-G MAb 4G4 (○) and 3F4 (△) were diluted 1/100 in phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 pH 7.4 and 10% (v/v) heat-inactivated foetal calf serum. The combination 4G4 + 3F4 (○–△) was prepared by mixing equal volumes of the 1/100 dilutions. The three preparations were spot tested 15 times in the wells of a microelisa plate, previously coated with HeLa cell-grown RS virus A2 strain capture antigen diluted in sodium carbonate-bicarbonate buffer pH 9.6. The antigen was used at a dilution sufficient to create conditions of antibody excess. Bound MAb was detected using rabbit anti-mouse IgG peroxidase conjugate (Miles) and the substrate o-phenylenediamine. The enzymic reaction was stopped with 3 M-sulphuric acid and the absorbance of each well's contents was read at 492 nm. Incubation periods for the capture antigen, MAb, conjugate and substrate steps were 90, 90, 60 and 30 min respectively at 37 °C and the plates were rinsed three times with PBS-Tween 20 in between each stage. Points represent mean spot test values corrected for non-specific binding to uninfected HeLa cell control antigen and the bars indicate the standard error of each mean. The data were analysed by Student's t-test.

Fig. 4. SDS-PAGE of RS virus anti-G protein immunoprecipitates. [3H]Glucosamine hydrochloride-labelled RIPA lysates of uninfected (lanes 2) and A2 strain RS virus-infected (lanes 1) HeLa (a) and Vero (b) cells were prepared and immunoprecipitated with two anti-G (4G4 and 3F4) and one anti-F (1A12) MAbs as described in the legend to Fig. 2. The immunoprecipitates were boiled in 200 µl of non-reducing sample buffer and, along with 1 in 5 dilutions of the cell lysates (LY), were electrophoresed on 10 % polyacrylamide gels. The molecular weights assigned to Ga and Gb were determined on reducing gels (not shown) due to the aberrant migration of the mol. wt. standards in non-reducing conditions.

tated with MAbs 4G4 and 3F4 (Fig. 6). No progression of radiolabel from Ga and Gb to G or vice versa was observed.

Minor changes in mobility of glycoproteins are commonly found between viruses replicating in different cell types and have been reported previously for RS virus (Lambert & Pons, 1983). These differences presumably relate to variations in the level or nature of glycosylation. Minor changes between virus strains are perhaps to be expected in view of the recent discovery of two to three subtypes of RS virus as defined by monoclonal antibodies specific for epitopes on both internal virion proteins and membrane glycoproteins (Mufson et al., 1985; Anderson et al., 1985). Such differences are likely to result from variation in the amino acid sequence of the polypeptide consequent upon genetic mutation. Anderson et al. (1985) have been able to differentiate the two strains of virus studied here by means of reaction to MAbs specific for the G protein.

The results described above indicate that in addition to the three glycoproteins (F₁, F₂ and G) usually resolved in lysates of RS virus cells, two others, here termed Ga and Gb, may also be
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Fig. 5. Immunoprecipitation of anti-G 3F4-absorbed Vero cell lysate with anti-G 4G4 monoclonal antibody. [3H]Glucosamine hydrochloride-labelled uninfected (lanes 2) and A2 strain (lanes 1) RS virus-infected Vero cell RIPA lysates were prepared as described in the legend to Fig. 2. The lysates were split into two aliquots, one of which was absorbed extensively with anti-G 3F4 MAb. Absorbed (b) and unabsorbed (a) lysates were then immunoprecipitated with 4G4 and the immunoprecipitates were electrophoresed on 10% polyacrylamide gels under non-reducing conditions.

Fig. 6. Immunoprecipitation of [3H]glucosamine pulse- and pulse-chase-labelled Vero cell lysates. At 18 h post-infection uninfected (lanes 2) or virus A2 strain-infected (lanes 1) Vero cell monolayers were pulse-labelled for 3 h with [3H]glucosamine hydrochloride as described in Fig. 2. Monolayers were then solubilized immediately in RIPA pH 9.5 (a) or, after rinsing three times in Hanks' balanced salt solution, incubated for a further 20 h in normal maintenance medium and then solubilized (b). Lysates were processed and immunoprecipitated with anti-G MAbs 4G4 and 3F4 as described in the legend to Fig. 2. Immunoprecipitates and 1 in 5 lysate dilutions (LY) were electrophoresed on 10% polyacrylamide gels under non-reducing conditions. Gels (a) and (b) were exposed for 20 and 60 days respectively. The longer exposure of (b) was necessary due to the loss of material from chased cell monolayers caused by advanced c.p.e.

detected in both human and monkey cells. This confirms the earlier reports of Wunner & Pringle (1976) who reported two glycoproteins in RS virus-infected BS-C-1 cells of molecular weight 48K and 42K. It is likely that at least one of these bands corresponded to Ga and/or Gb.

The immunoprecipitation data suggest that these two polypeptides are related to G, but the exact nature of the relationship is open to speculation. It is possible that the Ga and Gb molecules, seen most obviously in Vero cells, are generated by proteolysis of G after cell lysis, and are not components of intact infected cells. However, in these experiments cells were lysed in RIPA buffer at pH 9.5. This buffer completely inhibits the post-lysis breakdown of RS virus nucleoprotein from 42K to 40K which we have observed in lower pH buffer. In addition Ga and Gb were resolved from Vero cells solubilized in boiling reduced PAGE sample buffer, suggesting that these two molecules are pre-lysis components of infected cells (data not shown).

The protein backbone of G is 36K in size (Wertz et al., 1985) and a glycosylated precursor molecule of 45K has been identified in HeLa cells which was chased into G over a 2 h period (Gruber & Levine, 1985b; Fernie et al., 1985). This cannot account for Ga or Gb, because in the pulse-chase experiments described here they were still present after a 20 h chase period.
No evidence of intracellular G breakdown to yield Ga and Gb was observed; however, as effective glucosamine labelling necessitated a 3 h pulse period, a rapid post-synthesis breakdown of a proportion of G molecules cannot be ruled out. Perhaps G, like the N protein of RS virus (Cash et al., 1979), undergoes a transition from a protease-sensitive to a protease-resistant form. Certainly the G of RS virus A2 strain possesses at least two protease-sensitive sites, cleavage at which results in the generation of two differently sized fragments (Spring & Tolpin, 1983). Cleavage does not normally occur in HeLa cells, but a suitable enzyme, absent from HeLa cells, may be present in Vero cells. The possibility that Ga and Gb are cellular stress proteins coincidentally recognized by MAb 4G4 and 3F4 cannot be excluded; however, this is unlikely as polypeptides of equivalent molecular weight were not precipitated from *Herpesvirus hominis* type 1-infected Vero cells (data not shown). Partial transcription or translation of the G protein gene as a route to Ga and Gb formation has yet to be investigated. Whether these proteins are important in G protein biosynthesis or play any role in the biology of RS virus infection remains to be determined.

**REFERENCES**


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