Appearance of a Soluble Form of the G Protein of Respiratory Syncytial Virus in Fluids of Infected Cells

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SUMMARY

We examined the antigenic reactivities and virion associations of glycoproteins that were released into the culture fluids of cells infected with respiratory syncytial (RS) virus. Culture fluids and cell extracts were obtained from cells 24 to 30 h after they were infected with the Long strain of RS virus. Radioimmune precipitation of [3H]glucosamine-labelled glycoproteins by large glycoprotein (G)-specific or fusion protein (F)-specific monoclonal antibodies (MAbs) revealed that the G, F1 and F2 proteins were present in cell extracts but only the G protein was clearly evident in culture fluids. A glycoprotein (Mr 43K) which may be a precursor or a breakdown product of the G protein was also precipitated by the G-specific MAb from cell extracts and culture fluids. The G protein in culture fluids was slightly smaller (Mr 82K) than the G protein in cell extracts (Mr 88K). An abundant or heavily labelled, 18K glycoprotein in the fluids of virus-infected but not of mock-infected cells was weakly precipitated by the F-specific MAb; this suggested that the 18K protein shares epitopes with the fusion protein of RS virus. The absence of F1 and F2 polypeptides from culture fluids is evidence that the cells, which contained an abundance of these proteins, were intact.

To determine whether any of the viral glycoproteins released by infected cells were soluble (non-virion-associated), culture fluid was subjected to rate zonal centrifugation in a 10 to 50% sucrose gradient. An assay of fractions using a MAb-capture ELISA for the nucleocapsid (N) and F proteins revealed a peak of activity, due to virions, in the centre of the gradient, and a strong signal for the N protein at the top of the gradient suggesting that N protein was released from intact cells. Radioimmune precipitation of glycoproteins from the fractions at the top of the gradient using a hyperimmune guinea-pig serum revealed the G protein and a heterogeneous band which had the electrophoretic mobility of the 43K protein. Neither the F1 nor the F2 protein was present in these fractions thus suggesting that virions had remained intact. These results showed that a soluble form of the G protein of RS virus is released into the culture fluids of intact, infected cells. Several theories concerning viral and non-viral origins for the 18K protein are discussed.

INTRODUCTION

Respiratory syncytial (RS) virus, a member of the family Paramyxoviridae, repeatedly infects individuals (Glezen et al., 1971; Kim et al., 1973; Suto et al., 1965) and causes considerable morbidity, especially in children. Efforts at preventing RS viral infections using conventional vaccines, either temperature-sensitive mutants (Wright et al., 1982) or formalin-inactivated preparations of concentrated virus (Fulginiti et al., 1969; Kapikian et al., 1969; Kim et al., 1969), were abandoned because protection was inadequate or because vaccinees developed more severe disease following their next natural infection. More recently, perhaps in response to the
vaccine failures, research has focused on elucidating the roles of viral proteins in eliciting protective humoral and cell-mediated immunity to RS virus.

The two glycoproteins of RS virus are major viral immunogens in the host and may be essential to its infectivity. The large (90K) glycoprotein, G, probably functions in attachment to cells (Walsh et al., 1984b). Sequence analysis suggests that its protein backbone is relatively small (33K) (Wertz et al., 1985; Satake et al., 1985) and that oligosaccharides, most of which may be O-linked, contribute more than 50% of the mass to this molecule (Wertz et al., 1985; Gruber & Levine, 1985a). Purified G protein elicits neutralizing antibodies in rabbits (Walsh et al., 1984b) and several monoclonal antibodies (MAbs) that are directed to the G protein neutralize virus in vitro (Taylor et al., 1984; Walsh & Hruska, 1983). Following passive transfer of these MAbs to mice or cotton rats, viral replication in the lungs is depressed (Taylor et al., 1984; Walsh et al., 1984a). The fusion (F) protein is a disulphide-linked, heterodimer composed of a small, distal polypeptide (Fz, 20K) and a larger proximal protein (F1, 48K) (Fernie & Gerin, 1982; Lambert & Pons, 1983; Gruber & Levine, 1983; Walsh et al., 1985). It causes fusion of infected cells (Walsh & Hruska, 1983) and probably fusion of adsorbed virions to cells. Polyclonal, monospecific rabbit sera to the F protein neutralize RS virus (Walsh et al., 1985) and passive transfer of certain F-specific MAbs depresses viral replication in mice and cotton rats (Taylor et al., 1984; Walsh et al., 1984a). At least four epitopes that are recognized by fusion-inhibiting and/or neutralizing antibodies have been defined (Walsh et al., 1986). Clearly, the F and G proteins are antigens to which protective, humoral immunity is directed.

Considerable information is accumulating concerning the synthesis and maturation of the G and F proteins in infected cells (Collins et al., 1984; Lambert & Pons, 1983; Fernie et al., 1985; Gruber & Levine, 1985b), but there is a paucity of information concerning the release of these glycoproteins from infected cells. In addition, little information is available concerning the amount and nature of RS viral glycoproteins to which the host immune system is exposed. Soluble forms of glycoproteins released from infected cell cultures have been reported for vesicular stomatitis virus (VSV; Kang & Prevec, 1971; Little & Huang, 1977, 1978), herpes simplex virus (Chen et al., 1978) and murine leukaemia virus (Bolognesi & Langlois, 1975), and a soluble 70K glycoprotein of murine leukaemia virus has been detected in sera of mice (Strand & August, 1976). The presence of a soluble form of the G protein of RS virus was suggested by the observation that the G protein was precipitated from virus-free culture fluid which was used as a source of antigen for the characterization of MAbs (Fernie et al., 1982). We have embarked on a study to examine the production and release of RS viral glycoproteins from infected cells. We describe here the release of a soluble form of G protein.

**METHODS**

*Cells.* HEp-2 cells were obtained from Flow Laboratories and grown either in 150 cm² flasks (Costar) or in 12-well culture plates (Costar) in Eagle's minimal essential medium (MEM) supplemented with 5% foetal bovine serum (FBS) and antibiotics.

*Virus.* The Long strain of RS virus was twice purified by limiting dilution.

*Radio-labelling viral glycoproteins.* Subconfluent monolayers of HEp-2 cells in 12-well culture plates or 150 cm² flasks were infected with virus at an m.o.i. of one. Virus was adsorbed to monolayers at 37 °C for 90 min and monolayers were overlaid with MEM plus 2% FBS. Four to 6 h later, the medium was replaced with either MEM plus 2% FBS or RPMI-1640 medium (with 1/10 concentration of glucose) supplemented with 2% dialysed FBS and 0.1% sodium deoxycholate, 1 m~-PMSF) and processed as for culture fluids.

*Monoclonal and polyclonal antibodies.* MAbs to the F protein (MAb 13-1), the nucleocapsid (N) protein (MAb 6-3), and the G protein (MAb 111-2) were provided as ascitic fluids (Cote et al., 1981; Fernie et al., 1982). The specificities of these antibodies were determined by radioimmunoprecipitation. A hyperimmune serum to the Long strain was prepared in a 300 g male Hartley guinea-pig by two intradermal inoculations of purified virus (Huang & Wertz, 1982) and two intradermal inoculations of stock virus over a period of 7 weeks. The first inoculation was a 1:1 mixture of immunogen and complete Freund's adjuvant and the other three inoculations...
were 1:1 mixtures of immunogen and incomplete Freund's adjuvant. This serum had a neutralization titre of 580 in an assay with an endpoint of 60% neutralization.

**SDS-PAGE.** Proteins were resolved by discontinuous SDS-PAGE (Laemmli, 1970). In brief, samples were boiled for 3 min in disruption buffer (final concentrations 0.0625 M-Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and layered on a vertical slab gel containing a 4% acrylamide stacking gel and a 10% acrylamide resolving gel. Electrophoresis was carried out with constant voltage (55 V) for 14 h, gels were soaked for 30 min in Enlightening (New England Nuclear) and dried, and autoradiograms were prepared using Kodak Royal X-Omat film exposed at -70 °C.

**ELISA.** A MAb capture ELISA described by Hendry et al. (1985) was used.

**Sucrose gradients.** One to 2 ml of culture fluids were made 100 mM with respect to MgSO₄ and 50 mM with respect to HEPES pH 7.5 and layered over a 10 ml continuous, 10 to 50% sucrose gradient in MHN (1 M-MgSO₄, 50 mM-HEPES pH 7.5, 150 mM-NaCl; Fernie & Gerin, 1980). After centrifugation (150000 g, SW41 rotor) for 1 h at 4 °C, 0.5 ml fractions were collected from the bottom of the tube and assayed for acid-precipitable counts and for viral antigens.

**Radioimmunoprecipitation (RIP).** This procedure was derived from the method described by Bernstein & Hruska (1981). Samples of labelled preparations containing 500000 to 1000000 c.p.m. were brought up to 250 µl with PBS and incubated overnight at 4 °C with 10 µl of normal guinea-pig serum or normal ascitic fluid and IgGsorb (formalin-fixed Staphylococcus aureus, Cowan strain; The Enzyme Center, Maiden, Mass., U.S.A.) was added to achieve a final concentration of 2%. Bacterial cells were pelleted by centrifugation at 12000 g and supernatant fluids were incubated with 10 µl of hyperimmune guinea-pig serum or MAb for 4 to 5 h at 4 °C. Then, 100 µl of IgGsorb was added to achieve a final concentration of 2.5% and incubation was continued for an additional hour. Bacterial cells were washed twice in RIPA buffer containing 0.5 M-NaCl and once in RIPA buffer containing 0.15 M-NaCl. Bacterial pellets were boiled for 10 min in electrophoresis disruption buffer and proteins in the supernatant fluids were analysed by SDS-PAGE.

**RESULTS**

**Identification of viral glycoproteins in culture fluids and cell extracts**

The arrays of viral glycoproteins in infected cells and culture fluids from these cells were compared by discontinuous SDS-PAGE (Fig. 1). Heavily labelled G proteins whose heterogeneous electrophoretic mobilities suggested mol. wt. between 80K and 90K were seen in both cell extracts and culture fluids, but the F₁ protein (46K) and the F₂ protein (20K) could only be readily discerned in cell extracts (Fig. 1, lane 3). Culture fluids (Fig. 1, lane 1) contained a putative F₁ protein which migrated slightly slower than the F₁ protein seen in cell extracts and a very faint band which may have been the F₂ protein. Surprisingly, an 18K glycoprotein which was heavily labelled or abundant was observed in culture fluids of infected cells but not in fluids from mock-infected cells (Fig. 1, lane 2) or in cell extracts (Fig. 1, lanes 3 and 4). The presence of protease inhibitors, PMSF (final concentration of 1 µl) or a mixture of chymostatin, pepstatin and leupeptin (each at a final concentration of 50 µl), in the media of cells during infection and labelling did not affect the appearance or intensity of the 18K band or the three putative viral glycoproteins (data not shown).

In order to identify viral glycoproteins in cell extracts and culture fluids, immunoprecipitations with G- or F-specific MABs were carried out and precipitated proteins were resolved by SDS-PAGE (Fig. 2). The G protein and a 43K glycoprotein, perhaps representing either a precursor (Gruber & Levine, 1985b; Fernie et al., 1985) or a degradation product (Routledge et al., 1986) of G, were precipitated by a G-specific MAb (111-2) from both cell extracts and culture fluids (Fig. 2, lanes 3 and 7). In contrast, while both F₁ and F₂ proteins were precipitated by an F-specific MAb (13-1) from cell extracts (Fig. 2, lane 5) only traces of F₁ and F₂ polypeptides and an 18K glycoprotein (Fig. 2, lane 1) were precipitated from culture fluids. Recognition of the 18K protein by an F-specific MAb suggests three possibilities: that this protein is viral in origin, that it cross-reacts antigenically with the F protein, or that it forms a complex with F in culture fluids. These results demonstrated that large amounts of the G protein but neither the F₁ nor the F₂ polypeptide were found in the medium of these RS virus-infected cells and that the cells were largely intact since F₁ and F₂ proteins that were abundant in cell extracts would be expected to be released by lysing cells. [³H]leucine-labelled F₁ and F₂ polypeptides were precipitated by this F-specific MAb from fluids of infected cells that were...
Fig. 1. Viral proteins in culture fluids and cell extracts. Lanes 1 and 2, [³H]glucosamine-labelled glycoproteins in culture fluids; lanes 3 and 4, [³H]glucosamine-labelled glycoproteins in cell extracts; lanes 5 and 6, [³H]leucine-labelled proteins in cell extracts; lanes 1, 3 and 5, RS virus-infected; lanes 2, 4 and 6, mock-infected. The letters on the left side of the figure refer to viral proteins which are designated by arrows: G, large glycoprotein; F₁, proximal portion of fusion protein; N, nucleocapsid protein; P, phosphoprotein; M, matrix protein; F₂, distal portion of fusion protein. The numbers on the right side of the figure refer to the locations of mol. wt. standards.

Fig. 2. Precipitation of glycoproteins from culture fluids and cell extracts by MAbs. [³H]Glucosamine-labelled glycoproteins were precipitated by MAbs to the F protein (MAb 13-1, lanes 1, 2, 5 and 6) or to the G protein (MAb 111-2, lanes 3, 4, 7 and 8) from culture fluids (lanes 1 to 4) or from cell extracts (lanes 5 to 8). Samples of culture fluids (lane 9) and cell extracts (lanes 10 and 11) which are not immunoprecipitated are included to show the arrays of glycoproteins that were present in these preparations. Lanes 1 to 4 and 7 and 8 were exposed to X-ray film for 42 days and lanes 5 and 6 and 9 to 11 were exposed for 9 days. Odd numbered lanes, RS virus-infected; even numbered lanes, mock-infected.
Fig. 3. Resolution of viral proteins in a 10 to 50% sucrose gradient. (a) Detection of viral antigens in fractions from sucrose gradient using MAb capture ELISA. Protein in the fluids from RS virus-infected cells that were incubated with [3H]glucosamine were resolved in a 10 to 50% sucrose gradient and N (●) and F (○) proteins in each fraction were detected in an ELISA and expressed as A490 values. Total acid-precipitable counts (■) in each fraction are shown. (b) Radioimmune precipitation of viral glycoproteins from top fractions of sucrose gradient. [3H]Glucosamine-labelled proteins in fluids of RS virus-infected or mock-infected cells were resolved in 10 to 50% sucrose gradients. Immunoprecipitation of glycoproteins (lanes 1 to 4 and 9 to 12) from the top four gradient fractions (19 to 22) by a hyperimmune serum to the Long strain are shown; (lanes 1 to 5 and 7, RS virus-infected; lanes 6, 8 to 12, mock-infected). Samples of culture fluids which were not immunoprecipitated (lanes 5 and 6) are included to show the arrays of glycoproteins that were present in the culture fluids before they were layered on the gradients. [3H]Leucine labelled viral proteins from cell extracts are included (lanes 7 and 8) for reference. (c) Precipitation of viral glycoproteins by hyperimmune guinea-pig serum to the Long strain. Radioimmune precipitation of [3H]glucosamine labelled glycoproteins from fluids (lanes 1 and 2) and extracts (lanes 3 and 4) of RS virus-infected cells (lanes 1 and 3) or mock-infected cells (lanes 2 and 4).
allowed to progress to an advanced cytopathology (data not shown). Thus, the absence of F₁ and F₂ proteins in culture fluids may be used as evidence that the infected cells were relatively intact.

Detection of a soluble form of G in fluids of infected cell cultures

Because soluble (non-virion-associated) forms of viral glycoproteins have been shown to be released by intact cells infected with other viruses, an effort was made to detect and resolve a possible soluble form of the G protein of RS virus from virion-associated G protein by sucrose density gradient centrifugation (Fig. 3). A sample (1·0 ml) of clarified culture fluid that contained 1·5 × 10⁵ c.p.m. was layered over a 10 to 50% sucrose gradient and after centrifugation at 150000 g for 1 h, 0·5 ml fractions were collected from the bottom of the centrifuge tube. Assays of fractions for radioactivity and for viral antigen by ELISA (Fig. 3a) revealed a peak of activity at the top of the gradient (fractions 20 to 23) suggesting the presence of soluble proteins. The more rapidly sedimenting, homogeneous peak of activity (fractions 9 to 11) in the middle of the gradient most likely represents whole virions since (i) infectivity co-sediments with this activity, (ii) activity from this peak bands at 1·17 g/ml after isopycnic centrifugation in a 10 to 60% sucrose gradient, and (iii) isopycnically banded material from this peak elicits antibodies to G, F₁, N, phospho (P), matrix (M), F₂ and the 22K protein in rabbits and guinea-pigs (data not shown). Surprisingly, fractions from the top of the gradient contained strong ELISA signals for the N protein, thus suggesting that intact, infected cells released N protein, a finding that has also been reported for intact VSV-infected cells (Little & Huang, 1977). A weak ELISA signal for the F protein in this peak also suggested that a small amount of this protein was soluble (Little & Huang, 1978). It was not possible to assay fractions for the G protein using the monoclonal-capture ELISA since the bovine anti-bovine RS viral antiserum that was used as a detector antibody does not recognize the soluble G protein of the Long strain (J. Burns, M. Hendry & D. Hendricks, unpublished results).

To further clarify these results, the top fractions of a 10 to 50% sucrose gradient were assayed for [³H]glucosamine-labelled viral glycoproteins by immunoprecipitation using a hyperimmune guinea-pig serum to the Long strain. Results from a representative experiment are shown in Fig. 3(b). These fractions contained the G protein and a smaller protein (Mr 40K to 45K) both of which were heterogeneous (Fig. 3b, lanes 1 to 4) and absent from radioimmunoprecipitates of mock-infected preparations (Fig. 3b, lanes 9 to 12). The hyperimmune guinea-pig serum precipitated only these two viral glycoproteins from the original culture fluid (Fig. 3c, lane 1) but it clearly showed that the G, F₁, and F₂ proteins were present in the infected cells (Fig. 3c, lane 3) from the same cultures. The G protein, a 40K to 45K protein, and an 18K protein could be visualized in samples of these fractions which were not subjected to immunoprecipitations (data not shown). The identity of the diffuse 40K to 45K protein was not ascertained since F- and G-specific MAbs were not used for these immunoprecipitations; however it had the migration mobility expected for the 43K protein precipitated by a G-specific MAb from culture fluid (Fig. 2). The relative amounts of soluble and virion-associated G proteins in culture fluids could not be determined from these experiments because the low levels of radioactivity in the peak containing whole virions precluded visualization of glycoproteins by SDS-PAGE and autoradiography. These results show that G is the major soluble viral glycoprotein released from infected cells.

Different electrophoretic mobilities of the intracellular and extracellular forms of G

Since the G glycoprotein in cell extracts and extracellular fluids appeared to have different electrophoretic mobilities in 10% polyacrylamide gels in Fig. 1 and 2, proteins from these two sources were resolved on a 7·5% polyacrylamide gel using an extended running time (Fig. 4). The G protein from cell extracts could be assigned a mol. wt. of 88K in contrast to the mol. wt. of 82K for the extracellular G protein. Differences in electrophoretic mobilities could not be ascribed to the presence of RIPA buffer in cell extracts, since addition of this buffer to extracellular fluids did not affect the migration of proteins (data not shown). These data suggest that the most abundant form of the G protein in extracellular fluids is smaller than the intracellular form of the G protein.
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Fig. 4. Resolution of the G protein in culture fluids and cell extracts. [3H]Glucosamine-labelled proteins from fluids (lanes 1 and 2) and extracts (lanes 3 and 4) of RS virus-infected (lanes 1 and 3) or mock-infected (lanes 2 and 4) cells were resolved in a gel containing 7.5% polyacrylamide.

DISCUSSION

This is the first report that a soluble form of the G glycoprotein of RS virus is present in the extracellular fluids of intact, infected cells. Differences in the electrophoretic mobilities of the G protein in extracellular and intracellular preparations were reported by Bernstein & Hruska (1981) and Dubovi (1982) thus suggesting that there were different forms of this glycoprotein. These reports also showed that the G protein is the most abundant viral glycoprotein in fluids of infected cells which contained the G, F₁, and F₂ proteins. Our data were consistent with these reports. Demonstration of a soluble glycoprotein in fluids of infected cells should be accompanied by evidence that neither released virions nor cells were degraded. The low level or absence of F₁ and F₂ proteins in culture fluids in this work shows that cells which contained an abundance of these glycoproteins were intact. In addition, the hypothesis that degraded virions contributed soluble G to culture fluids is invalid since F₁ and F₂ proteins were not present. These data were similar to those of Fernie et al. (1982).

The soluble form, Gs (s for shedding), of the G protein of VSV has been well characterized (Kang & Prevec, 1971; Little & Huang, 1977, 1978; Garris-Wabnitz & Kruppa, 1984) and may serve as a model for the characterization of the soluble G protein of RS virus. The Gₛ protein of VSV is antigenically related to the G protein but is smaller due to the proteolytic removal of about 60 amino acids from the carboxy terminus in an intracellular compartment. The Gₛ protein contains both of the carbohydrate chains of the G protein. About one in six G molecules is Gₛ. Little & Huang (1977) showed that the Gₛ protein is released from infected cells when the synthesis of progeny virus was inhibited by (i) defective interfering particles, or (ii) utilization of a temperature-sensitive, replication-minus mutant or a glycoprotein mutant.

Although a soluble form of the 70K glycoprotein of murine leukaemia virus has been found as free glycoprotein (non-virion-associated; non-antibody-complexed) in the sera of New Zealand...
black mice, it is not known whether or not release of soluble viral glycoproteins during infection of humans or experimental animals is a general phenomenon. If soluble forms of viral glycoproteins are released during infection, then it would be important to study their possible role in absorbing neutralizing antibodies, in mediating immunopathological mechanisms, or perhaps in saturating viral receptors on susceptible cells or antigen receptors on immune cells.

A G-specific MAb precipitated two virus-specific glycoproteins from both infected cells and their culture fluids. The larger molecule was probably the mature, 80K to 90K, endoglycosidase H-resistant form of the glycoprotein whereas the 43K protein may have been the predominant precursor of G detected by several groups (Gruber & Levine, 1985b; Fernie et al., 1985). Routledge et al. (1986) could not exclude the possibility that a 45K to 50K glycoprotein that is precipitated by G-specific MAbs from lysates of RS virus-infected simian or human cells is a proteolytic cleavage product of G. The origin of the 43K glycoprotein in this work could not be ascertained from the data.

The appearance in the fluids of infected cells of a heavily labelled 18K glycoprotein that was precipitated by an F-specific MAb was surprising. The only evidence that this glycoprotein was a viral protein was its modest precipitation by an F-specific MAb from an apparently abundant and/or heavily labelled pool in culture fluids. It is possible that this glycoprotein is a degradation product of the G, F1 or F2 protein that has so few native epitopes that it is no longer recognized by either the F- or the G-specific MAbs or the hyperimmune guinea-pig serum. More likely, this protein is either a cellular stress (heat shock) protein and/or a cellular protein that cross-reacts with the F-specific MAb used here. Cellular stress proteins including glucose-regulated proteins have been shown to be induced by herpes simplex virus (Notarianni & Preston, 1982), adenovirus (Nevins, 1982), Sindbis virus, VSV (Garry et al., 1983), Newcastle disease virus (Collins & Hightower, 1982), parainfluenza type 1 virus and simian virus 5 (Peluso et al., 1978). These proteins, some of which are glycoproteins, can be induced by viral infection and physical and chemical stress; predominant forms have molecular weights of 99K, 87K, 80K and 70K although forms as small as 25K (Notarianni & Preston, 1982) have been reported. This 18K glycoprotein is smaller than the previously reported stress proteins. Sheshberadaran & Norrby (1984) recently reported that three of 11 MAbs to the fusion protein of measles virus cross-react with an array of stress proteins of which the predominant form is a 79K protein. In addition, viral proteins of simian virus 40 (Crawford et al., 1982; Fujinami et al., 1983), herpes simplex virus (Fujinami et al., 1983) and Japanese encephalitis virus (Gould et al., 1983) have been shown by MAbs to share determinants with host components, many of which are cytoskeletal elements.

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