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Rous Sarcoma Virus Glycoproteins Contain Hybrid-Type Oligosaccharides

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Examination of [^{3}H]mannose-labeled glycopeptides from Prague C Rous sarcoma virus gp85 with gel filtration and sequential glycosidase digestions demonstrated the presence of hybrid-type asparaginyl-oligosaccharides. The major hybrid species had an oligomannosyl core ($Man_5GlcNAc_2$-ASN) characteristic of neutral structures, plus "branch" sugars (NeuNAc-Gal-GlcNAc-) characteristic of complex, acidic structures.

Previous gel filtration and glycosidase digestion studies with [^{3}H]mannose- and [^{3}H]glucosamine-labeled glycopeptides from Rous sarcoma virus, Prague C strain (PrC RSV), indicated that the viral glycoprotein contained asparagine-linked oligosaccharides of both the complex, acidic type [$(NeuNAc\text{-}Gal\text{-}GlcNAc)_{2\text{-}4}Man_3GlcNAc_2$-ASN] and the mannose-rich, neutral type ($Man_{5\text{-}9}GlcNAc_2$-ASN) (6) and that these oligosaccharides were more extensively processed in virus glycoprotein from transformed than from untransformed chicken embryo fibroblasts (5). An unusual oligosaccharide product of endo-β-*N*-acetylglucosaminidase H (endo-H)-digested glycopeptides was observed, but not characterized, in these earlier gel filtration analyses. The objective of the present studies was to characterize the composition and structure of these RSV-specific asparaginyl-oligosaccharides, and to demonstrate their similarity to unusual, "hybrid"-type asparaginyl-oligosaccharides reported recently by Kobata and coworkers for ovalbumin (10, 11) and rhodopsin (7). These oligosaccharides were designated as hybrid-type (7, 10, 11) because they possessed an oligomannosyl core and endo-H sensitivity characteristic of mannose-rich, neutral structures ($Man_{4\text{-}5}GlcNAc_2$-ASN) and outer chain sugars (Gal±GlcNAc) characteristic of complex, acidic-type structures.

[^{3}H]mannose- or [^{3}H]glucosamine-labeled PrC RSV was purified from RSV-infected and transformed chicken embryo fibroblast cultures and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (5, 6). The only major sugar-labeled protein detected in purified virus preparations was gp85, the major viral envelope glycoprotein (Fig. 1). [^{3}H]mannose-labeled gp85 was subsequently isolated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3), digested with trypsin (tolylsulfonyl phenylalanyl chloromethyl ketone-treated; Worthington) (9), and analyzed by gel filtration (Bio-Gel P-4, minus 400 mesh; Bio-Rad) before and after digestion with endo-H (*Streptomyces griseus*; Miles Laboratories) as described in detail in previous studies (5, 6). As previously demonstrated with total virion glycoprotein from PrC RSV (5, 6), the larger glycopeptides from gp85 were of the complex, acidic type and were resistant to endo-H, whereas the smaller glycopeptides were sensitive (Fig. 2). The radiolabel in the endo-H-sensitive glycopeptides was converted to a series of smaller-size peaks eluting in the position of neutral oligomannosyl cores ($Man_nGlcNAc_1$) with five to nine mannoses, plus a larger-size peak designated "X". This unusual oligosaccharide peak contained 12% of the total radiolabel in the RSV gp85 glycopeptides and oligosaccharides.

To further characterize this oligosaccharide and the glycopeptides from which it was derived, pronase (grade B; Calbiochem)-digested glycopeptides from total [^{3}H]mannose-labeled virion glycoprotein (greatly enriched for gp85; Fig. 1) were fractionated by preparative gel filtration, and individual size classes were reanalyzed after endo-H digestion (Fig. 3A). A fraction of the medium-size glycopeptides (fractions 61 to 70) was resistant to endo-H, but the majority of radiolabel was converted to a major peak eluting in the same position as the unusual "X" oligosaccharides from purified gp85 and a second peak in the position of $Man_{8\text{-}9}GlcNAc_1$ neutral

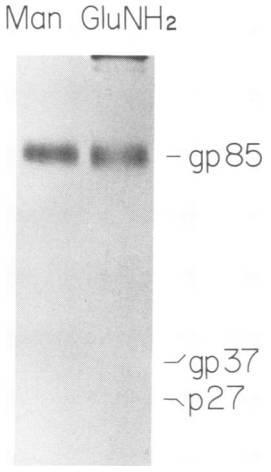

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{3}H-sugar-labeled PrC RSV. Purified RSV labeled with either [^{3}H]mannose (Man) or [^{3}H]glucosamine ($GluNH_2$) was analyzed by electrophoresis in a 10% polyacrylamide slab gel, and the gel was then subjected to fluorography. gp85 and gp37 refer to the major and minor envelope glycoprotein species, with the position of gp37 detected by longer autoradiography of the gel. p27 refers to the major internal protein, detected by staining with Coomassie brilliant blue.*

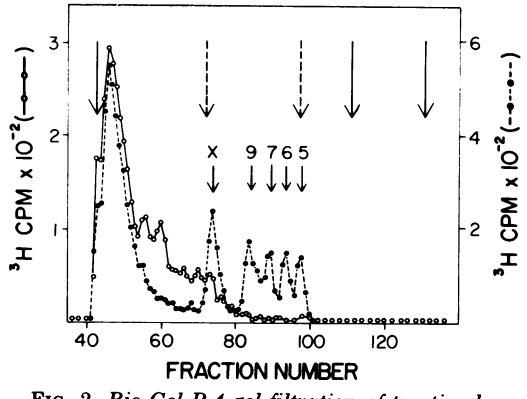

FIG. 2. *Bio-Gel P-4 gel filtration of tryptic glycopeptides from purified gp85 of [^{3}H]mannose-labeled PrC RSV. Untreated (○) and endo-H-treated (●) glycopeptides were separately analyzed on the same column, and the profiles of radiolabel were superimposed by alignment of the peak elution positions of the five gel filtration standards. The three solid vertical arrows represent, from left to right, the peak elution positions of blue dextran (void volume), stachyose, and mannose; the two dashed vertical arrows refer to [^{14}C]glucosamine-labeled glycopeptides ($Man_{8-9}GlcNAc_2$ peptides) and neutral oligosaccharides ($Man_5GlcNAc_1$). 9, 7, 6, and 5 refer to the elution positions of neutral oligomannosyl core products of endo-H digestion ($Man_nGlcNAc_1$) with n = 9, 7, 6, and 5, respectively; and X refers to the peak of hybrid-type oligosaccharides released by endo-H.*

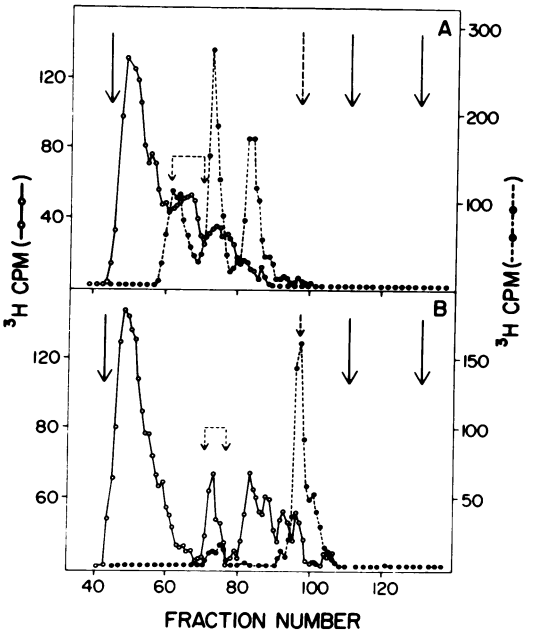

FIG. 3. *Isolation and analysis of hybrid-type glycopeptides and oligosaccharides from [^{3}H]mannose-labeled PrC RSV by Bio-Gel P-4 gel filtration. Pronase-digested glycopeptides derived from total virion glycoprotein were subjected to preparative gel filtration either without further treatment (A) or after treatment with both endo-D and endo-H (B), and the profiles of radiolabel (○) were determined from aliquots (0.1 ml from 1.2 ml total) of each fraction. Medium-size glycopeptides from the preparative gel filtration in (A) (fractions 61 to 70, bracketed by arrows) were subjected to digestion with endo-H and rechromatographed on the same column (●). A sample of the hybrid-type oligosaccharides from the preparative gel filtration of the endo-H- and endo-D-digested glycopeptides in (B) (fractions 71 to 77, bracketed by arrows) was further digested with the mixture of exoglycosidases and endo-D and rechromatographed on the same column (●). The profiles from the corresponding preparative (○) and analytical (●) analyses were superimposed by alignment of the three unlabeled gel filtration standards described in the legend to Fig. 2 (the [^{14}C]glucosamine-labeled oligosaccharide [$Man_5GlcNAc_1$] was also included in the analytical gel filtration analyses).*

oligosaccharides. The larger-size glycopeptides (fractions 40 to 60) were resistant to endo-H, and the smaller-size glycopeptides (fractions 71 to 90) were converted by endo-H to a series of peaks eluting in the position of $Man_{5-9}GlcNAc_1$ neutral oligosaccharides (data not shown). These results indicated that the unusual large oligosaccharides had originated from slightly larger glycopeptides, consistent with the removal of the unlabeled peptide and innermost

N-acetylglucosamine (GlcNAc-asparaginyl peptide) by endo-H digestion.

To determine the actual size of the oligomannosyl core, these oligosaccharides were isolated by preparative gel filtration of glycopeptides digested with endo-H and endo-D (*Diplococcus pneumoniae*; Miles Laboratories) (Fig. 3B). A portion of the pooled sample (fractions 71 to 77, Fig. 3B) was further digested with a mixture of exoglycosidases from *D. pneumoniae* containing neuraminidase, galactosidase, and glucosaminidase (1). The major product was a much smaller oligosaccharide that coeluted with a [^{14}C]glucosamine-labeled oligosaccharide ($Man_5GlcNAc_1$) included as an internal gel filtration standard (Fig. 3B). In addition, minor amounts of radiolabel eluted in the positions expected for an oligomannosyl core with four mannoses ($Man_4GlcNAc_1$; fractions 101 to 104) and undigested oligosaccharides (fractions 71 to 76). The combined results of endo-H sensitivity of these glycopeptides and the exoglycosidase sensitivity and gel filtration properties of the oligosaccharide products suggested that they were hybrid-type structures similar to those previously described for ovalbumin (10, 11) and rhodopsin (7).

The possible presence of terminal sialic acid was investigated by digestion of another sample of the hybrid oligosaccharides with neuraminidase (*Clostridium perfringens*, fraction IX; Sigma) (2) and subsequent gel filtration chromatography (Fig. 4A). All of the radiolabel was shifted by neuraminidase digestion to slightly smaller structures that were equivalent in elution to $Man_{8-9}GlcNAc_1$-size neutral oligosaccharides. The removal of negatively charged sialic acid was also confirmed by chromatography on Dowex AGl-X2 (9): the [^{3}H]mannose-labeled hybrid oligosaccharides behaved as acidic structures before neuraminidase digestion (bound to Dowex) but were converted to neutral structures (unbound) after digestion with neuraminidase alone or the mixture of glycosidases from *D. pneumoniae*. Additional digestion of the desialylated oligosaccharides with jack bean and beef kidney β-*N*-acetylglucosaminidase (Sigma) had no effect on the elution position, suggesting that branch *N*-acetylglucosamine was not in an accessible, terminal position in these oligosaccharides prior to β-galactosidase digestion. These eucaryotic glucosaminidases have been shown to be even more efficient than the bacterial glucosaminidases in removing branch *N*-acetylglucosamines from complex, acidic-type asparaginyl-oligosaccharides (Hunt, unpublished data).

The desialylated oligosaccharides were also extensively digested with jack bean α-mannosidase (Sigma) (2). The resulting radiolabeled products eluted from the Bio-Gel P-4 column (Fig. 4B) as a somewhat smaller and heterogeneous peak of oligosaccharides (60% of the ^{3}H label) and a second peak coeluting with free mannose (40% of the ^{3}H label; equivalent to the removal of approximately two of the five man-

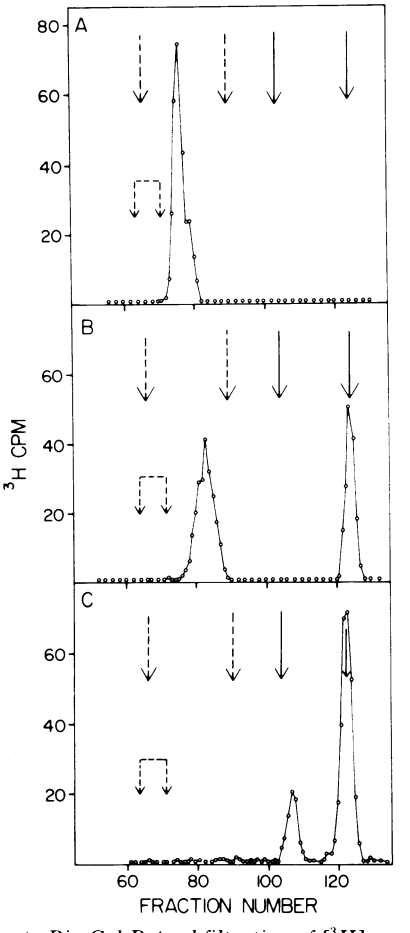

FIG. 4. *Bio-Gel-P-4 gel filtration of [^{3}H]mannose-labeled hybrid-type oligosaccharides after sequential exoglycosidase treatments. Samples of the hybrid-type oligosaccharides (obtained by preparative gel filtration as shown in Fig. 3B) were subjected to further digestions with glycosidases and rechromatographed along with the five gel filtration standards described in the legend to Fig. 2: (A) neuraminidase treated; (B) neuraminidase and α-mannosidase treated; and (C) treated with the mixture of exoglycosidases followed by α-mannosidase. The small bracketed arrows between fractions 60 and 80 represent the elution position of the hybrid-type oligosaccharides before further digestion with exoglycosidases. The peak elution position for blue dextran (void volume, approximately fraction 40) is not shown in these profiles along with the other four standards.*

noses). Another sample of the desialylated oligosaccharides was treated sequentially with the mixture of *D. pneumoniae* glycosidases and α-mannosidase. The result was a major peak of free mannose and a minor peak eluting in the position expected for a β-linked mannose-glucosamine disaccharide (Fig. 4C), with a ratio of 3.8:1.0 for radiolabel in free mannose versus disaccharide.

The presence of asialo hybrid structures in the original endo-H digestion products was examined by α-mannosidase digestion of the $Man_{8-9}GlcNAc_1$-size oligosaccharides from the preparative gel filtration profile in Fig. 3B (fractions 81 to 86). The digestion products included a major peak of free mannose (73% of radiolabel), a disaccharide peak (8% of radiolabel), and a third peak (18% of radiolabel) in the same position as the neuraminidase- and α-mannosidase-treated oligosaccharides in Fig. 4B (data not shown). Thus, the radiolabel in the region of large oligomannosyl core structures may actually have been a mixture of asialo forms of the hybrid oligosaccharides and $Man_{8-9}GlcNAc_1$ neutral-type structures. The absence of an intermediate size peak between the sialic acid-containing and asialo forms of the hybrid oligosaccharides (Fig. 2 and 3A and B) was consistent with the presence of only a single terminal sialic acid residue for the major species. Similar-size oligosaccharides were also isolated from the endo-H digests of [^{3}H]glucosamine-labeled PrC RSV glycopeptides and analyzed by gel filtration after further digestion with the mixture of *D. pneumoniae* glycosidases; radiolabel was recovered in $Man_{4-5}GlcNAc_1$-size oligosaccharides, free sialic acid, and free *N*-acetylglucosamine in a ratio of approximately 0.7:1.01.1 (data not shown).

The results of sequential glycosidase digestion and gel filtration analysis of ^{3}H-sugar-labeled glycopeptides from PrC RSV were most consistent with the following composition for the hybrid-type asparaginyl-oligosaccharides: $NeuNAc^+Gal\text{-}GlcNAc\text{-}(Man)_{4-5}GlcNAc_2\text{-}ASN$, with the major species containing terminal sialic acid and a five-mannose core. Because the presence of an additional branch *N*-acetylglucosamine affected the elution position of an oligomannosyl core from a Bio-Gel P-4 column to a greater extent than two additional mannose residues [$GlcNAc_1(Man)_3GlcNAc_1$ versus $Man_5GlcNAc_1$] (5; unpublished data), the coelution of the neuraminidase-treated hybrid oligosaccharides [Gal-GlcNAc-$(Man)_5GlcNAc_1$] with the $Man_{8-9}GlcNAc_1$-size neutral oligosaccharides was not unexpected.

The combined results of the present studies (especially the endo-H, neuraminidase, and α-mannosidase sensitivities) were consistent with the detailed structure (7) of oligosaccharide C of rhodopsin (Fig. 5), with an additional terminal sialic acid and galactose present on the major hybrid-type oligosaccharide species from PrC RSV glycoprotein. The apparent removal of only two of the five mannoses of the desialylated oligosaccharides by jack bean α-mannosidase (Fig. 4), rather than the three mannoses expected with the structure shown in Fig. 5, was identical to other recent observations on the inability of this enzyme to remove the inner α-1,6-linked mannose when the inner α-1,3-linked mannose was substituted by another sugar (12). The hybrid structures for ovalbumin contained an additional *N*-acetylglucosamine linked to the innermost, β-linked mannose (10, 11), but the results of exoglycosidase digestions of PrC RSV oligosaccharides were most consistent with the presence of only a single branch *N*-acetylglucosamine. The sialic acid-containing hybrid oligosaccharides represented approximately 9% of the total normalized radiolabel ([^{3}H]mannose counts per minute divided by the number of mannoses per oligosaccharide) in purified PrC RSV gp85 (Fig. 2), but it is not known whether these structures were localized at one major glycosylation site or present at multiple sites that normally contained complex, acidic-type oligosaccharides.

Although these studies are the first demonstration of hybrid-type asparaginyl-oligosaccharides in viral envelope glycoproteins, [^{3}H]mannose-labeled glycopeptides with similar endo-H sensitivity and gel filtration properties have also been observed in relatively minor amounts in pronase digests of Sindbis virus (5) and cellular

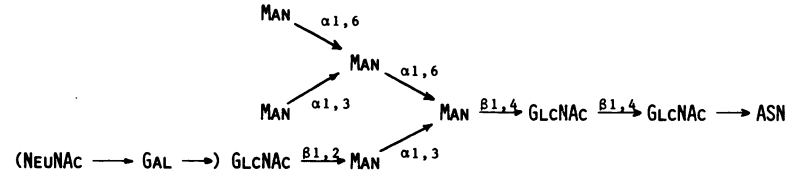

FIG. 5. *Proposed structure of hybrid-type oligosaccharides. The structure was adapted from the results for oligosaccharide C of rhodopsin (7) by the addition of terminal galactose and sialic acid.*

membrane glycoproteins (4). Hybrid-type structures have also been suggested very recently for the HA glycoprotein of influenza virus (1, 8), but these influenza glycopeptides were quite distinct from the RSV and ovalbumin (10, 11) hybrid-type glycopeptides and oligosaccharides on the basis of both opposite sensitivity to endo-H (influenza glycopeptides mostly resistant [1, 8]; RSV glycopeptides sensitive) and affinity for *Lens culinaris* lentil lectin (influenza glycopeptides mostly bound [8]; RSV oligosaccharides completely unbound to lentil lectin, but completely bound [five mannose species] to concanavalin A [unpublished data] as expected from the concanavalin A binding of ovalbumin hybrid-type structures with five mannoses [11]).

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