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Role of Oligosaccharides in the Structure and Function of Respiratory Syncytial Virus Glycoproteins

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The contribution of oligosaccharides to the structural and functional make-up of respiratory syncytial (RS) virus G and F proteins was investigated by observing the effects of various oligosaccharide-specific enzymes on their molecular size as well as on virus infectivity. The N-linked oligosaccharides of the F protein were completely removed by endoglycosidase F and *N*-glycanase. Addition of oligosaccharides to F protein during synthesis was completely inhibited by the drug tunicamycin (TM), an inhibitor of N-linked glycosylation. Glycosylation of the G protein was partially resistant to TM resulting in an 80-kDa form designated G_{TM}. The G protein was estimated to contain approximately 3% N-linked and 55% O-linked carbohydrates, based on migration of G and G_{TM} in polyacrylamide gels. Furthermore, treatment of detergent-extracted G protein with endoglycosidase F and endo-α-*N*-acetylgalactosaminidase, enzymes that specifically cleave N-linked and O-linked oligosaccharides, respectively, generated a variety of partially unglycosylated species, ranging in molecular weight from approximately 80 to 40 kDa. Virus infectivity was sensitive to limited removal of N-linked or O-linked oligosaccharides by endoglycosidases under conditions which did not greatly alter the molecular weight of the G protein. Thus, G and F protein oligosaccharides readily accessible to enzymatic removal are presumed to play an important role in the infectious process.

INTRODUCTION

Respiratory syncytial (RS) virions and infected cells contain seven major proteins (Lambert and Pons, 1983; Pringle *et al.*, 1981; Levine, 1977; Wunner and Pringle, 1976) and three small minor nonstructural polypeptides, totaling 10 unique viral proteins (Huang *et al.*, 1985). The two glycoproteins, designated G and F, are responsible for cell attachment (Levine *et al.*, 1987) and cell fusion, respectively (Walsh and Hruska, 1983). The unglycosylated G (36 kDa) and F (59 kDa) polypeptides synthesized during *in vitro* translation have been identified (Collins *et al.*, 1984). The *in vivo* synthesized G (84–90 kDa) and F (68 kDa) glycoproteins have been identified by two-dimension nonreducing and reducing SDS–PAGE of purified virions and immunoprecipitates of infected cells (Lambert and Pons, 1983). RS virus F protein consists of two smaller disulfide-bonded subunits, F_1 (48 kDa) and F_2 (19–21 kDa) (Fernie and Gerin, 1982; Gruber and Levine, 1983; Lambert and Pons, 1983). Monoclonal antibody to F inhibits cell fusion and partially neutralizes virus infectivity (Walsh and Hruska, 1983). Polyclonal monospecific antibody raised to G protein neutralizes RS virus (Walsh *et al.*, 1984) and inhibits virus attachment to cells (Levine *et al.*, 1987). Thus, F and G proteins appear to be functionally similar to the F (fusion) and HN or H (attachment) proteins of other paramyxoviruses except that RS virus has no hemagglutinin or neuraminidase activity (Richman *et al.*, 1971).

Tunicamycin (TM) inhibits lipid–dolichol-mediated N-linked glycosylation of asparagine residues in nascent proteins (Takatsuki *et al.*, 1971, 1975). The carbohydrate moieties (either "high mannose" or "complex") of viral glycoproteins are most commonly N-linked to asparagine residues via the dolichol phosphate pathway and are thus sensitive to the inhibitory effects of TM (Leavitt *et al.*, 1977). TM completely inhibits glycosylation of the HN and F proteins of a number of paramyxoviruses and has allowed identification of unglycosylated forms of viral glycoproteins (Nakamura *et al.*, 1982). However, it has been shown that several animal viruses contain tunicamycin-resistant O-linked carbohydrates on their envelope glycoproteins (Holmes *et al.*, 1981; Johnson and Spear, 1983; Niemann *et al.*, 1984, 1982; Olofsson *et al.*, 1981; Shida and Dales, 1981). O-Linked oligosaccharides are less structurally complex, compared to N-linked oligosaccharides, and are found linked to serine, threonine, hydroxylysine, or hydroxyproline residues in proteins (Sharon and Lis, 1981). We have found that glycosylation of RS virus G protein was partially resistant to tunicamycin treatment resulting in an 80-kDa form of this protein (Lambert and Pons, 1984). The G protein has been shown to contain O-linked oligosaccharides and a high number of potential O-linked glycosylation sites were present in its sequence (Gruber and Levine, 1985; Wertz *et al.*, 1985).

Several laboratories have investigated the effects of both tunicamycin and monensin on synthesis and pro-

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cessing of RS virus glycoproteins (Lambert and Pons, 1983; Gruber and Levine, 1985; Wertz *et al.*, 1985; Satake *et al.*, 1985; Fernie *et al.*, 1985). Monensin, an ionophore which inhibits membrane flow through the Golgi apparatus, and thus nonspecifically inhibits O-linked glycosylation (Griffiths *et al.*, 1983), did not inhibit transport of either G or F of RS virus glycoproteins to the cell surface (Satake *et al.*, 1985). Fernie *et al.* (1985) demonstrated that several N-linked glycosylated precursor species (29 kDa to a predominant 45 kDa) of the G protein are processed into mature G protein (VP84). No intermediate forms of G protein were observed between 45 and 84 kDa, leading these investigators to suggest that the G protein might be a dimer of the 45-kDa intermediate form.

Since the immune response to the G and F proteins appears to be important in protection and recovery from RS virus infection, it is important to further examine the structure and function of these proteins so that their roles in the pathology and immunology of this virus can be better understood. In this report, the contribution of N-linked and O-linked oligosaccharides to the structural and functional make-up of the G and F proteins was investigated, using endoglycosidases specific for these carbohydrate side chains. These experiments allowed determination of the role these oligosaccharides play both in the structural integrity of G and F proteins, as determined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and in virus infectivity.

MATERIALS AND METHODS

Cells and virus

HEp2 and CV-1 cells were used in this study. Cells were grown in minimum essential medium (MEM) supplemented with heat-inactivated fetal bovine serum. The Long strain of RS virus was prepared as previously described (Lambert and Pons, 1983). Virus infectivity assays were carried out in HEp2 cell monolayers using the methylcellulose overlay method as previously described (Lambert *et al.*, 1980).

SDS–PAGE purification of RS virus G, F, and N proteins

RS virions, grown in CV-1 cells labeled with either [^{3}H]glucosamine ([^{3}H]Glu) or ^{3}H-amino acids (^{3}H-aa) at 50 μCi/ml, were purified as previously described (Lambert and Pons, 1983). Virus pellets were resuspended in SDS–PAGE sample buffer (Laemmli, 1970) without 2-mercaptoethanol and run on preparative 10% polyacrylamide gels (Laemmli, 1970). Recovery of proteins from gels was done essentially as described by Shida and Dales (1981). Briefly, 1-cm-wide longitudinal strips removed from gels were cut into 2-mm sections and counted in a liquid scintillation counter to locate labeled protein bands. Protein bands cut from the remainder of the gel were eluted into 0.1% SDS in dH_2O, for 18 hr at room temperature, and precipitated with 6 vol of cold 100% acetone at −20° overnight. Purified proteins were pelleted at 20,000 *g*, air dried, and dissolved in dH_2O containing 0.5% NP-40.

Glycosidase digestion of RS virus glycoproteins

For studies with gel-purified glycoproteins, oligosaccharide-specific enzymes were added to 10 μl of gel-purified glycoproteins (G or F) or nucleocapsid (N) protein (as described above) in appropriate digestion buffers (see below). Enzyme concentrations indicated are amounts in the final reaction mixture. Influenza virus neuraminidase (Calbiochem, 0.1 U/ml final concentration) and endoglycosidase F (Endo F, 8.0 U/ml, NEN) digestion was carried out in 0.02 *M* sodium phosphate buffer, pH 6.1, 50 m*M* EDTA, 1.0% NP-40, 0.1% SDS, 5 μg/ml phenylmethylsulfonyl fluoride (PMSF). Digestion with peptide:*N*-glycosidase F (*N*-glycanase™, Genzyme, Boston, MA; 26 mU/μl) was carried out in 0.02 *M* sodium phosphate buffer, pH 8.6, 20 m*M* EDTA, 2.0% NP-40, 0.5% SDS, 5 μg/ml PMSF. Digestion with endo-α-*N*-acetylgalactosaminidase, (*O*-glycanase™, Genzyme, Boston, MA; 33 mU/μl) was carried out in 0.02 *M* sodium phosphate, pH 6.1, 2 m*M* calcium acetate, 1.0% NP-40, 0.1% SDS, and 5 μg/ml PMSF. Migration of untreated samples in each buffer was identical (not shown). Proteins, in the appropriate buffer, were heated in a boiling water bath for 2 min and then chilled on ice before addition of enzyme for 24 hr at 37°. An equal volume of 2× SDS–PAGE sample buffer containing 2-mercaptoethanol was added and samples were resolved in 10% polyacrylamide gels.

For studies of virion-associated G and F glycoproteins or G protein from virions extracted with 20 m*M* *n*-octylglucoside, enzyme digestions were carried out in Dulbecco's phosphate-buffered saline lacking calcium and magnesium ions (PBS). Neuraminidase (from *A. ureafaciens*, 0.1 U/ml, Boehringer-Mannheim) was used for removal of terminal 5-*N*-acetylneuraminic acid (NANA) or 5-*N*-glycolylneuraminic acid (NGNA) residues. Endo F (17 U/ml), from *F. meningosepticum* (New England Nuclear (NEN) or Boehringer-Mannheim), and Endo F free of peptide *N*-glycosidase F [Endo F(-PNGase F) 17 U/ml, Boehringer-Mannheim] are enzymes which cleave both complex and high-mannose N-linked oligosaccharides. As designated in the text, either *O*-glycanase™ (33 mU/μl, Genzyme)

or endo-α-*N*-acetylgalactosaminidase (1.9 U/ml, Boehringer-Mannheim) was used to digest O-linked oligosaccharides (Umemoto *et al.*, 1977). Concentrations and combinations of enzymes are also indicated in the figure legends.

Western blot analysis of RS glycoproteins

SDS–PAGE of enzyme-treated proteins and Coomassie blue prestained protein molecular weight markers (Diversified Biotech, Newton Centre, MA, or Bethesda Research Laboratories, BRL, Bethesda, MD) were carried out in 10% gels (Laemmli, 1970). Western blots were performed essentially as described by Towbin *et al.* (1979) except that non-fat dried milk (5% solution in PBS) was used to block binding sites on nitrocellulose membranes (1 hr at room temperature) after electroblotting. Blots were probed with monoclonal antibody (MAb) directed against G or F proteins. MAb L7, L9 (anti-RS virus G protein), and L4 (anti-RS virus F protein) were kindly provided by Dr. Edward Walsh (Rochester University Medical School, Rochester, NY). Antibodies were used at a dilution of 1:2000 in 5% dried milk dissolved in PBS. MAb–viral protein bands were detected using a Western blot kit containing horseradish peroxidase-labeled goat anti-mouse IgG (Bio-Rad).

RESULTS

Endoglycosidase digestion of gel-purified virion glycoproteins

To accurately determine the effects of enzymes specific for N-linked or O-linked oligosaccharides on the structure of radiolabeled RS virus G and F proteins, it was necessary to first gel purify virion proteins. Analysis of gel-purified proteins demonstrated that they were not contaminated with other proteins and that they comigrated with virion proteins (Fig. 1). Data presented in Fig. 2 show that purified G protein was relatively unaffected by neuraminidase treatment (^{3}H-aa G and [^{3}H]Glu G, lanes 2). Both endoglycosidase F and *N*-glycanase treatments (lanes 3 and 4, respectively), which remove N-linked oligosaccharides, decreased the size of G protein from 84 to approximately 80 kDa (^{3}H-aa and [^{3}H]Glu). Removal of N-linked oligosaccharides from ^{3}H-amino acid-labeled F_2 polypeptide (^{3}H-aa F, lanes 3 and 4) migrated more rapidly, whereas [^{3}H]-glucosamine-labeled F_2 polypeptide was not detectable ([^{3}H]Glu F, lanes 3 and 4). The migration of the F_1 subunit was unaffected by these enzyme treatments suggesting that the F_2 subunit contained the bulk of oligosaccharides present on the fusion protein.

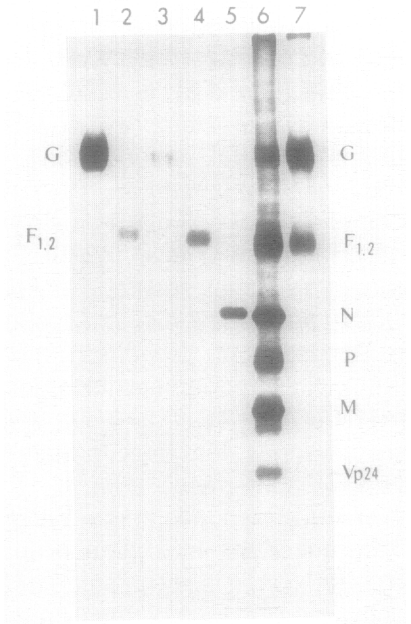

FIG. 1. Polyacrylamide gel purification of RS virus G, F, and N proteins. [^{3}H]glucosamine and ^{3}H-amino acid-labeled proteins of purified RS virions were separated by electrophoresis in preparative 10% polyacrylamide–SDS-containing gels. G, F, or N proteins were isolated from gel fractions as described under Materials and Methods. To test purity and condition of extracted proteins, aliquots of isolated proteins were electrophoresed in 10% gels. Lane 1, [^{3}H]-glucosamine-labeled G. Lane 2, [^{3}H]glucosamine-labeled $F_{1,2}$. Lane 3, [^{3}H]amino acid-labeled G. Lane 4, ^{3}H-amino acid-labeled $F_{1,2}$. Lane 5, ^{3}H-amino acid-labeled N protein. Purified virions labeled with [^{3}H]amino acids (lane 6) or [^{3}H]glucosamine (lane 7) were run as controls. Viral proteins are designated G (large glycoprotein), $F_{1,2}$ (fusion protein), N (nucleocapsid protein), P (phosphoprotein), M (matrix protein), and Vp24.

^{3}H-aa G (lane 5) and [^{3}H]Glu G (lane 5) show that treatment with endo-α-*N*-acetylgalactosaminidase (*O*-glycanase), which cleaves O-linked oligosaccharides, decreased the amount of the detectable mature G protein (84 kDa) and generated a variety of smaller species of [^{3}H]glucosamine-labeled G protein. This mixture of smaller forms of G protein most probably represented G protein containing N-linked oligosaccharides and varying amounts of O-linked carbohydrate side chains. This most likely accounts for the lack of detectable labeled bands in ^{3}H-aa G protein (lane 5). The absence of complete degradation of G to a discrete protein band, containing only N-linked oligosaccharides, was likely due to incomplete enzymatic removal of O-linked oligosaccharides. *O*-glycanase treatment had no apparent effect on the migration of subunits of F protein (^{3}H-aa F and [^{3}H]Glu F, lanes 5) or the N protein (^{3}H-aa N, lane 5) indicating that no protease activity was present in these enzyme preparations.

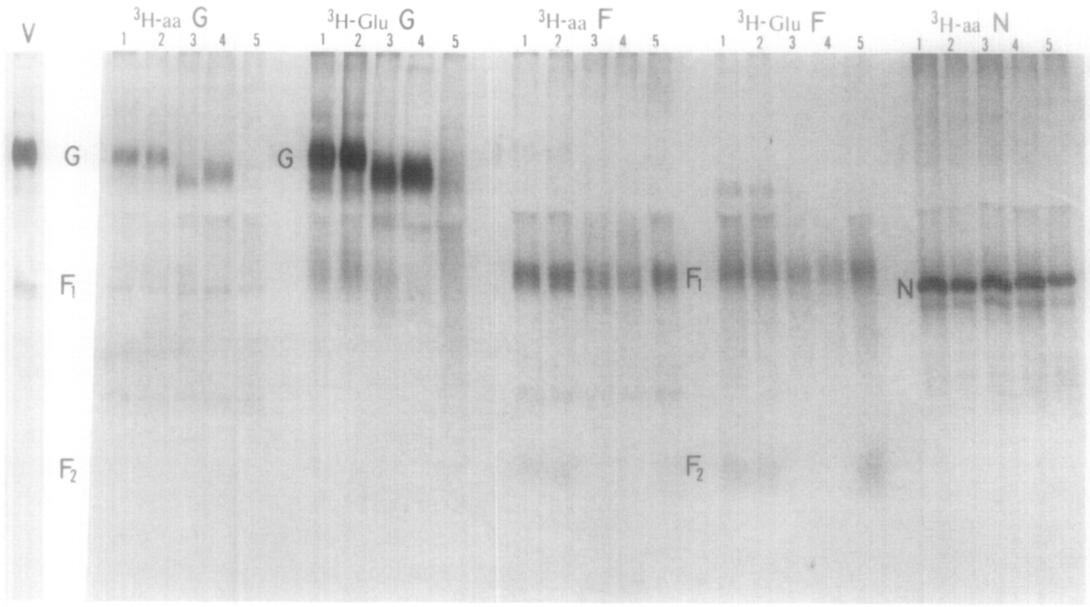

FIG. 2. Glycosidase digestion of purified RS virus glycoproteins. Purified proteins were treated with various endoglycosidases and neuraminidase. Lanes 1, untreated. Lanes 2, neuraminidase-treated. Lanes 3, endoglycosidase F-treated. Lanes 4, *N*-glycanase-treated. Lanes 5, *O*-glycanase-treated. Purified protein preparations, ^{3}H-aa and [^{3}H]Glu G, F, or N proteins are designated at the top of each panel of five gel lanes.

Effects of endoglycosidase digestion on the structure of G and F proteins in virus particles

Because of the difficulties in identifying protein components of labeled G protein digests (Fig. 2), Western blot analysis of purified virion proteins was used (1) to further define the contribution made by oligosaccharides to the structure of G and F proteins and (2) to compare the susceptibility of TM-treated virion glycoproteins to endoglycosidase treatments. Nonreducing 10% polyacrylamide gels were used so that unreduced G and F proteins could be compared by Western blots probed with monoclonal antibody (MAb) directed against the G (Fig. 3A) or the F protein (Fig. 3B).

Results presented in Fig. 3A show that in the untreated sample (lane 1), two protein bands of approximately 175 kDa (G_{ag}) and 90 kDa (G) reacted with MAb against G protein suggesting that dimeric aggregates of the 90 kDa G protein were resolved in these nonreducing gels. Dimers of G or G_{TM} proteins were not observed in reducing gels. TM-treated virus contained two sets of doublet bands of approximately 175/155 and 86/80 kDa which reacted with anti-G MAb (Fig. 3, lane 8). Note that both endoglycosidase F (lane 3) and *N*-glycanase (lane 5) treatments also generated the 86/80 kDa doublet G from normal G protein. Thus, the two forms (86 and 80 kDa) of O-linked glycosylated G protein were incorporated into both untreated RS virions and virions grown in the presence of TM.

A 36-kDa band (G_{36}), representing totally unglycosylated G protein, was detected in virion preparations from TM-treated HEp2 cells (Fig. 3A, lanes 8–14). G_{36} in untreated (lane 8) and neuraminidase-treated (lane 14) cells did not react as well with MAb. The presence of this band in TM-treated virions suggests that a portion of G protein was not subject to either N-linked or O-linked glycosylation when synthesized in the presence of TM and moreover that these unglycosylated molecules were transported to the cell surface and incorporated into virion membranes. A 38-kDa form of G protein was observed in normal virions digested with Endo F free of peptide *N*-glycanase F [Endo F (-PNGase F)] (Fig. 3A, lane 4). The Endo F preparation used contained both enzymes. Less visible bands migrating at approximately 40 kDa were also observed in Endo F-treated (lane 3) and *N*-glycanase-treated (lane 5) samples. Most of the G protein treated with Endo F was reduced to approximately 80 kDa (lane 3), whereas Endo F (-PNGase F) had no visible effect on the migration of normal G protein (lane 4) other than causing the appearance of the 38-kDa G product. *N*-Glycanase also reduced the molecular weight of G protein to approximately 80 kDa (lane 5). Thus, under the conditions used, it appears that peptidase *N*-glycanase F (PNGase F) in the Endo F preparation was responsible for digestion of N-linked oligosaccharides from G protein.

None of the enzyme treatments altered the migration rate of the doublet G_{TM} bands (lanes 8–12) with the exception of *O*-glycanase (lane 13) indicating that G_{TM} contained no N-linked oligosaccharides. *O*-Glycanase hydrolyzed normal G protein (lane 6) to a mixture of smaller species of partially unglycosylated proteins. Digestion of G_{TM} with *O*-glycanase resulted in smaller species of partially unglycosylated G proteins migrating between 43 and 60 kDa.

In Fig. 3B, identical blots of nonreducing gels probed with MAb directed against the RS virus fusion (F) protein showed that Endo F (lane 3) digested the bulk of the glycosylated form of $F_{1,2}$ (68 kDa) to its unglycosylated form which migrated at approximately 59 kDa, similar to TM-treated F protein (F_{TM}, lanes 8–14). F protein treated with Endo F (-PNGase F) (Lane 4) was not affected. Migration of F_{TM} in these gels was not affected by any of the endoglycosidase treatments (Fig. 3B, lanes 9–14) demonstrating that F_{TM} contained no N-linked oligosaccharide. *O*-Glycanase treatment of $F_{1,2}$ decreased the molecular weight of this protein from 68 to approximately 65 kDa (Fig. 3B, lane 6) and indicates that a small amount of some contaminating enzyme was present in the *O*-glycanase preparation used since this was not seen in other experiments using different preparations of *O*-glycanase. Dimeric aggregates of $F_{1,2}$ (F_{ag}, Fig. 3B, lanes 1 and 2) but not of enzyme-treated cells (lanes 3–7) or in F_{TM} (lanes 8–14) were resolved in these gels.

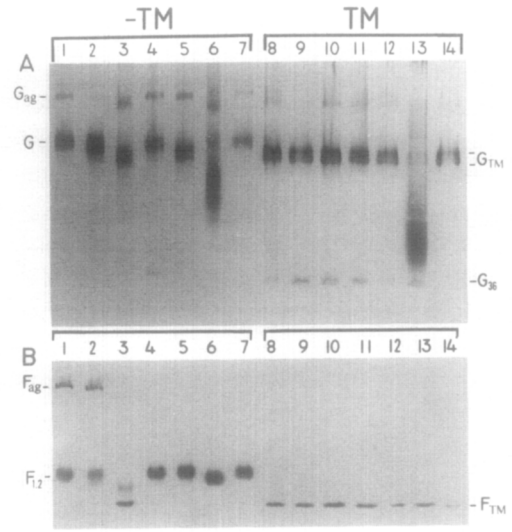

FIG. 3. Effects of endoglycosidase digestion on RS G and F proteins in whole virion membranes. Ten-microliter aliquots of purified virions, grown in untreated (–TM, lanes 1–7) or TM-treated (TM, 1 μg/ml; lanes 8–14) HEp2 cells, were incubated for 24 hr at 37° in PBS (lanes 1 and 8). Virions were treated with various enzymes in PBS: Lanes 2 and 9 with Endo F (NEN, 8 U/ml); lanes 3 and 10 with Endo F (Boehringer-Mannheim, 60 U/ml); lanes 4 and 11 with Endo F (-PNGase) (Boehringer-Mannheim, 60 U/ml); lanes 5 and 12 with *N*-glycanase (0.28 U/ml); lanes 6 and 13 with *O*-glycanase (1.15 U/ml); and lanes 7 and 14 with neuraminidase (Calbiochem, 0.1 U/ml). After enzyme digestions, virions were disrupted in 2× SDS–PAGE sample buffer without 2-mercaptoethanol and electrophoresed in 10% polyacrylamide gels at 30 mA/gel. Proteins were then blotted onto nitrocellulose for 17 hr at 20 V. Blots were blocked with 5% dried milk in PBS for 1 hr and probed with MAb against either the G protein (A) or the F protein (B). Monoclonal antibodies were detected with peroxidase-labeled rabbit anti-mouse IgG.

Analysis of the oligosaccharide structure of detergent-extracted G and G_{TM} proteins

Detergent extracted glycoproteins were used to determine whether complete removal of oligosaccharides on the G protein could be accomplished. RS virus glycoproteins extracted from purified virions with 20 m*M* octylglucoside were adjusted to 1% NP-40 and 0.1% SDS and digested with endoglycosidase F and with endo-α-*N*-acetylgalactosaminidase (*O*-glycan-peptide hydrolase, Boehringer-Mannheim Biochemicals) in the presence of neuraminidase to facilitate O-linked oligosaccharide removal (Fig. 4). Reducing gels were used to decrease the potential for aggregation and to more completely denature unglycosylated G protein species for optimal separation.

Western blots, probed with monoclonal antibody L9 specific for RS virus G protein, showed that neuraminidase digestion alone (Fig. 4, lane 1) generated a more diffuse (82–90 kDa) G protein band compared to untreated G protein (lane 5) which migrated at approximately 85 kDa. Removal of N-linked oligosaccharides with endoglycosidase F alone (lane 2) generated at least five bands migrating in the range of 75–85 kDa. Endo-α-*N*-acetylgalactosaminidase treatment, which specifically removes O-linked oligosaccharides, generated at least seven bands in the molecular weight range of 55–85 kDa (lane 3). Treatment with a mixture of Endo F and endo-α-*N*-acetylgalactosaminidase generated at least eight detectable bands having molecular weights of 40, 44, 48, 51, 54, 55, 60, and 81 kDa (lane 4) with the 55-kDa band being the most abundant. Faint bands representing partially unglycosylated forms of G protein were also detected between 60 and 80 kDa (lane 4). Similar treatment of G_{TM} protein generated five glycoprotein bands having molecular weights of approximately 40, 45, 48, 50 and 55 kDa (lanes 8 and 9). Control experiments with F protein and, more importantly, G_{36} demonstrated that no protease activity was detectable in these enzyme preparations (Figs. 3A, B). Thus, the smaller bands generated by glycosidase digestions in Fig. 4 represent various partially unglycosylated species of RS virus G protein.

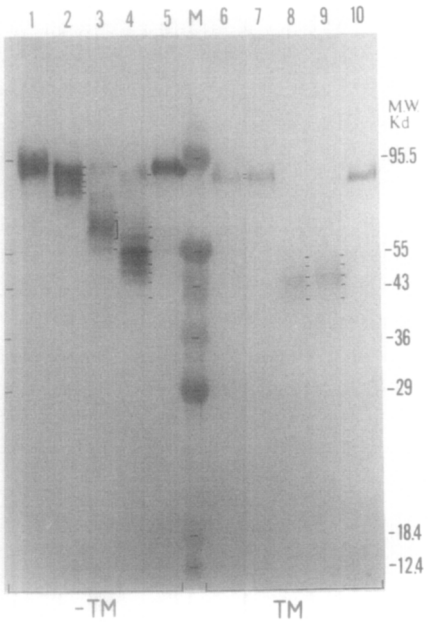

FIG. 4. Endoglycosidase digestion of *n*-octylglucoside-extracted RS virus G protein. Purified virions, from untreated HEp2 cells (−TM, lanes 1–5) or cells treated with 1 μg/ml TM (lanes 6–10), were extracted with 20 m*M* octylglucoside in PBS. Samples were adjusted to 1% NP-40 and 0.1% SDS, and then heated in a boiling waterbath for 2 min. After cooling to room temperature, 20-μl aliquots of virion glycoproteins was digested for 24 hr at 37° with neuraminidase (0.1 U/ml; lanes 1 and 6), neuraminidase plus Endo F (Boehringer-Mannheim, 17 U/ml; lanes 2 and 7), neuraminidase plus endo-α-*N*-acetylgalactosaminidase (Boehringer-Mannheim, 1.9 U/ml; lanes 3 and 8), neuraminidase plus a mixture of Endo F and endo-α-*N*-acetylgalactosaminidase (lanes 4 and 9), and PBS alone (lanes 5 and 10). Digested glycoproteins were electrophoresed in 10% polyacrylamide gels and electroblotted onto nitrocellulose. The lane marked M represents prestained marker proteins having molecular weights indicated on the right margin of the figure. Blots were probed with MAb L9 specific for RS virus G protein and detected by peroxidase-labeled rabbit anti-mouse IgG.

Effects of Endoglycosidase Treatments on Virus Infectivity

The importance of oligosaccharide side chains to the function of RS virus glycoproteins was evaluated by investigating the effects of several endoglycosidase enzymes on RS virus infectivity. The effects of the specific endoglycosidases were compared by incubation of virions in PBS at room temperature for 3 hr either without enzyme or with neuraminidase, *N*-glycanase, *O*-glycanase or a mixture of both *N*- and *O*-glycosidases. These conditions were chosen as a compromise between efficient enzyme digestion and thermal inactivation of virus infectivity. Corresponding aliquots of virus, disrupted in 0.5% NP-40, were diluted in appropriate buffers optimized for the individual enzymes (see Materials and Methods) and incubated under the same conditions to ensure that the enzyme preparations were active.

Proteins of virus samples treated with endoglycosidases, either in disruption buffers (Fig. 5, lanes 1–6) or in PBS (Fig. 5, lanes 7–12), were run in polyacrylamide gels, electroblotted onto nitrocellulose filters, and probed with a mixture of two monoclonal antibodies (L7 and L9) specific for RS virus G protein (Fig. 5). Lanes 6 and 12 represent the G protein of disrupted or whole virus, respectively, which was incubated on ice rather than at room temperature. Disrupted virus samples digested under denaturing conditions showed extensive changes in molecular weight as a result of oligosaccharide removal (Fig. 5, lanes 2–5). In addition, decreased MAb binding observed for several samples may indicate either protein denaturation or loss of protein (lanes 1 and 2). However, less dramatic alterations in electrophoretic mobility were seen for whole-virus G protein digested in PBS (Fig. 5, lanes 8–11). As one might predict, these results demonstrate that removal of oligosaccharides from G protein was more efficient, during the 3-hr incubation period used, with detergent-disrupted virus glycoproteins than with whole virus as evidenced by the differences in G protein migration rates.

Aliquots of each virus sample treated with endoglycosidases in PBS (Fig. 5, lanes 7–12) were taken, immediately after treatment, for virus titration on HEp2 cell monolayers (Fig. 6). Untreated virus (UNTx) incubated under identical conditions had a titer of 1.75×10^5 PFU/ml which represented 100% infectivity.

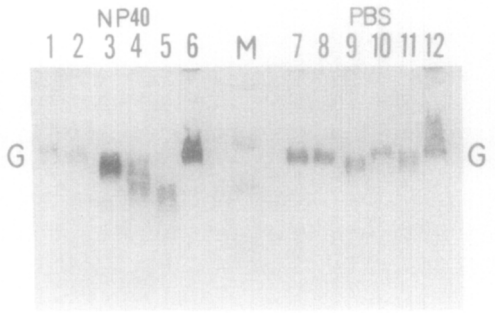

FIG. 5. Comparison of endoglycosidase treatments on the G protein of NP-40-lysed virus and virions in PBS by Western blot analysis. Blots were probed with a mixture of anti-G protein monoclonal antibodies L7 and L9. Virion G proteins represented in lanes 1–6 (NP-40) were treated in PBS containing 0.5% NP-40 and virions represented in lanes 7–12 (PBS) were in PBS alone. Lanes 1 and 7, were untreated. Lanes 2 and 8, neuraminidase (0.1 U/ml). Lanes 3 and 9, *N*-glycanase (14 U/ml). Lanes 4 and 10, *O*-glycanase (16.45 U/ml). Lanes 5 and 11, a mixture of *N*-glycanase (14 U/ml) and *O*-glycanase (16.45 U/ml). Lanes 6 and 12 are control lanes of virion preparations held on ice during the incubation period. Lane M indicates Coomassie blue prestained molecular weight marker proteins (BRL).

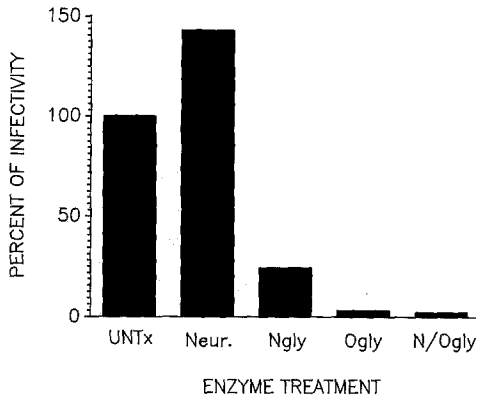

FIG. 6. Effects of endoglycosidase treatments on virus infectivity. Aliquots (10 μl) of virions treated with endoglycosidases in PBS (Fig. 5, lanes 8–13) were diluted with 990 μl Earle's saline containing 1% fetal bovine serum. Ten-fold dilutions were made in Earle's saline and virus was plaque assayed in duplicate on HEp2 cell monolayers in 35-mm tissue culture plates and overlayed with 0.75% methylcellulose in MEM. Virus titers were compared to untreated virus (100%) to obtain percentage of control virus. Treatments are represented by UNTx (untreated), Neur. (neuraminidase, 0.1 U/ml), Ngly (*N*-glycanase, 14 U/ml), Ogly (*O*-glycanase, 16.45 U/ml), N/Ogly (*N*-glycanase, 14 U/ml, and *O*-glycanase, 16.45 U/ml).

Treatment with neuraminidase increased the infectivity of RS virions by about 143% (Neur.), possibly by disaggregation of infectious virus particles. However, virus infectivity was reduced by 76% after treatment with *N*-glycanase (Ngly), which cleaves N-linked oligosaccharides. This indicates that removal of readily accessible N-linked oligosaccharides from the G and F proteins, had deleterious effects on virus infectivity. Treatment with *O*-glycanase alone (Ogly) or a combination of *N*-glycanase and *O*-glycanase (N/Ogly) caused reductions of 97–98% of RS virus infectivity. Thus, both N-linked and, to a greater extent, O-linked oligosaccharides appear to be necessary for RS virus infectivity. These are presumed to be only a few readily accessible oligosaccharide side chains because enzyme digestion of whole virions at room temperature caused relatively little alteration in migration rates of the Western blotted G proteins (Fig. 5, lanes 7–12).

DISCUSSION

In this report it was shown that RS virus G protein contained both N-linked and O-linked carbohydrate moieties which could be removed from the protein backbone with specific endoglycosidases generating discrete smaller molecular weight forms of G. These endoglycosidase studies support previous findings with tunicamycin and monensin, which showed that the G protein contains both N-linked and O-linked oligosaccharides (Lambert and Pons, 1984; Gruber and Levine, 1985; Wertz *et al.*, 1985; Satake *et al.*, 1985; Fernie *et al.*, 1985). The migration rate of the protein moiety of RS virus G protein in SDS–PAGE is approximately 36,000 Da (Collins *et al.*, 1984). Based on calculated molecular weights determined by SDS–PAGE, the TM and endoglycosidase data presented here indicate that the G protein contained approximately 58% carbohydrate (55% O-linked and 3% N-linked). Although subject to error due to potential SDS–PAGE migration anomalies, these calculations are consistent with nucleotide sequence analysis of the G protein gene (A2 strain) which indicates that there are four potential glycosylation sites for N-linked oligosaccharides and 91 serine and threonine residues which could serve as possible O-linked glycosylation sites (Wertz *et al.*, 1985).

Because of difficulties in labeling the protein moiety of RS virus G protein to high specific activity (Pringle *et al.*, 1981; Dubovi, 1982), Western blot analysis with monoclonal antibody was used in most of the experiments reported here in order to follow the migration of G protein endoglycosidase digestion products. Results demonstrated that discrete, smaller molecular weight forms of the G protein were obtained after endo-α-*N*-acetylgalactosaminidase (*O*-glycanase) treatments. Treatment with a mixture of Endo-F and endo-α-*N*-acetylgalactosaminidase produced eight partially unglycosylated G protein bands having molecular weights of 40, 45, 48, 51, 54, 55, 60, and 81 kDa and faint bands of G protein were also detected between 60 and 81 kDa. Similar treatment of G_{TM} generated five detectable glycoprotein bands having molecular weights of approximately 40, 45, 48, 50, and 55 kDa. Since control experiments with F protein demonstrated that no protease activity was detectable in these enzyme preparations, the smaller bands generated must represent various partially to almost totally unglycosylated species of RS virus G protein.

Endoglycosidase-generated intermediate forms of G protein (40–85 kDa) suggest that the 85- to 90-kDa G protein is not a dimer of the intracellular 45-kDa intermediate form as suggested by Fernie *et al.* (1985) but more likely represents a highly glycosylated monomer of mostly O-linked oligosaccharides. Results reported here strongly suggest that sufficient O-linked and N-linked oligosaccharides are added to the 36-kDa G protein during cellular processing to contribute to its 85–90 kDa fully glycosylated form. Attempts to dissociate possible 90-kDa dimeric forms of 45-kDa G protein using strong denaturing agents, such as 4 *M* urea and 6 *M* guanidine–HCl, had no effect on G protein migration in SDS–PAGE (data not shown). The molecular size of G protein O-linked oligosaccharides, released by endo-α-*N*-acetylgalactosaminidase and analyzed on BioGel P6 columns had an average molecu-

lar weight of about 1 kDa (Lambert, in preparation). Thus, on average, only about half of the possible O-linked glycosylation sites need be used to increase the molecular weight of G protein from the 45-kDa (N-linked intermediate form) to the 84- to 90-kDa "mature" form. Complete conversion of 85- to 90-kDa G protein to totally unglycosylated G protein (G_{36}) was not efficiently produced by endoglycosidase treatments of normal G protein used in this study, although G_{36} protein was identified in some TM-treated virion preparations. A possible explanation could be that "blocked" oligosaccharide cleavage sites or protein conformational constraints inhibited complete oligosaccharide removal using the conditions employed here. Interestingly, a faint band representing a 38-kDa form of G protein was observed in whole virions digested with Endo F(-PNGase F) and 40-kDa forms of G were also observed in Endo F-treated and *N*-glycanase-treated samples (Fig. 3). During these experiments, two forms of O-linked glycosylated G protein were resolved in TM-treated virions as evidenced by a doublet band of G_{TM}. This suggests heterogeneity in either size or number of the O-linked oligosaccharide side chains.

In agreement with others (Gruber and Levine, 1985; Fernie *et al.*, 1985), it was demonstrated here that glycosylation of the F protein was completely sensitive to TM treatment since no further digestion of F_{TM} was accomplished by N-linked oligosaccharide-specific endoglycosidases. Endo F and *N*-glycanase digestion of purified labeled fusion protein, demonstrated that the F_2 subunit was more highly glycosylated than F_1 (Fig. 2), and essentially all the N-linked oligosaccharides could be removed from the F protein by Endo F treatment. Comparison of molecular weight differences of F and F_{TM} proteins, and Endo F digested samples, indicates that F protein contained approximately 13% N-linked oligosaccharides.

The importance of oligosaccharide side chains in biologic and antigenic properties of viral glycoproteins has been reported. Neuraminic acid residues on the hemagglutinin protein are involved in adsorption of measles virus to the surface of cells (Dore-Duffy and Howe, 1978). Loss of a specific oligosaccharide side chain through mutation at a single glycosylation site on the hemagglutinin protein is responsible for increased virulence of avian (H5N2) influenza virus (Kawaoka *et al.*, 1984). Oligosaccharides might also contribute, either directly or indirectly, to antigenic sites on viral glycoproteins. For example, carbohydrate-dependent epitopes have been shown to exist in gC protein of herpes simplex virus type 1 (Sjoblom *et al.*, 1987).

Infectivity assays of RS virus, after treatment with enzymes which remove N-linked or O-linked oligosaccharides, demonstrated that although little alteration of the molecular weight of the G (attachment) protein was observed, virus infectivity was greatly reduced. Thus, limited removal of both N-linked and O-linked oligosaccharides greatly reduced virus infectivity and suggests that the few readily accessible N-linked or O-linked oligosaccharides on the G and F proteins may exert considerable influence on the attachment and/or penetration functions of RS virus glycoproteins. Studies to clarify the specific role of oligosaccharide-mediated virus–host cell interactions for RS virus are currently under way. It will be of interest to also determine whether these oligosaccharides play an important role in immunity to RS virus infections.

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