Respiratory Syncytial Virus Polypeptides. IV. The Oligosaccharides of the Glycoproteins

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SUMMARY

The cell-associated glycoproteins of respiratory syncytial (RS) virus included GP1 (90K), VP70 (70K), VGP48 (48K) and GP26 (26K). Although present in infected cells, there was no VP70 in purified virus. Trypsin treatment of infected cells removed 80 to 90% of VP70 as well as its products VGP48 and GP26. This suggested that most of the VP70 in the cell is located on the plasma membrane. The glycoproteins of purified RS virus (GP1, VGP48 and GP26) contain mannose, galactose and fucose as well as glucosamine, but the quantity of mannose in GP1 is low when compared to that of the other three sugars. The effects that follow the treatment of infected cells with the glycosylation inhibitors tunicamycin and monensin, and the treatment of the immunoprecipitated product of pulse-chase experiments with endonuclease H demonstrated that VP70 and its products contained N-linked oligosaccharides, and that the oligosaccharides of the mature VGP48 subunit were of the complex type, while GP1 contained both N- and O-linked oligosaccharides. The non-glycosylated forms of VP70 and GP1 have estimated mol. wt. of 50K and 33K respectively. Therefore, the carbohydrate contribution to the mol. wt. of VP70 and GP1, as determined by PAGE, was equivalent to 20K for the former and 57K for the latter. The majority of the GP1 oligosaccharides were O-linked, a form of sugar linkage not previously found among paramyxoviruses.

INTRODUCTION

Respiratory syncytial (RS) virus contains two envelope-associated glycoproteins. One (GP1) has a molecular weight of 90000 (90K) and the other has a molecular weight of 70K (Gruber & Levine, 1983; Peeples & Levine, 1979). The 70K glycoprotein consists of two disulphide-linked glycopeptides (VGP48 and GP26) (Fernie & Gerin, 1982; Gruber & Levine, 1983), and has been tentatively identified as the fusion protein (Walsh & Hruska, 1983).

RS virus proteins of 68K (Dubovi, 1982) or 73K (Bernstein & Hruska, 1981) were found in RS virus-infected cells and a viral protein of 66K was found in purified RS virus (Fernie & Gerin, 1982). Since these proteins were present after SDS dissociation under reducing conditions, they may represent the uncleaved precursor of VGP48 and GP26.

The above studies did not determine the intracellular location of this viral protein. In this paper, we show that VP70 is associated with the plasma membrane and is found in purified virus only in the processed form of VGP48 and GP26.

It has been demonstrated that the RS virus glycoproteins contain glucosamine (Bernstein & Hruska, 1981; Gruber & Levine, 1983; Levine, 1977; Peeples & Levine, 1979) and fucose (Bernstein & Hruska, 1981; Cash et al., 1979). However, no other details of the carbohydrate content of these glycoproteins are known. We therefore thought it useful to determine (i) the types of oligosaccharides that are associated with these glycoproteins, and (ii) the contribution by the oligosaccharides to the mol. wt. of the glycoproteins.

There are two main types of oligosaccharide linkage found in glycoproteins (N or O). The synthesis of N-linked oligosaccharides is initiated in the rough endoplasmic reticulum (RER) by
the transfer of a high-mannose core to an asparagine residue of a newly synthesized polypeptide chain (Schachter, 1974; Waechter & Lennarz, 1976). The N-linked oligosaccharides are then processed to a high-mannose form as the polypeptide moves from the RER to the Golgi apparatus, or to a complex form within the Golgi apparatus (Bretz et al., 1980; Tabas & Kornfeld, 1979; Tulsiani et al., 1982). The antibiotic tunicamycin prevents the addition of N-linked oligosaccharides to nascent polypeptides (Lehle & Tanner, 1976; Takatsuki et al., 1971), but does not block the attachment of O-linked oligosaccharides to glycoproteins (Holmes et al., 1981; Shida & Dales, 1981).

O-linked oligosaccharides are synthesized within the Golgi apparatus by the addition of individual monosaccharides to serine or threonine residues of the polypeptide chain (Bretz et al., 1980; Hanover et al., 1980; Schachter, 1974). Monensin, a carboxylic ionophore, blocks Golgi apparatus functions, including the synthesis of O-linked oligosaccharides and the processing of the N-linked oligosaccharides to their complex form, but not the addition of N-linked oligosaccharides to the polypeptide (Alonso & Compans, 1981; Johnson & Schlesinger, 1980; Johnson & Spear, 1982, 1983; Niemann et al., 1982; Pressman, 1976; Tartakoff & Vassalli, 1977).

The enzyme endo-β-N-acetylglucosaminidase H (endo-H) cleaves between the two N-acetylglucosamine residues in N-linked high-mannose oligosaccharides of glycoproteins. However, N-linked complex oligosaccharides are resistant to this enzymic cleavage (Arakawa & Muramatsu, 1974; Tarentino & Maley, 1974).

We used tunicamycin, monensin and endo-H to determine whether the RS viral glycoproteins contained N- and/or O-linked oligosaccharides, whether the N-linked oligosaccharides of the mature glycoproteins were of the high-mannose or complex type and to determine the molecular weights of the non-glycosylated proteins. We found that the VP70 precursor as well as the VGP48 and GP26 products had only N-linked oligosaccharides of the complex type and that GP1 had both N- and O-linked oligosaccharides.

METHODS

Cells. HeLa cells were maintained in continuous cultures and grown in Eagle’s MEM supplemented with 10% foetal bovine serum (FBS).

Virus. The Long strain of RS virus was grown in HeLa cell monolayers maintained in MEM with 5% FBS, and assayed on HeLa monolayers by the plaque method previously described (Levine et al., 1971; Levine & Hamilton, 1969). Virus was labelled by growth in the presence of [3H]glucosamine (5 μCi/ml), [3H]mannose (5 μCi/ml), [3H]galactose (5 μCi/ml), [3H]fucose (5 μCi/ml), [14C]glucosamine (2-5 μCi/ml) or [35S]methionine (10 μCi/ml), incorporated into MEM containing 5% FBS. Radiolabelled virus was purified on sucrose gradients as previously described (Levine, 1977; Peeples & Levine, 1979; Gruber & Levine, 1983).

Trypsinization and pulse chase experiments. HeLa cells were grown to monolayers in 35 mm plastic tissue culture dishes and were infected at a m.o.i. of greater than 3 or mock-infected with Hanks’ balanced salt solution (HBSS). At 2 h post-infection, 2 ml/plate of MEM containing 5% FBS was added. At 24 h, the medium was replaced with 1 ml/plate of HBSS and the plates were incubated for 45 min at 37 °C, to reduce the intracellular pools of amino acids.

For the [35S]methionine pulse-chase experiments, infected HeLa cells were preincubated with HBSS for 45 min at 24 h post-infection, as above. Then cells were pulsed for 10 min at 37 °C, with [35S]methionine (100 μCi/ml) in methionine-free MEM containing 2% FBS (1 ml/plate) and the monolayers were again incubated at 37 °C, until 29 h post-infection when the [35S]methionine-labelled medium was removed and the cell monolayers were washed twice in cold HBSS. Then cold HBSS, 1 ml/plate, was added to three mock-infected and three infected monolayers, after two washings with cold HBSS, were rinsed with 2 ml/plate trypsin solution (0.25~ trypsin and 0.2 mg/ml EDTA in 0.01 M-phosphate-buffered saline, pH 7.2), incubated at 37 °C for 5 min with 0.6 ml/plate of the trypsin solution, then suspended in 1.4 ml/plate of MEM containing 10% FBS and pooled. The pools of scraped and trypsinized cells were centrifuged at 1000 r.p.m. for 10 min at 4 °C, and the cell pellets were solubilized in dissociation buffer (see below) and sonicated. Equal quantities of protein from each pool were immunoprecipitated.

For the [35S]methionine pulse-chase experiments, infected HeLa cells were preincubated with HBSS for 45 min at 24 h post-infection as above. Then cells were pulsed for 10 min at 37 °C, with [35S]methionine (100 μCi/ml) in methionine-free MEM containing 2% FBS. For the chase at 37 °C, the cell monolayers were washed twice with HBSS and then fed with MEM containing 5% FBS and 20 times the normal concentration of methionine.
For the $[^{3}H]$leucine pulse-chase experiments, the cell monolayers were preincubated with leucine-free, FBS-free MEM for 4 h starting at 20 h after infection. Then, they were pulsed for 15 min at 37 °C with 0.4 ml/plate of $[^{3}H]$leucine (250 μCi/0.4 ml) in leucine-free, FBS-free MEM. The chase, at 37 °C, involved washing the monolayers twice with HBSS and feeding with MEM containing 5% FBS and 20 times the normal concentration of leucine.

The pulse-chase procedures were terminated by washing the monolayers twice with cold HBSS. Then 1 ml/plate of cold HBSS was added to the monolayers, and the cells were scraped, pooled and pelleted at 1000 r.p.m. for 10 min. The pellets were resuspended and solubilized in 150 μl of dissociation buffer (see below) containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.5 trypsin inhibitor units (TIU)/ml aprotinin. The samples were sonicated for approximately 30 s in an MSE ultrasonic power unit, and aliquots from each pulse and chase were TCA-precipitated for radiolabel counting.

**Polyacrylamide gel electrophoresis (PAGE).** Polypeptides were separated on 9% SDS-polyacrylamide slab gels with 3% stacking gels, utilizing the discontinuous buffer system of Laemmli (1970), as previously described (Levine, 1977; Peeples & Levine, 1979). Samples for electrophoresis were suspended in dissociation buffer (50 mM Tris·HCl pH 7.0, 2% SDS, 8% sucrose and bromophenol blue) with 5% 2-mercaptoethanol and dissociated by heating for 2 min in boiling water.

**Preparation of antisera to viral glycoproteins.** Unlabelled virus was purified, and the protein from dissociated, non-reduced virus were separated on Laemmli SDS-polyacrylamide slab gels. To locate the position of the viral glycoproteins radiolabelled virus was loaded in the first and last wells, with the unlabelled virus loaded in the intervening wells.

The two unlabelled viral glycoproteins were cut out of the gel and the gel slices were emulsified in Freund's complete or incomplete adjuvant. Each glycoprotein, suspended either in Freund's complete or incomplete adjuvant, was then injected subcutaneously into a separate set of female New Zealand White rabbits at 2-week intervals. The serum was collected 14 to 18 weeks later. The antisera were absorbed with disrupted uninfected cells to remove contaminating antibody.

**Western blot analysis.** The proteins to be analysed were first separated by PAGE. The proteins in the gel were then transferred to a nitrocellulose sheet as follows (Towbin et al., 1979; Vaessen et al., 1981). The sheet of nitrocellulose (0.45 μm pore size, Bio-Rad) was soaked in transfer buffer (24 mM-Tris-HCl pH 8.3, 192 mM-glycine, 20% methanol) for 10 min and then laid on top of two pre-soaked Scotch-Brite pads which had been placed on the anode grid of an E-C electrophoret apparatus. The gel was placed on the nitrocellulose sheet, followed by a sheet of nitrocellulose (0.45 g/M pore size, Bio-Rad) was soaked in transfer buffer (24 mM-Tris-HCl pH 8.3, 192 mM-glycine, 20% methanol) for 10 min. Then the antibody solution was added to each sample. The mixture was incubated for 4 h at 4 °C with mixing every 15 min. Next, Protein A-Sepharose beads (50 μl of 40% (w/v)) were added to the immunoprecipitation reaction, and incubation was continued at 4 °C for 1 h, with mixing every 10 min. The immunoprecipitates were pelleted in an Eppendorf microfuge and then washed, first in 140 mM-NaCl, 20 mM-Tris·HCl pH 7.8, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mg/ml ovalbumin, 0.5 TIU/ml aprotinin, and 0.5% Na2S and gently shaken for 24 h at 25 °C on a rotating shaker. Then, the BSA solution was replaced with fresh BSA solution containing the antibody, and the nitrocellulose sheet was again shaken at 25 °C for approximately 16 h. The antibody solution was removed and the nitrocellulose sheet was washed six times, at 5 min intervals, in TDN buffer without BSA. The last wash was done with TDN containing BSA and the nitrocellulose was shaken for 15 min. Then $^{125}$I-labelled protein A (0.25 μCi/gel slot) was added to the BSA solution and the nitrocellulose was shaken for an additional 4 h, after which the $^{125}$I-Protein A solution was removed by washing. The nitrocellulose was washed in TDN buffer six times at 10 min intervals, air-dried, wrapped in plastic-wrap, and placed overnight at −70 °C in a metal X-ray cassette with an intensifying screen and Kodak X-OMAT RP film.

**Immunoprecipitation.** Radiolabelled cell lysates solubilized in dissociation buffer were immunoprecipitated according to the following procedure (Lee et al., 1982). All samples were brought to 0.4 ml in immunoprecipitation buffer (140 mM-NaCl, 20 mM-Tris·HCl pH 7.8, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mg/ml ovalbumin, 0.5 TIU/ml aprotinin) and cleared of particulate matter by centrifugation at 12000 g for 1 min. Antibody (80 μl) was added to each sample. The mixture was incubated for 4 h at 4 °C with mixing every 15 min. Next, Protein A-Sepharose beads (50 μl of 40% (w/v)) were added to the immunoprecipitation reaction, and incubation was continued at 4 °C for 1 h, with mixing every 10 min. The immunoprecipitates were pelleted in an Eppendorf microfuge and then washed, first in 140 mM-NaCl, 20 mM-Tris·HCl pH 7.8, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% SDS, then in 140 mM-NaCl, 20 mM-Tris·HCl pH 7.8, 0.1% Triton X-100 and finally in 10 mM-Tris·HCl pH 7.8, 0.1% Triton X-100. The immunoprecipitates were either boiled in 30 μl dissociation buffer containing 5% 2-mercaptoethanol or treated as in the endo-H experiment described below, and 1 μl was removed for counting. The eluted, precipitated proteins were analysed by PAGE.

**Tunicamycin and monensin treatment.** HeLa cell monolayers were infected with RS virus at a m.o.i. greater than 3 or vesicular stomatitis virus at a m.o.i. greater than 2. For virus yields and Western blots, tunicamycin at 5 μg/ml, or as indicated, was added to infected cells in MEM containing 5% FBS at 2 h. Infected cells that were to be radiolabelled were incubated with tunicamycin from 2 h to 20 h post-infection at which time MEM with 5% FBS was added to the monolayers.
containing tunicamycin and either [\(^{14}\)C]glucosamine (5 \(\mu\)Ci/plate) or [\(^{3}\)H]mannose (5 \(\mu\)Ci/plate), was added. For virus yields, the cultures were harvested when the control cells exhibited extensive c.p.e. For the radiolabel experiments, and for Western blots, the medium containing tunicamycin was removed at 48 h, the cells were washed twice with cold HBSS and were solubilized in dissociation buffer containing the protease inhibitors PMSF and aprotinin.

Monensin, dissolved in absolute ethanol to make a stock solution of 1 mM, was added in MEM containing 5% FBS, at 0·1 mM, or as indicated, to RS virus-infected cells at 2 h after infection. For virus yields, the cells were harvested when control cells exhibited extensive c.p.e. For Western blots, the medium containing monensin was removed at 48 h post-infection, the cells were washed twice with cold HBSS and were solubilized as above.

**Endo-H digestion.** Immune precipitates were resuspended in an equal volume of 0·1 M-sodium acetate buffer pH 5·5 containing 0·1% SDS and 0·5 TIU/ml aprotinin. Ten Itl of sodium acetate buffer either with or without 0·01 units endo-H was added to duplicate immune precipitates. The enzyme mixtures were incubated at 37°C with constant rotation for 20 h. The reaction was terminated by adding an equal volume of twice-concentrated dissociation buffer, containing 10% 2-mercaptoethanol followed by heating in boiling water for 2 min. The dissociated samples were analysed by PAGE (Herrler & Compans, 1983).

**Fluorography.** The method used was that of Bonner & Laskey (1974) as previously described (Peeples & Levine, 1979).

**Chemicals and radioisotopes.** The tunicamycin was a gift from Dr J. Benjamin of Wayne State University and from Dr R. L. Hamill of Eli Lilly Research Laboratories. The trypsin was purchased from Difco, the bovine albumin powder Fraction V from Schwarz/Mann and the monensin, the endo-H and the Protein A-Sepharose CL-4B from Sigma. The [\(^{15}\)I-Protein A (> 30 mCi/mg), D-[\(^{14}\)C]glucosamine (55.5 mCi/mmol), [\(^{35}\)S]methionine (840 to 1385 Ci/mmol), D-[\(^{3}\)H]glucosamine (20 Ci/mmol), D-[\(^{3}\)H]mannose (2·1 Ci/mmol), D-[\(^{3}\)H]galactose (5·2 Ci/mmol), L-[\(^{6}\)H]fucose (18·5 Ci/mmol), L-[\(^{4,5}\)H]leucine (131 Ci/mmol) were all purchased from Amersham.

**RESULTS**

**Characterization of rabbit antisera to RS virus glycoproteins**

Rabbit antisera were made to the two viral glycoproteins as described in Methods. Two antisera were obtained, one, designated E028, was directed primarily against GP1 (Fig. 1), but at a 1:50 dilution it also reacted with denatured BSA as well as with another viral component which may be the nucleocapsid protein 44K (see Fig. 6). That the reactivity was directed against denatured BSA was determined by Western transblot analysis with E028 against reduced, SDS-denatured BSA (data not shown). The second antiserum, E311, was directed against VGP48 and GP26, the subunit products of the 70K glycoprotein (VP70) as shown in Fig. 2. We do not know the nature of the slowest band that migrates near GP1 (Fig. 2, lanes 2 and 3). However, we do not believe it represents cross-reactivity with GP1 because (i) it migrated slower than GP1, (ii) we saw this band in some, but not all preparations of purified virus, (iii) we did not see a band in this position in RS virus-infected cells (Fig. 3, lane 2 and Fig. 6, lanes 2 to 6). The two antisera, E028 and E311, did not neutralize virus without complement, but did neutralize virus with complement at a dilution of 1:15, as determined by a 90% plaque reduction. However, in the Western transblot procedure with 20 \(\mu\)g of purified virus E311 had a titre of at least 1:62·500 and E028 had a titre of 1:250 against their specific glycoprotein antigens.

**Location of the 70K viral glycoprotein**

The 70K RS virus glycoprotein was reported in 'purified virus' (Fernie & Gerin, 1982). We have never seen a protein with this molecular weight in either purified [\(^{14}\)C]glucosamine- or [\(^{35}\)S]methionine-labelled RS virus (Gruber & Levine, 1983; Levine, 1977; Peeples & Levine, 1979). Western transblot analysis, in our hands, is a very sensitive procedure, so it was used to determine if VP70 could be found in purified virus. As shown in Fig. 3, lane 1, only VGP48 and GP26 were found in purified virus. However, VP70 could be found in infected cells, as can be seen in Fig. 3, lane 2. This protein could also be found in [\(^{35}\)S]methionine-labelled infected cells and was identified by one-dimensional peptide mapping to be related to VGP48 (data not shown).

Next, the location of VP70 within infected cells was investigated, starting with the cellular plasma membrane. Trypsin removes the glycoproteins from RS virions and also releases the cell-associated RS virus from infected cells as non-infectious, glycoprotein-deficient particles
Fig. 1. Western transblot of purified unlabelled RS virus and unlabelled uninfected HeLa cells from a reduced Laemmli polyacrylamide gel. Lanes 2 and 3 were reacted with antiserum E028 at 1:50 followed by 125I-Protein A. Lane 1, transblot of purified [14C]glucosamine-labelled RS virus marker; lane 2, uninfected HeLa cells (160 µg); lane 3, unlabelled RS virus (20 µg); lane 4, transblot of purified [35S]methionine-labelled RS virus marker. (The figure is a composite of different X-ray film exposures of the same transblot.)

Fig. 2. Western transblot of purified unlabelled RS virus and unlabelled uninfected HeLa cells from a reduced Laemmli polyacrylamide gel. Lanes 2, 3 and 4 were reacted with E311 at 1:50 followed by 125I-Protein A. Lane 1, transblot of purified [14C]glucosamine-labelled RS virus marker; lane 2, unlabelled RS virus (20 µg); lane 3, unlabelled RS virus (60 µg); lane 4, uninfected HeLa cells (160 µg).

(Peeples & Levine, 1979, 1980). Therefore, trypsin was used to determine whether VP70 was present on the plasma membrane of infected cells. Infected cell monolayers, labelled with [35S]methionine, were treated with trypsin or left untreated. The cells were solubilized, equal quantities of protein were added to the immune precipitation reaction mixture and the VP70-related proteins were immunoprecipitated with antisem E311. Fig. 4 shows that trypsin treatment reduced the quantity of VP70 and its products, VGP48 and GP26, that were immunoprecipitated from infected cells. When compared to untreated cells, only 10 to 20% of
Fig. 3. Western transblot of purified unlabelled RS virus and RS virus-infected HeLa cells from a reduced Laemmli polyacrylamide gel. Lanes 1 and 2 were reacted with antiserum E311 at 1:50 and 125I-Protein A. Lane 1, purified unlabelled RS virus (20 µg); lane 2, infected HeLa cells 18 h post-infection (80 µg); lane 3, transblot of purified [35S]methionine-labelled RS virus marker.

Fig. 4. Fluorogram of Laemmli polyacrylamide gel of [35S]methionine-labelled RS virus-infected cells either untreated or trypsin-treated and immunoprecipitated with antiserum E311. Lane 1, [14C]glucosamine-labelled RS virus; lane 2, uninfected cells; lane 3, untreated infected cells; lane 4, trypsin-treated infected cells; lane 5, [35S]methionine-labelled RS virus. (The figure is a composite of different X-ray film exposures of the same gel.)

VP70, 10% of VGP48 and 10% of GP26 remained in trypsin-treated infected cells, as determined by densitometry tracing. This suggests that most of the VP70 in the cell is located on the plasma membrane.

$^3$H-labelled sugar incorporation into viral glycoproteins

Oligosaccharides of RS virus glycoproteins

viral glycoproteins of reduced, SDS-dissociated virus were analysed by SDS–PAGE. The three viral glycopeptides (GP1, VGP48 and GP26) were labelled by all four sugars, though the quantity of radiolabelled mannose incorporated into GP1, relative to the incorporation into VGP48, was much smaller than the quantity incorporated with the other three sugars (Fig. 5). These results suggested that the oligosaccharides of GP1 differed from those of VGP48 and GP26. The minor bands apparent in lanes 2, 3, 4 and 5 might be due to metabolic cycling of the radiolabelled monosaccharides, with the label becoming incorporated into non-glycosylated viral proteins. The heavier labelling of these minor bands in lane 4 might then be due to greater metabolic cycling of mannose. More likely, these differences are more apparent than real and arise from our use of equal counts of purified radiolabelled virus for this comparison. Since at least 50% of the radiolabelled monosaccharide (e.g. glucosamine) incorporated into virus is found in GP1, if a radiolabelled monosaccharide is not directly incorporated into this glycoprotein (as is the case with mannose) the contribution of these minor protein bands is increased.

Fig. 5. Fluorogram of Laemmli polyacrylamide gel of different ³H-sugar-labelled RS virus preparations. Each lane contains the same number of c.p.m. Lane 1, [³H]fucose-labelled RS virus; lane 2, [³H]galactose-labelled RS virus; lane 3, [³H]glucosamine-labelled RS virus; lane 4, [³H]mannose-labelled RS virus; lane 5, [¹⁴C]glucosamine-labelled RS virus.
Effect of tunicamycin on infectious virus yields and radioisotope incorporation

Tunicamycin, which inhibits the N-linked glycosylation of proteins, was used to determine the types of oligosaccharide chains associated with the RS virus glycoproteins. This antibiotic is known to inhibit the yields of enveloped viruses (Holmes et al., 1981; Leavitt et al., 1977; Nakamura & Compans, 1978; Schwarz et al., 1976; Stallcup & Fields, 1981). The effect of different concentrations of tunicamycin on RS virus yields was compared to their effect on vesicular stomatitis virus (VSV) yields. Maximum inhibition of the yields of both viruses, 99.8% for RS virus and 95.6% for VSV, occurred at a tunicamycin concentration of 5 μg/ml. This concentration selectively inhibited protein glycosylation, since it reduced the level of [14C]glucosamine incorporation into TCA-precipitable material in both infected and mock-infected HeLa cells to 12 to 16%, while reducing [35S]methionine incorporation to 47%. Therefore, this concentration of tunicamycin (5 μg/ml) was used in subsequent experiments, because it maximally inhibited the RS virus yield and inhibited protein glycosylation much more than protein synthesis.

Synthesis of viral glycoproteins in tunicamycin-treated infected cells

Infected and uninfected cells were either mock-treated or treated with 5 μg/ml tunicamycin 2 h after infection. At 48 h, the cell monolayers were washed twice with cold HBSS and the cells were solubilized in dissociation buffer containing protease inhibitors. The cellular proteins were dissociated and reduced, separated by SDS-PAGE, transferred from the gel to nitrocellulose and the nitrocellulose was treated with antiserum E028 or E311, as described in Methods. The results of this experiment are shown in Fig. 6. The related viral proteins that react to E028, GP1 (90K) and 46K, could be seen in infected cells (Fig. 6, lane 7). The 46K protein is the precursor to GP1 as determined by pulse-chase studies (not shown). (The other bands are BSA and probably N protein, as mentioned above.) In contrast, in tunicamycin-treated infected cells two differently migrating viral protein bands were found (Fig. 6, lane 8). One band migrated slightly faster than GP1 (83K) and the other band (approx. 33K) migrated faster than the 46K precursor to GP1. In the Western blot of untreated cells with serum E311, four viral protein bands were found (Fig. 6, lane 2). The largest protein was VP70, then there appeared to be two forms of VGP48, which migrated as a doublet, and GP26. In the presence of tunicamycin, three differently migrating viral proteins were detected by E311 (Fig. 6, lane 3). The largest mol. wt. protein (50K) migrated faster than VP70. The next largest protein migrated with the faster moving form of the VGP48 doublet and the smallest protein (45K) migrated slightly faster than VGP48. We did not find any protein migrating at 26K or faster. Therefore, GP1 appears to be relatively resistant to tunicamycin treatment, while VP70 and its products are sensitive to tunicamycin. The simplest interpretation of the Western blot showing the effect of tunicamycin on VP70 and its products (Fig. 6, lane 3) would be to equate the 50K protein with oligosaccharide-free VP70, the 45K protein with oligosaccharide-free VGP48 and the absence of a protein corresponding to the sugar-free GP26 to its being too small to be retained on the gel.

[14C]Glucosamine incorporation into viral glycoproteins in tunicamycin-treated infected cells

To determine if any of the RS virus glycoproteins synthesized in the presence of tunicamycin were glycosylated, we used [14C]glucosamine to label infected and uninfected cells in the presence and absence of tunicamycin. The protocol of tunicamycin treatment used above was also used here, except that in this instance we added [14C]glucosamine (2.5 μCi/ml) at 20 h post infection. The cells were washed, solubilized at 48 h, and aliquots containing equal TCA-precipitable counts were reduced and analysed by SDS–PAGE. GP1, VGP48 and GP26 were labelled with [14C]glucosamine in the absence of tunicamycin (Fig. 7, lane 4), but only the differently migrating GP1 appears to be labelled in the presence of tunicamycin (Fig. 7, lane 5). (Refer to Fig. 6 for the identification of the differently migrating viral protein bands in the presence of tunicamycin.) In addition, tunicamycin prevented the incorporation of mannose into GP1 as well as the other viral glycoproteins (Fig. 8, lanes 4 and 5). These results suggest that GP1 is glycosylated by another mechanism, in addition to that of N-linked oligosaccharide attachment.
Oligosaccharides of RS virus glycoproteins

Fig. 6. Western transblot of tunicamycin- and monensin-treated RS virus-infected HeLa cells. The nitrocellulose, minus lane 1 containing the virus marker, was reacted against antiserum E028 or E311 at 1:50, followed by 125I-Protein A. Lane 1, transblot of purified [14C]glucosamine-labelled RS virus; lanes 2 and 7, infected cells (40 µg); lanes 3 and 8, tunicamycin-treated infected cells (80 µg) lanes 4 and 9, monensin-treated infected cells (160 µg); lanes 5 and 10, monensin-treated uninfected cells (160 µg); lanes 6 and 11, uninfected cells (160 µg). Lanes 2 to 6 were reacted with E311 and lanes 7 to 11 were reacted with E028. (The figure is a composite of different X-ray film exposures of the same transblot.)

**Effect of monensin on infectious virus yields**

The ionophore monensin was used to determine whether GP1 contained O-linked oligosaccharides. Monensin blocks O-linked oligosaccharide addition to glycoproteins as well as the processing of N-linked oligosaccharides to the complex type (Johnson & Spear, 1983; Niemann et al., 1982). Monensin also reduces infectious virus yields (Alonso & Compans, 1981; Chatterjee et al., 1982; Johnson & Schlesinger, 1980; Johnson & Spear, 1982; Payne & Kristensson, 1982; Pesonen & Kääriäinen, 1982). To determine the minimum monensin concentration that maximally affected RS virus yields, concentrations of monensin ranging from 0.1 µM to 5.0 µM were added to infected cells 2 h after infection. At 46 h, when the control plates showed a great deal of c.p.e., all the plates were first frozen, then thawed and assayed. At 0.1 µM-monensin, the infectious RS virus yield was inhibited 99.6%, so that higher monensin concentrations were not much more inhibitory.

**Synthesis of viral glycoproteins in monensin-treated infected cells**

To determine the effect of monensin on RS virus glycoprotein synthesis, infected and uninfected cells were either mock-treated or treated with 0.1 µM-monensin at 2 h post-infection. At 48 h, the cells were washed, solubilized and analysed by Western transblot, by the same procedure as used above with the tunicamycin-treated cells. The results are presented in Fig. 6 (lanes 2, 4, 7 and 9). Monensin treatment had no effect on the migration of VP70 and VGP48 (though the VGP48 band was less diffuse in the monensin-treated cells), while GP26 migrated
slightly faster than in mock-treated cells (Fig. 6, lanes 2 and 4). However, monensin treatment did affect GP1, i.e. this protein disappeared and instead a widely diffuse band appeared in the region of the GP1 precursor (46K) (Fig. 6, lane 9). Therefore, in contrast to the results following tunicamycin treatment, VP70 and its products VGP48 and GP26 are relatively resistant, while GP1 is sensitive to monensin treatment.

Treatment of RS virus glycoproteins with endo-H

The results with tunicamycin and monensin suggested that GP1 contains both O- and N-linked oligosaccharides, while VP70 and its products contain only N-linked oligosaccharides. To confirm that GP1 had N-linked as well as O-linked oligosaccharides and to determine the molecular weights of the non-glycosylated proteins, as well as provide additional information about the types of "linked oligosaccharides attached to the RS virus glycoproteins, we used endo-H. This endoglycosidase cleaves high-mannose, but not complex N-linked oligosaccharides or O-linked oligosaccharides, from glycoproteins.

Because GP1 incorporates methionine poorly but does incorporate adequate quantities of leucine (Dubovi, 1982), it was labelled with [3H]leucine, while VP70 was labelled with [35S]methionine in pulse-chase experiments in RS virus-infected cells. The immature (pulsed cells) and mature (chased cells) form of the glycoproteins were immunoprecipitated and either mock-treated or treated with endo-H.

The results of these experiments are shown in Fig. 9. The precursor of GP1 (46K), found after the pulse (lane P, ) was sensitive to treatment with endo-H and migrated with an estimated
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Fig. 8. Fluorogram of Laemmli polyacrylamide gel of $[^3H]$mannose-labelled infected and mock-infected HeLa cells in the presence and absence of tunicamycin. Each lane of cells contains equal TCA-precipitable counts. Lane 1, $[^14C]$glucosamine labelled RS virus; lanes 2 to 5, $[^3H]$mannose-labelled cells; lane 2, mock-infected cells; lane 3, tunicamycin-treated mock-infected cells; lane 4, infected cells; lane 5, tunicamycin-treated infected cells; lane 6, $[^35S]$methionine-labelled RS virus. (The X-ray film was exposed to the gel for 6 weeks (overexposing the marker viruses) in order to show GP1 in the untreated infected cells (lane 4).)

mol. wt. of 33K (lane P$_H$ +). However, the mature form, GP1, found at the chase, was resistant to this enzyme (lanes C$_3$H$-$ and C$_3$H$+$). The other precursor, VP70, found in the pulse (lane P$_S$ $-$), was also endo-H-sensitive and migrated with an estimated mol. wt. of 55K after treatment (P$_S$ +). The mature form VGP48, in the chase, was endo-H resistant (lanes C$_2$S$-$ and C$_2$S$+$). GP26 was not seen in the lanes (C$_2$S $-$ and C$_2$S$+$) containing the proteins of infected cells following the chase, probably because the small size of its polypeptide chain (approx. 9K) limited the quantity of $[^35S]$methionine that could be incorporated.

These results provided additional proof that GP1 contains N-linked as well as O-linked oligosaccharides and that the unglycosylated forms of GP1 and VP70 have estimated mol. wt. of 33K and 50K, respectively. (The difference in mol. wt. between the VP70 forms of 55K in this experiment and 50K in the tunicamycin experiment was probably due to the residual glucosamine residues remaining after cleavage.) The results with endo-H also suggested that the mature forms of the N-linked oligosaccharides of the RS virus glycoproteins are mainly of the complex type.
Fig. 9. Fluorogram of Laemmli polyacrylamide gel of endo-H-treated RS virus glycoprotein immune precipitates. Infected cells were pulsed for 15 min with $[^3H]$leucine ($P_H$) and then chased for 3 h ($C_{3H}$) or pulsed for 10 min with $[^35S]$methionine ($P_S$) and then chased for 2 h ($C_{2S}$). The cells were solubilized, immunoprecipitated with either E028 (H) or E311 (S) and the immune precipitates were either mock-treated (lane $-$) or treated (lane $+$) with endo-H for 20 h at 37 °C. Lane 1, $[^14C]$glucosamine-labelled RS virus; lane 10, $[^35S]$methionine-labelled RS virus.

DISCUSSION

We have prepared rabbit antisera to the SDS-denatured forms of the two RS virus glycoproteins GP1 and VP70 for the express purpose of identifying and analysing these proteins within infected cells. We have determined the location of VP70 and the types and relative quantities of each type of the oligosaccharides attached to the polypeptide backbones of the viral glycoproteins. Our results argue against the conclusion of a previous study that a viral protein of 66K is a structural polypeptide (Fernie & Gerin, 1982). We present conclusive evidence both by radiolabel and Western transblot analysis that such a protein is not associated with purified virus. However, as reported by other laboratories, a 70K protein (VP70) was detected when infected cells were analysed in SDS-polyacrylamide gels under reducing conditions (Bernstein & Hruska, 1981; Dubovi, 1982). Our results, with trypsin treatment of infected cells, indicated that 80 to 90% of this protein is located on the surface of the plasma membrane. Trypsin treatment also removed 90% of its cleavage products, VGP48 and GP26, from infected cells. We have found by pulse-chase studies that the precursor VP70 chases into the products VGP48 and GP26 only after 30 min of chase (unpublished results), ample time for a viral glycoprotein to reach the plasma membrane (Herrler & Compans, 1983; Strous & Lodish, 1980). In addition to VP70 and its products, 90% or more of the mature RS virus produced in the infected cell is found associated with the plasma membrane. These virions remain attached to the plasma membrane after budding (Peeples & Levine, 1980). Since our preparations of purified virus, which consist
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Almost entirely of released virus, contain no VP70, but only its proteolytically processed products, VGP48 and GP26, it is possible that proteolytic processing of VP70 is involved in the release of budded virions, and that this is a limiting step in infected cells. However, it is not possible to test the validity of this hypothesis with the data presented in this paper.

One of the differences between N-linked and O-linked oligosaccharides is their sugar content. N-linked oligosaccharides contain mannose whereas O-linked oligosaccharides do not (Herp et al., 1979; Schachter, 1974; Slomiany & Slomiany, 1978; Thomas & Winzler, 1969; Tulsiani et al., 1982; Waechter & Lennarz, 1976). The finding that the RS virus glycoproteins were labelled to different extents with mannose suggested that different types of oligosaccharide chains were added to each glycoprotein. We used the glycosylation inhibitors tunicamycin and monensin, and one endoglycosidase, endo-H, to determine the types and the relative amounts of each type of oligosaccharide that were associated with these glycoproteins.

Tunicamycin treatment increased the electrophoretic mobility of the precursor VP70 and its products. The migration of VP70 was shifted to \( M_r 50K \) in the presence of tunicamycin, suggesting that the precursor protein contained 20K of N-linked oligosaccharides. One product of VP70, VGP48, migrated with \( M_r 45K \) in the presence of tunicamycin, suggesting that VGP48 contains 3K of oligosaccharides and that GP26 contains the remaining 17K of N-linked oligosaccharides. If so, the removal of 17K of oligosaccharides from GP26 would result in a 9K non-glycosylated polypeptide which would not have been retained on the gel (Fig. 6). However, at present we cannot exclude the possibility that we do not find oligosaccharide-free GP26 because the antibody is directed against the sugar determinants of this polypeptide.

Tunicamycin has been used in other studies as a tool for determining the polypeptide contribution to the mol. wt. of glycoproteins (Diggelmann, 1979; Morrison & Simpson, 1980; Oker-Blom et al., 1983). Endo-H treatment has substantiated this particular use of tunicamycin and provided more evidence for an estimated mol. wt. of 50K for the non-glycosylated VP70. Of particular interest, in this regard, is the report that a mRNA isolated from RS virus-infected cells, RNA5, codes for a 59K polypeptide, i.e. approximately the anticipated size for the non-processed, non-glycosylated VP70 (Huang & Wertz, 1983). Therefore, RNA5 may code for the unmodified 70K protein which is subsequently processed, by removal of the leader sequence, glycosylation and cleavage.

The other virus-associated glycoprotein, GP1, was relatively resistant to tunicamycin. This protein migrated slightly faster in the presence of the drug, with a shift in \( M_r \) from 90K to 83K, indicating the presence of 7K mol. wt. of N-linked oligosaccharides. However, this altered GP1 was still glycosylated, suggesting the presence of O-linked oligosaccharides, since tunicamycin does not prevent the addition of oligosaccharides with this type of sugar linkage. We tested this assumption by treating RS virus-infected cells with monensin. Monensin prevents the attachment of O-linked oligosaccharides as well as the processing of N-linked oligosaccharides to the complex type, functions known to reside in the Golgi complex (Bretz et al., 1980; Hanover et al., 1980; Johnson & Spear, 1982, 1983; Niemann et al., 1982; Schachter, 1974). GP1 was very sensitive to monensin treatment, and in monensin-treated infected cells it migrated as a diffuse band at the approximate position of its 46K precursor, i.e. with a \( M_r \) range of from 46K to 50K. The diffuse nature of this band suggests a heterogeneity that could result from variation in the post-translational removal of mannose residues, which can occur in the presence of monensin (Johnson & Schlesinger, 1980). The effect of the monensin treatment on the migration of GP1 is greater than would be expected from a block that involved only the processing of N-linked oligosaccharides to their complex form and therefore the majority of the mol. wt. loss must be attributable to a block in the formation of O-linked oligosaccharides. Thus, O-linked oligosaccharides may make up from 40K to 44K of the mol. wt. of GP1. These O-linked oligosaccharides contained glucosamine (Fig. 5) as is also true of other glycoproteins with O-linked oligosaccharides (Niemann & Klenk, 1981; Slomiany & Slomiany, 1978). We have found by pulse-chase studies that the precursor of GP1 (46K) is chased into GP1 only after 30 min (unpublished results). This suggests a late processing event, i.e. the addition of O-linked oligosaccharides to this precursor, which occurs in the Golgi region. In the presence of tunicamycin, one protein band migrated with an estimated mol. wt. of 33K, i.e. faster than the...
46K GP1 precursor. In addition, when the 46K GP1 precursor was treated with endo-H, it also migrated with a mol. wt. of 33K. When considered together, these results suggest that the non-glycosylated form of GP1 has a mol. wt. of 33K and that the shift in the migration that results from the maturation of GP1 is due to the addition of carbohydrate. The mRNA for GP1 could be the recently isolated RNA2b which is translated in vitro into a 36K polypeptide not found in RS virus-infected cells (Collins et al., 1984).

It has been suggested that VP70 is the fusion protein of RS virus (Fernie & Gerin, 1982; Gruber & Levine, 1983; Walsh & Hruska, 1983), and like a classical paramyxovirus fusion protein, it is proteolytically processed with its two polypeptides held together by disulphide bonds. The results reported here demonstrate that, like the other paramyxovirus glycoproteins, the precursor, VP70, and its products contain only N-linked oligosaccharides (Herrler & Compans, 1983; Kohama et al., 1978; Schwalbe & Hightower, 1982). It would seem that the GP26 product contains nearly all of the N-linked oligosaccharides of the precursor VP70, thereby making the carbohydrate contribution to its Mr greater than the polypeptide contribution. In this regard, GP26 resembles the F2 glycopeptide of other paramyxoviruses, which also have a high carbohydrate per unit protein ratio (Hardwick & Bussell, 1978; Scheid & Choppin, 1977). The molecular weight of GP26 is also close to that reported for the F2 glycopeptides of other paramyxoviruses.

In contrast to VP70, GP1 differs radically from the glycoproteins reported for other paramyxoviruses. Though the function of GP1 has not yet been determined, the designation of VP70 as the fusion protein carries the implication that GP1 is the attachment protein. However, GP1 is not present in the virion as disulphide-linked dimers or tetramers (Gruber & Levine, 1983), which are the usual states for the attachment proteins of other paramyxoviruses (Graves, 1981; Hardwick & Bussell, 1978; Herrler & Compans, 1983). In addition, it does not appear to be the major glycoprotein of the envelope, as one would expect for an attachment protein. Only N-linked oligosaccharides have been found associated with the attachment proteins of other paramyxoviruses and the polypeptide contribution to their Mr is much greater than the carbohydrate contribution (Herrler & Compans, 1983; Kohama et al., 1978; Morrison & Simpson, 1980; Schwalbe & Hightower, 1982). The situation with GP1 is just the reverse. The carbohydrate appears to contribute more to the Mr of GP1 than does the polypeptide backbone, although the migration of GP1 may not be due entirely to its size, but also to its shape. In addition, GP1 possesses both N- and O-linked oligosaccharides. Although glycoproteins with O-linked or both O- and N-linked oligosaccharides have been found in other viruses (Holmes et al., 1981; Johnson & Spear, 1982, 1983; Niemann & Klenk, 1981; Shida & Dales, 1981; Wenske & Courtneyn, 1983), glycoproteins with O-linked oligosaccharides have not been found in any paramyxoviruses, until now. Taken together, these differences may point to some fundamental difference between RS virus and the other members of the Paramyxoviridae.

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REFERENCES


JOHNSON, D. C. & SCHLESINGER, M. J. (1980). Vesicular stomatitis virus and Sindbis virus glycoprotein transport to the cell surface is inhibited by ionophores.

JOHNSON, D. C. & SPEAR, P. G. (1982). Monensin inhibits the processing of herpessimplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells.


LEE, G. T.-Y., PARA, M. F. & SPEAR, P. G. (1982). Location of the structural genes for glycoproteins gD and gE and for other polypeptides in the S component of herpes simplex virus type 1 DNA.


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