JOURNAL OF VIROLOGY, Jan. 1979, p. 336-343
0022-538X/79/01-0336/08$02.00/0

Vol. 29, No. 1

Oligosaccharide Chains of Avian RNA Tumor Virus Glycoproteins Contain Heterogeneous Oligomannosyl Cores

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Received for publication 7 June 1978

Chicken embryo fibroblasts (C/E phenotype) infected with subgroups B and C of the Prague strain of Rous sarcoma virus were radiolabeled with either [6-^{3}H]-glucosamine or [2-^{3}H]mannose, and virus was purified from the growth medium. The large envelope glycoprotein, gp85, was the only major radiolabeled component of purified virus. Pronase-digested glycopeptides from purified virus were analyzed by a combination of (i) gel filtration with columns of Sephadex G15/G50 and Bio-Gel P4 and (ii) enzymatic digestion of the oligosaccharide chains with specific exoglycosidases and endo-β-*N*-acetylglucosaminidases. The rather broad molecular weight distribution (approximately 2,000 to 4,000) for glycopeptides in these studies and previous studies in other laboratories was shown to represent actual heterogeneity in the carbohydrate moieties: (i) the glycopeptides contained both mannose-rich, neutral chains and complex, acidic chains with terminal sialic acid; and (ii) both classes of asparagine-linked carbohydrate structures exhibited heterogeneity in the size of the oligomannosyl core (a mixture of approximately 5 to 9 mannose units for the neutral structures, and 3 or 5 mannose units for the acidic structures). With the [2-^{3}H]mannose-labeled glycopeptides from Rous sarcoma virus, Prague strain subgroup C, most of the oligosaccharide chains were high-molecular-weight, acidic structures, with similar numbers of 3-mannose and 5-mannose core structures.

The major and minor glycoproteins of the avian RNA tumor viruses, designated gp85 and gp37 (also gp35), are the envelope components that define subgroup- or type-specific antigenicity and determine properties of viral host range, neutralization, and interference (1, 2, 6, 21). Recent studies indicate that gp85 and gp37 are distinct polypeptides, rather than gp37 being a cleavage product of gp85 (22), and the two glycoprotein species may exist as a disulfide-linked dimer in the virus envelope (20). Both glycoproteins may arise from the proteolytic cleavage of a common higher-molecular-weight precursor polypeptide (pr90) that is only partially glycosylated (7).

The sugar composition of purified gp85 from several subgroups of Rous sarcoma virus (RSV) is indicative of asparagine-linked oligosaccharides typical of those found on serum glycoproteins [(NeuNAc-Gal-GlcNAc)$_x$Man$_n$GlcNAc-GlcNAc(±Fucose)-Asn], with the carbohydrate representing approximately 40% by weight of subgroup A virus glycoprotein and 15% by weight of subgroups B and C virus glycoproteins (15-17). Gel filtration of Pronase-digested virus glycoprotein indicated that the radioactive sugar-labeled glycopeptides had a very heterogeneous size distribution and were generally larger than corresponding glycopeptides from Sindbis and vesicular stomatitis (VSV) viruses (10, 19, 26). Minor differences have been detected between the glycopeptides from viruses of the same subgroup released from transformed (sarcoma virus-infected) and untransformed (leukosis virus-infected or uninfected) chicken embryo fibroblasts, with additional terminal sialic acid in the sarcoma virus glycopeptides accounting for most of this difference (19, 26). Studies of the exoglycosidase sensitivity of unlabeled glycoprotein from subgroup A RSV suggested that gp85 contained both major classes of asparagine-linked oligosaccharide chains (17): the complex, acidic structures that contain sialic acid, galactose, *N*-acetylglucosamine, mannose, and fucose, and the mannose-rich, neutral structures that contain only mannose and *N*-acetylglucosamine. The possible role(s) of the glycoprotein oligosaccharides in the biosynthesis, maturation, and stability of the envelope glyco-

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proteins, or in viral infectivity and host range, is not clear.

The objective of these studies was twofold: (i) to determine whether the heterogeneity and large size of the glycopeptides were due to actual heterogeneity in the oligosaccharide moieties or due to heterogeneity in the residual peptide moiety, or possibly two oligosaccharides connected by a Pronase-resistant peptide; and (ii) to determine the size of the oligomannosyl cores and their distribution between the complex, acidic oligosaccharides and the high-mannose, neutral oligosaccharides, using the very informative techniques involving exoglycosidase and endo-β-*N*-acetylglucosaminidase digestion coupled with high-resolution analytical gel filtration.

MATERIALS AND METHODS

Preparation of radiolabeled virus. Standard cell culture techniques were used (31). Chicken embryo fibroblasts of C/E phenotype (prepared from fertile eggs obtained from Life Sciences Inc., St. Petersburg, Fla.) infected with subgroups B and C of the Prague strain of RSV (PrB- and PrC-RSV, obtained from M. Linial and P. E. Neiman, originally from P. Vogt) were grown to confluence in 75-cm^2 culture flasks. Labeling with [^{35}S]methionine was done in Eagle minimal essential medium lacking unlabeled methionine, supplemented with 2% fetal calf serum and 1% dimethyl sulfoxide, and containing 50 μCi of [^{35}S]methionine (100 to 200 Ci/mmol, New England Nuclear Corp.) per ml. Labeling with radioactive sugars was done in minimal essential medium with one-third the normal concentration of glucose, supplemented with 2% fetal calf serum and 1% dimethyl sulfoxide, and containing 50 μCi of either D-[6-^{3}H]glucosamine (5 to 15 Ci/mmol; New England Nuclear) or [2-^{3}H]mannose (2 Ci/mmol; Amersham) per ml. After 6 to 8 h at 37°C the medium was harvested (5 ml per 75-cm^2 flask), fresh medium lacking radiolabeled methionine or sugars was added to the cells (10 ml per 75-cm^2 flask), and this medium was harvested after an additional 14 to 16 h at 37°C.

Virus purification. Combined harvests of culture medium were clarified by centrifugation at 1,500 rpm for 3 min, followed by centrifugation at 10,000 rpm for 10 min in a Sorvall HB-4 rotor. Virus was pelleted by centrifugation at 25,000 rpm for 2 h in a Beckman SW27 rotor. Virus was resuspended in several milliliters of NET buffer (0.1 M NaCl-1 mM EDTA-10 mM Tris, pH 7.4) and layered over a 16-ml 10 to 50% or 20 to 60% linear sucrose gradient in an SW27 rotor. The gradient was fractionated, and peak radioactive fractions were determined by assaying samples for trichloroacetic acid-precipitable radioactivity. Fractions corresponding to the peak of radioactive virus were pooled, diluted with buffer, and layered over a 21-ml 20 to 50% sucrose gradient in an SW27 rotor. After centrifugation overnight at 18,000 rpm, the gradient was fractionated, peak radioactive fractions were determined, and the peak fractions were pooled and frozen at −20°C. Radiolabeled VSV was grown and purified as previously described (12).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel electrophoresis of samples of purified virus that had been radiolabeled with methionine, glucosamine, or mannose was performed in 10% polyacrylamide slab gels using the discontinuous buffer system described by Laemmli (18). The slab gels were then subjected to fluorography (3) to detect bands of radiolabeled protein.

Preparation and gel filtration of Pronase-digested glycopeptides. Radiolabeled glycoprotein was extracted from detergent-treated virus and digested with Pronase as described earlier (12). Pronase-digested glycopeptides were analyzed by gel filtration through either a Bio-Gel P4 or a Sephadex G15/G50 column (12). Asparaginyloligosaccharides were prepared from ovalbumin and radiolabeled by acetylation with [^{14}C]acetic anhydride (New England Nuclear) in 0.1 M $NaHCO_3$ (14).

Glycosidase digestions of glycopeptides. Digestion of glycopeptides with *Clostridium perfringens* neuraminidase or a mixture of exoglycosidases and endo-β-*N*-acetylglucosaminidase D from *Streptococcus pneumoniae* (14) was performed as described earlier (9). Endo-β-*N*-acetylglucosaminidase H was prepared from *Streptomyces plicatus* (30). Digestion with endo-β-*N*-acetylglucosaminidase H (5 to 10 mU/ml) was carried out in 0.05 M sodium citrate buffer (pH 5.5) for 24 h at 37°C. The oligomannosyl neutral core products of the endo-β-*N*-acetylglucosaminidase digestions were isolated by passing the digests through a column of Dowex AG1-X2 (formate) as described elsewhere (25).

RESULTS

Purification of radiolabeled RSV. PrB- and PrC-RSV was radiolabeled with either [^{3}H]glucosamine, [^{3}H]mannose, or [^{35}S]methionine, and virus was purified from the growth medium of virus-infected chicken embryo fibroblasts. The relative purity of the virus was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the results are shown in Fig. 1. The major internal virus protein, p27, was the predominant band in the electrophoretic profiles of ^{35}S-labeled PrB-RSV and PrC-RSV. The only major sugar-labeled protein in both viruses was gp85, the major envelope glycoprotein of the avian RNA tumor viruses. The minor envelope glycoprotein, gp37, was only detected with longer autoradiographic exposures or larger amounts of radioactive label (middle panel of PrB-RSV in Fig. 1). This electrophoretic analysis indicated that the virus preparations were relatively free of contaminating cell membranes and glycoproteins, which would have also been radiolabeled, since host-cell protein synthesis is not inhibited by virus infection.

Analysis of virus glycopeptides by Sephadex G15/50 and Bio-Gel P4 chromatography. Because the ^{3}H-sugar label present in protein of purified virus was greatly enriched for the oligosaccharide moieties of a single glycopro-

tein species, the initial comparative studies of the oligosaccharide moieties of the envelope glycoproteins of PrB- and PrC-RSV involved Pronase digestion of total protein from purified virus. Gel filtration of the Pronase digest products on a Sephadex G15/G50 column resulted in a relatively broad distribution for the [^{3}H]glucosamine-labeled peptidyloligosaccharides (Fig. 2a and b), as previously reported (10, 19, 26). The profile for glycopeptides from PrC-RSV was shifted toward a higher apparent molecular weight than the profile for the PrB-RSV glycopeptides. Enzymatic removal of terminal sialic acid reduced the heterogeneity and the differences between the glycopeptide distributions of the two subgroups of RSV, but the glycopeptides were still more heterogeneous than the glycopeptides from another enveloped animal virus, VSV (Fig. 2c to e) (9). The peak molecular weights for the asialoglycopeptides were approximately 3,000 for PrC-RSV, 2,500 for PrB-RSV, and 2,300 for VSV. A radioactive peak corresponding to free sialic acid was observed in the PrB- and PrC-RSV samples because of the intracellular conversion of [^{3}H]glucosamine into sialic acid and incorporation into oligosaccharides. A similar peak was not detected with the VSV sample because most of the radiolabel was in [^{3}H]mannose instead of glucosamine, and the glycoprotein from HeLa-grown VSV is deficient in terminal sialic acid (8, 9, 12).

Gel filtration of the same three asialoglycopeptide samples on a Bio-Gel P4 column resulted in even greater heterogeneity for the avian virus glycopeptides (Fig. 3). The increased heterogeneity relative to the VSV glycopeptides could be partially explained by the increased resolution with Bio-Gel P4 in the 1,000- to 4,000-molecular-weight range. In addition, the negative charge exclusion property of Bio-Gel resins (9, 12) would result in an earlier than expected elution for the fraction of glycopeptides containing aspartic or glutamic acid in their residual peptides. The significance of the negative charge exclusion was demonstrated by the altered elution of the [^{3}H]sialic acid removed by the neuraminidase treatment: sialic acid eluted between the stachyose and mannose gel filtration markers with the Sephadex G15/50 column, but eluted well ahead of the stachyose marker with the Bio-Gel P4 column (compare Fig. 2d and e with Fig. 3b and c).

Analysis of the products of endo-β-*N*-acetylglucosaminidase digestion of virus glycopeptides. To eliminate the contribution of the peptide moieties to the heterogeneous distribution of virus glycopeptides and to determine the actual heterogeneity of the oligomannosyl core region ($Man_nGlcNAc_2$-Asn) of the oligosaccharide chains, the glycopeptides from [^{3}H]glucosamine-labeled PrB-RSV were digested either with a mixture of exoglycosidases and endo-β-

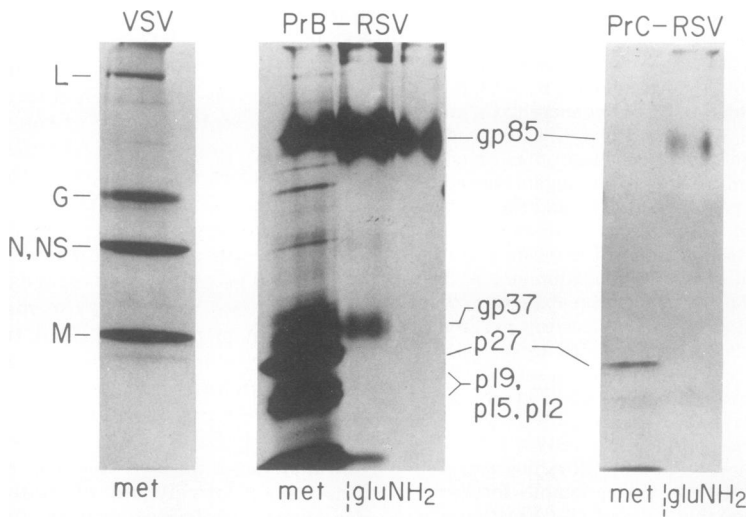

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of radiolabeled PrB and PrC strains of RSV. Purified PrB- and PrC-RSV, labeled with either [^{35}S]methionine or [^{3}H]glucosamine, were subjected to electrophoresis on a polyacrylamide slab gel along with [^{35}S]methionine-labeled VSV (left panel). The approximate molecular weights of the VSV proteins are: L. 180,000; G, 67,000; N(NS), 50,000; and M, 25,000 to 30,000 (32). gp85 and gp37 refer to the two avian RNA tumor virus glycoproteins; p27, p19, p15, and p12 refer to the internal virus proteins (1). The audoradiograph of the [^{35}S]methionine-labeled PrB-RSV was overexposed to clearly reveal the gp85 band. The two profiles of [^{3}H]glucosamine-labeled PrB-RSV represent different amounts of the same purified virus sample.*

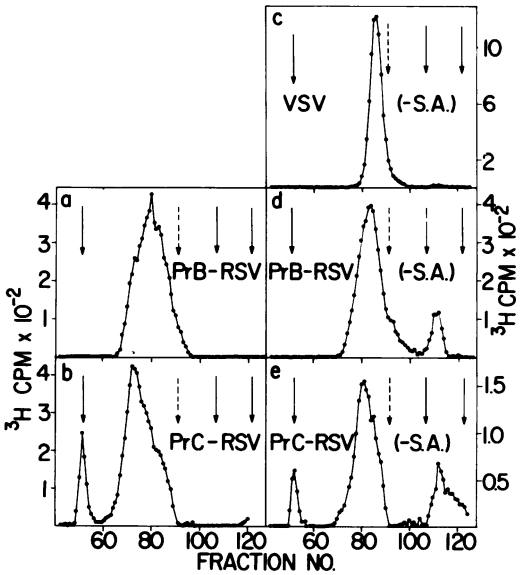

FIG. 2. *Sephadex G15/G50 gel filtration of virus glycopeptides before and after neuraminidase treatment. Solid arrows indicate, from left to right, the peak elution positions of blue dextran (void volume, approximately 30,000 molecular weight exclusion), stachyose (molecular weight, 667), and mannose (molecular weight, 180). Dotted vertical arrow indicates the peak elution position of an ovalbumin glycopeptide (Asn[acetyl-^{14}C]-GlcNAc$_2$Man$_5$; molecular weight, 1,391) co-chromatographed with the ^{3}H-labeled virus glycopeptides. (a), (b) Pronase-digested glycopeptides from [^{3}H]glucosamine-labeled PrB- and PrC-RSV; (c) neuraminidase-treated, Pronase-digested glycopeptides from [^{3}H]mannose and [^{3}H]-glucosamine-labeled VSV glycoproteins; (d), (e) neuraminidase-treated, Pronase-digested glycopeptides from [^{3}H]glucosamine-labeled PrB- and PrC-RSV, respectively. The radiolabeled material that eluted with the void volume in (b) and (e) represented residual undigested glycoprotein. Free sialic acid eluted between the stachyose and mannose markers.*

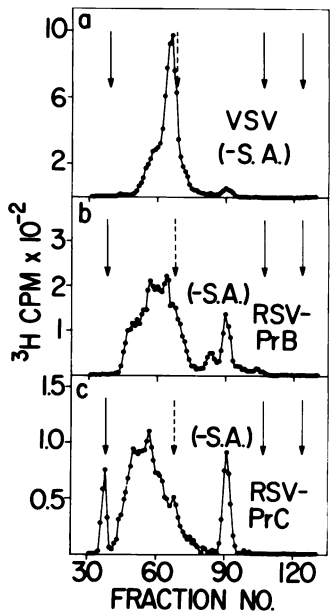

FIG. 3. *Bio-Gel P4 gel filtration of neuraminidase-treated glycopeptides from RSV and VSV. The gel filtration markers are identical to those in Fig. 2, except that the void volume corresponds to a molecular weight of approximately 4,000 or larger. The profiles in (a), (b), and (c) correspond to the same virus glycopeptide samples in Fig. 2 (c), (d), and (e). The peak elution fraction of free sialic acid was approximately fraction 90.*

N-acetylglucosaminidase D, or with endo-β-*N*-acetylglucosaminidase H alone. Both endo-β-*N*-acetylglucosaminidases are capable of cleaving between the two *N*-acetylglucosamine residues proximal to the peptide moiety of mannosyl di-*N*-acetylchitobiose core structures, but their substrate specificities are different. Endo-β-*N*-acetylglucosaminidase D will only cleave smaller cores (3 to 5 mannose units) characteristic of the complex, acidic chains which have been predigested with exoglycosidases to remove branch sugars (NeuNAc-Gal-GlcNAc) (29), whereas endo-β-*N*-acetylglucosaminidase H will cleave larger cores (≥4 mannose units) that are typical of neutral chains (27, 28). The gel filtration profiles of the VSV and RSV glycopeptides after digestion with the mixture of exoglycosidases and endo-β-*N*-acetylglucosaminidase D are shown in Fig. 4. Two new peaks of radioactivity were observed in the VSV sample (Fig. 4a): a major peak corresponding to $Man_3GlcNAc_1$ (labeled in both the mannose and glucosamine residues) and a minor peak corresponding to the free *N*-acetylglucosamine released by the exoglycosidase treatment. Two similarly eluting peaks were observed in the profile of the PrB-RSV digestion products, as well as a broad peak of undigested glycopeptides and a peak of radioactive material eluting in the position of sialic acid (Fig. 4b). Because of the heterogeneity of these labeled products, a Dowex ion-exchange column was used to separate the oligomannosyl neutral core products from the ^{3}H-labeled sialic acid, *N*-acetylglucosamine-peptide, and undigested glycopeptides. Gel filtration of the neutral material from the Dowex column resulted in three peaks (Fig. 4c): a major peak corresponding to free *N*-acetylglucosamine and two smaller peaks eluting in the positions expected for $Man_5GlcNAc_1$ and $Man_3GlcNAc_1$. The corresponding acidic material from the Dowex column eluted from the P4 column as a sharp peak

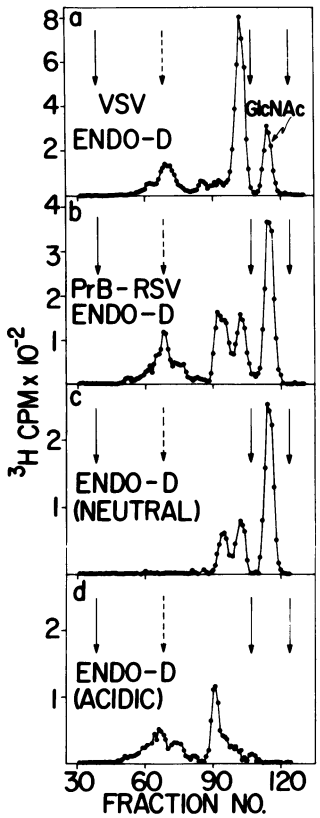

FIG. 4. *Bio-Gel P4 gel filtration of exoglycosidase- and endo-β-N-acetylglucosaminidase-treated glycopeptides from VSV and PrB-RSV. Pronase-digested glycopeptides from [^{3}H]mannose and [^{3}H]glucosamine-labeled VSV (a) or [^{3}H]glucosamine-labeled PrB-RSV (b) were treated prior to gel filtration with a mixture of exoglycosidases and endo-β-N-acetylglucosaminidase D. (c) and (d) correspond to the same glycosidase digestion of PrB-RSV glycopeptides displayed in (b), except that the neutral and acidic products were separated on a Dowex column prior to gel filtration. Free N-acetylglucosamine eluted between the stachyose and mannose markers.*

of sialic acid, a broad distribution of undigested glycopeptides, and smaller, heterogeneous material, presumably corresponding to the *N*-acetylglucosamine-peptide product of endo-β-*N*-acetylglucosaminidase D digestion (Fig. 4d).

These gel filtration results indicated that: (i) a large fraction of the glycopeptides from purified PrB-RSV were sensitive to the combined exoglycosidase and endo-β-*N*-acetylglucosaminidase D digestion, and (ii) the oligosaccharide chains on these glycosidase-sensitive glycopeptides were heterogeneous with respect to the size of their oligomannosyl cores, with similar amounts of 3-mannose and 5-mannose structures. The large amount of radioactivity released in the form of *N*-acetylglucosamine and sialic acid was indicative of complex, acidic oligosaccharides, but some of the radiolabeled oligomannosyl neutral core products (especially $Man_5GlcNAc_1$) could have originated from mannose-rich, neutral chains. A small fraction of the [^{3}H]glucosamine-labeled PrB-RSV glycopeptides were also sensitive to digestion with endoglycosidase H alone (data not shown), suggesting that at least some of the residual, undigested glycopeptides in Fig. 4d contained neutral chains with large oligomannosyl cores.

In further studies of the structural heterogeneity of the oligosaccharide side chains, glycopeptides from [^{3}H]mannose-labeled PrC-RSV were used because the oligomannosyl neutral cores would be the only radiolabeled products from the endoglycosidase digestions. The additional separation of digestion products by ion exchange chromatography would be unnecessary, except to confirm the neutral oligosaccharide property of the digestion products. The gel filtration profiles on Bio-Gel P4 of [^{3}H]mannose-labeled glycopeptides before and after digestions with various exoglycosidases and endo-β-*N*-acetylglucosaminidases is displayed in Fig. 5. Most

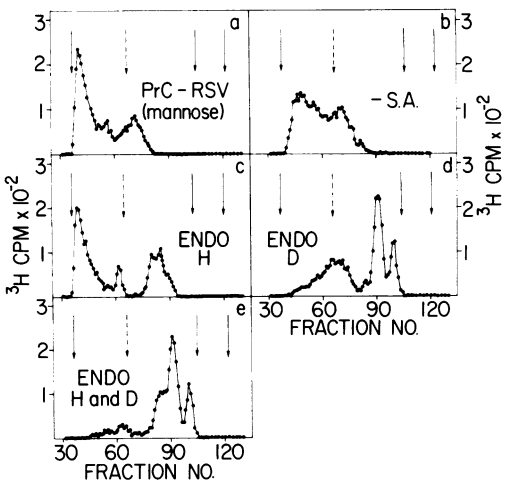

FIG. 5. *Bio-Gel P4 gel filtration of glycosidase-treated glycopeptides from [^{3}H]mannose-labeled PrC-RSV. Equal portions of a sample of Pronase-digested glycopeptides from [^{3}H]mannose-labeled PrC-RSV were analyzed by gel filtration, either without further treatment or after treatment with various glycosidases. (a) Untreated glycopeptides; (b) neuraminidase-treated glycopeptides; (c) endo-β-N-acetylglucosaminidase H-treated glycopeptides; (d) exoglycosidase and endo-β-N-acetylglucosaminidase D-treated glycopeptides; and (e) glycopeptides after treatment first with endo-β-N-acetylglucosaminidase H, and then with the mixture of exoglycosidases and endo-β-N-acetylglucosaminidase D.*

of the radioactivity in the untreated glycopeptides (Fig. 5a) eluted either in a major peak just after the void volume marker or in a broad peak after the [^{14}C]ovalbumin glycopeptide marker. The glycopeptides in the first large peak were sensitive to digestion with neuraminidase (Fig. 5b), insensitive to digestion with endo-β-*N*-acetylglucosamine H (Fig. 5c), and sensitive to digestion with the mixture of exoglycosidases and endo-β-*N*-acetylglucosamine D (Fig. 5d). Digestion with the mixture of glycosidases converted the radioactivity in the large glycopeptides to two new peaks that eluted in the positions expected (11) for $Man_5GlcNAc_1$ and $Man_3GlcNAc_1$ (fractions 87 to 95 and 97 to 102, respectively, in Fig. 5d). In contrast, the glycopeptides in the second major peak (Fig. 5a, fractions 60 to 80) were insensitive to digestion with either neuraminidase alone (Fig. 5b) or the mixture of exoglycosidases and endo-β-*N*-acetylglucosaminidase D (Fig. 5d), but sensitive to digestion with endo-β-*N*-acetylglucosaminidase H (Fig. 5c). The products of this endoglycosidase digestion, and also the products of the endo-β-*N*-acetylglucosaminidase D digestion, were shown to be neutral oligosaccharides by Dowex ion exchange chromatography (data not shown). The heterogeneous gel filtration profile (fractions 74 to 93, Fig. 5c) corresponded in size to oligomannosyl cores with approximately 5 to 9 mannose units ($Man_{5-9}GlcNAc_1$).

Digestion first with endo-β-*N*-acetylglucosaminidase H and then with the mixture of glycosidases (Fig. 5e) converted almost all of the radioactivity in glycopeptides to neutral oligosaccharides with a gel elution profile identical to the sum of the neutral oligomannosyl core products from the individual endoglycosidase digestions (Fig. 5c and d), as expected if essentially all of the radiolabel was in mannose units. A comparison of the gel filtration profiles in Fig. 5c and 5e indicated that the additional incubation with the mixture of exoglycosidases did not affect the elution of the large neutral oligomannosyl cores released by the original endo-β-*N*-acetylglucosaminidase H digestion. This suggested that these neutral oligosaccharides were large oligomannosyl cores ($Man_{5-9}GlcNAc_1$) that lacked any additional branch *N*-acetylglucosamine, as is the case with a fraction of ovalbumin glycopeptides (27). In addition, since most of the radiolabeled products of the endo-β-*N*-acetylglucosaminidase H digestion (Fig. 5c) eluted ahead of the expected position for $Man_5GlcNAc_1$, the majority of radioactivity in the major peak in Fig. 5e ($Man_5GlcNAc_1$) must have resulted from the combined exoglycosidase and endo-β-*N*-acetylglucosaminidase D digestion of the large-molecular-weight, sialic acid-enriched glycopeptides rather than the smaller-molecular-weight glycopeptides with neutral oligosaccharide chains.

In summary, the Pronase-digested glycopeptides from [^{3}H]mannose-labeled PrC-RSV contained two major classes of oligosaccharide side chains: (i) a mixture of complex, acidic oligosaccharides with either 3 or 5 mannose units, represented by the large peak of glycopeptides eluting after the void volume marker in Fig. 5a; and (ii) a mixture of mannose-rich oligosaccharide chains containing $\geq$5 mannose units, represented by the broad peak of glycopeptides eluting after the [^{14}C]ovalbumin glycopeptide marker. It should be noted that the acetylated glycopeptide marker is not an accurate composition marker, since the acetylation of the asparagine amino group results in an increase in apparent molecular weight due to the resulting net negative charge on the glycopeptide and the negative-charge exclusion property of small-pore Bio-Gel columns (9, 12). It is used here as an internal standard, which allows an accurate comparison of elution characteristics in the several column analyses shown.

DISCUSSION

In these initial studies of the oligosaccharide moieties of avian RNA tumor virus glycoproteins, Pronase-digested glycopeptides from purified virus were analyzed by gel filtration coupled with specific exoglycosidase and endo-β-*N*-acetylglucosaminidase digestions. The [^{3}H]glucosamine- or [^{3}H]mannose-labeled glycopeptides from PrB- and PrC-RSV contained a mixture of both mannose-rich, neutral oligosaccharide chains ($Man_{5-9}GlcNAc_2$-Asn) and complex, acidic oligosaccharide chains [$(NeuNAc\text{-}Gal\text{-}GlcNAc)_nMan_{3-5}GlcNAc_2$-Asn] with a heterogeneous number of mannose units in the oligomannosyl cores. These studies confirmed previous results from exoglycosidase digestions of unlabeled oligosaccharide chains of purified gp85 from RSV of subgroups A and C, which also suggested the presence of both acidic and neutral oligosaccharides (17).

The broad distribution of radiolabeled glycopeptides observed in the gel filtration profiles of Pronase-digested avian RNA tumor virus glycoproteins in these studies (Fig. 2 to 4) and previous studies (10, 19, 26) could be attributed to actual heterogeneity in the composition and structure of the oligosaccharide chains, as well as possible heterogeneity in the residual peptide moieties. Although these initial studies utilized total virus glycopeptides rather than glycopeptides from purified gp85 or gp37, the population was greatly enriched for radiolabeled oligosaccharides from gp85 (Fig. 1). Similar carbohy-

drate heterogeneity might, therefore, be expected with purified, radiolabeled gp85.

The Pronase-digested glycopeptides of another enveloped animal virus, Sindbis virus, have also been shown to contain both complex, acidic oligosaccharide chains and mannose-rich, neutral oligosaccharide chains (5, 13), but the size distribution of the oligomannosyl cores has not been determined directly by endo-β-*N*-acetylglucosaminidase digestion and gel filtration. The ratio of the amounts of acidic and neutral oligosaccharide chains varied with both the host cell and the two major envelope glycoprotein species in Sindbis virus (5, 13). In contrast, the single species of envelope glycoprotein of VSV has two glycosylation sites that contain complex, acidic oligosaccharides with a uniform size oligomannosyl core (8, 9, 12, 24). Recent studies with VSV-infected and uninfected animal cells (11) suggested that the heterogeneous mixture of acidic and neutral oligosaccharide chains on these virus envelope glycoproteins could be derived from a single, common precursor oligosaccharide ($Man_{\geq 7}GlcNAc_2$) by intracellular trimming of the oligomannosyl structure and addition of branch sugars (NeuNAc-Gal-GlcNAc).

Although the number of mannose units in the oligomannosyl core structure of the acidic (3 or 5) and neutral oligosaccharide chains (a mixture of 5 to 9) could be deduced from the gel filtration profiles (Fig. 4c and 5c to e) and the known substrate specificities of the two endo-β-*N*-acetylglucosaminidases (27–29), other quantitative data, such as the number of branch chains (NeuNac-Gal-GlcNAc) per acidic oligosaccharide or the ratio of acidic chains to neutral chains, could not be directly determined. Approximate values for the ratio of acidic to neutral chains or the ratio of 3-mannose to 5-mannose cores in the complex, acidic chains in the [^{3}H]-mannose-labeled glycopeptides from PrC-RSV could be calculated if one assumed that all of the mannose units were equally labeled, and if the radioactivity in each size of oligomannosyl neutral core product ($Man_nGlcNAc_1$) was normalized by dividing by the number of mannose units. Such calculations suggested that: (1) the population of complex, acidic chains contained approximately equal numbers of 3-mannose and 5-mannose cores; and (ii) the ratio of complex, acidic chains to mannose-rich, neutral chains was approximately 3:1.

With the [^{3}H]glucosamine-labeled oligosaccharides derived from PrB-RSV, a correction for the specific activity of labeling of the neutral oligomannosyl core products was not necessary, because the different-size cores from the exoglycosidase and endo-β-*N*-acetylglucosaminidase digestions should have contained radioactivity only in the single proximal *N*-acetylglucosamine residue ($Man_nGlcNAc_1$). Similar amounts of the 3-mannose and 5-mannose cores were also observed with these [^{3}H]glucosamine-labeled glycopeptides (Fig. 4). An estimate of the number of branch chain *N*-acetylglucosamine and sialic acid residues per complex, acidic chain could be calculated if one neglected the fraction of the $Man_5GlcNAc_1$ cores in Fig. 4c that were derived from mannose-rich, neutral chains, and assumed that the branch *N*-acetylglucosamine and terminal sialic acid were radiolabeled to the same extent as the proximal *N*-acetylglucosamine present in the neutral oligomannosyl core products: two branch *N*-acetylglucosamine residues and one and one-half terminal sialic residues per complex oligosaccharide chain. These speculative estimates represent average values for a heterogeneous mixture, because one might expect the number of branch chains and terminal sialic acid residues to vary with the size of the oligomannosyl core (3 or 5 mannose units).

Estimates of the number of carbohydrate attachment sites for the major avian RNA tumor virus glycoprotein (10) which are based upon an "average size" oligosaccharide chain (derived from gel filtration of Pronase-digested glycopeptides) should be reconsidered in light of the extensive oligosaccharide heterogeneity demonstrated in these studies. A more direct method of determining the number of attachment sites on the polypeptide would be the ion exchange chromatography of trypsin-digested glycopeptides following the removal of terminal sialic acid, a method successfully applied to the analysis of the VSV glycoprotein (24).

One interesting possibility raised by these studies is the presence of either different-type structures (neutral or acidic) or different-size oligomannosyl cores (3 or 5 for acidic chains) at a single attachment site on the virus polypeptide. If site-specific heterogeneity was possible and the core structure of the oligosaccharide varied with the growth conditions or viral transformation of the host cell, differences in the sialic acid content of glycopeptides from sarcoma and leukosis viruses (19) might be explained by increases in virus-transformed cells in either (i) sialyl transferase activity (4, 13, 23) or (ii) penultimate galactose substrates, resulting from a shift from neutral to acidic chains or an increase in oligomannosyl core size and number of branch structures in acidic chains.

ACKNOWLEDGMENTS

We thank M. Linial and P. E. Neiman for supplying starter cultures of PrB- and PrC-RSV; the Virus Cancer Logistic Program for supplying C/E fertile eggs through Life Sciences, Inc.; and Bill Lamph for technical assistance.

L.A.H. was supported by a Public Health Service research

training grant to the Department of Medicine, Division of Hematology and Oncology, from the National Institute of Arthritis, Metabolism and Digestive Diseases (5-T32-AM-07115-02). This research was supported by the Medical Research Service of the Veteran's Administration, the Dean's Research Advisory Committee, and an Institutional American Cancer Society Grant; by Public Health Service grant 1-RO1-AI12316-01 from the National Institute of Allergy and Infectious Diseases; and by grant PCM-74-21128 from the National Science Foundation.

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