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Sialylated Oligosaccharides O-Glycosidically Linked to Glycoprotein C from Herpes Simplex Virus Type 1

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Glycoprotein C (gC) was purified by immunoabsorbent from herpes simplex virus type-1-infected BHK cells labeled with [^{14}C]glucosamine for 11 h and chased for 3 h. Glycopeptides obtained by pronase digestion of gC were fractionated by Bio-Gel filtration and concanavalin A-Sepharose chromatography. Each glycopeptide fraction was analyzed for amino sugar composition by thin-layer chromatography. The majority of radioactivity was recovered as *N*-acetylglucosamine, but a significant amount of labeled *N*-acetylgalactosamine was detected and recovered preferentially in some glycopeptide species. Mild alkaline borohydride treatment of the glycopeptides resulted in the release of small degradation products which contained *N*-acetylgalactosaminitol as the major labeled component and a drastic reduction of *N*-acetylgalactosamine in the residual glycopeptides. These results demonstrated that gC carries O-glycosidically linked oligosaccharides in addition to the N-linked di- and triantennary glycans previously described (F. Serafini-Cessi, F. Dall'Olio, L. Pereira, and G. Campadelli-Fiume, J. Virol. 51:838–844, 1984). Chromatographic behavior on DEAE-Sephacel chromatography and neuraminidase digestion of O-linked oligosaccharides indicated the presence of two major sialylated species carrying one and two sialic acid residues, respectively. The characterization of a peculiar glycopeptide species supported the notion that some of the O-linked oligosaccharides are bound to a cluster of hydroxyamino acids located near an N-glycosylation site which carries one N-linked diantennary oligosaccharide.

Glycoprotein C (gC) is one of the major herpes simplex virus type 1 (HSV-1) glycoproteins (11, 29, 30). Recently, interest in this glycoprotein increased since it was reported that it acts as a receptor for the C3b complement component on infected cell membranes, and this interaction may be relevant to the pathogenesis of HSV-1 infections (8). In a previous study (25), we reported on the processing and characterization of N-linked oligosaccharides of gC. The results of that study showed a high heterogeneity of complex-type chains present in the mature form of gC, which arise from different processing of the common high-mannose chains carried by the immature form. The occurrence of O-linked glycans in gC has been reported by other authors (13, 20), who observed a change in the gel filtration profile of glucosamine (GlcN)-labeled gC after mild alkaline borohydride treatment or after digestion with endo-*N*-acetylgalactosaminidase. It has also been reported (21, 22) that a portion of gC interacts with *N*-acetylgalactosamine (GalNAc)-binding lectins, such as *Helix pomatia* and soybean lectins. These results do not prove the occurrence of O-linked chains in gC in that (i) the two lectins also bind to *N*-acetylglucosamine (GlcNAc) and galactose (10) and (ii) GalNAc has also been found at the nonreducing end of N-linked oligosaccharides (15, 24).

Although it is generally accepted that O-linked chains are cleaved by mild alkaline borohydride treatment, whereas N-linked glycans are more stable, in the literature (5, 12, 19, 23) there are several reports that this treatment also results in substantial changes of glycoproteins carrying *N*-asparagine-linked oligosaccharides. These changes consist in: (i) partial cleavage of the linkage between the carbohydrate chains and asparagine, (ii) a partial split of the chitobiosyl core of N-linked chains, and (iii) multiple breaks in the peptide backbone. All of these events affect the gel filtration profile of mild alkaline borohydride-treated glycoproteins independently on the release of the O-linked chains. Hounsell et al. (12) found that the gel filtration profile of fetuin after mild alkaline borohydride treatment is very similar to that obtained after pronase digestion. Kumarasamy and Blough (16) observed no change in the gel filtration profile of GlcN-labeled gC subjected to mild alkaline borohydride degradation as described by Spiro (31).

The purpose of the present study was to obtain a clear demonstration of the presence of oligosaccharides O-glycosidically linked to gC. First, we fractionated the pronase glycopeptides from [^{14}C]GlcN-labeled gC by different chromatographic systems to select glycopeptide species rich in GalNAc. It is well known that GalNAc is the typical sugar of O-linked chains, being involved in the linkage between the carbohydrate chain and the hydroxyamino acids. Second, we analyzed mildly alkaline borohydride-treated glycopeptides to detect *N*-acetylgalactosaminitol (GalNAc-ol), which is formed by reductive action of borohydride on the reducing terminal GalNAc of cleaved O-linked oligosaccharides. Our results clearly indicate that different species of O-linked chains are present in gC in addition to the heterogenous forms of N-linked complex-type oligosaccharides previously described (16, 25).

MATERIALS AND METHODS

Virus infection of cells and glycoprotein labeling. Monolayer cultures of BHK cells were infected with HSV-1(F) at an input multiplicity of infection of 10 PFU per cell. Infected cells were continuously labeled from 7 to 18 h after infection with minimum essential medium containing (per milliliter) 0.5 mg of glucose and 20 μCi of [^{14}C]GlcN (specific activity, 56.8 mCi/mmol), rinsed five times, and chased for 3 h with minimal essential medium. At the end of the label-chase period, the cells were washed three times with ice-cold phosphate-buffered saline before extraction of glycoproteins with 0.5%

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Nonidet P-40–0.15 N NaCl–0.01 M Tris hydrochloride (pH 7.5)-10^{-5} M each tolylsulfonyl phenylalanyl chloromethylketone and tosyl-L-lysine chloromethylketone. Cell lysate was then scraped and centrifuged at low speed. The supernatant was centrifuged at 100,000 × *g* for 1 h and dialyzed extensively against 0.1% Nonidet P-40–0.5 N NaCl–0.01 M Tris hydrochloride (pH 7.5)–0.1 mM phenylmethylsulfonyl fluoride.

Purification of [^{14}C]GlcN-labeled gC. gC was purified from the supernatant of cell lysate by affinity chromatography to HC_1 monoclonal antibodies-Sepharose as previously described (25). To check the purity of the glycoprotein, we subjected it to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and compared its mobility with that of molecular weight (MW) calibration standards.

Pronase digestion of gC. A 1% solution of pronase purified by acetone precipitation was autoincubated in 0.2 M Tris hydrochloride (pH 8) containing 1 mM $CaCl_2$ for 1 h at 37°C to inactive possible contaminating enzymes. The glycoprotein in 0.025 M Tris hydrochloride (pH 8) containing 0.1% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride was digested with pronase at 60°C for 24 h. Three additions of enzymes were done during the incubation period, achieving a final concentration of 0.13%. Digestion was stopped by boiling the sample for 3 min.

Column chromatography. Chromatography of glycopeptides on a concanavalin (ConA)-Sepharose column was done as previously described (26). Chromatography on Bio-Gel P-10 was performed on a column (1 by 70 cm) equilibrated with 0.1 M pyridine-acetate buffer (pH 5). The voided volume (V_0) of the column was determined with blue dextran, the elution volume of N-linked complex type glycopeptides with a ^{3}H-labeled tetra-antennary glycopeptide marker prepared as previously described (28), and the total included volume (V_t) with GlcNAc as detected by the Morgan-Elson reaction (17). DEAE-Sephacel chromatography was performed as previously described (1).

Mild alkaline borohydride treatment of glycopeptides. Glycopeptides were treated with 1 M $NaBH_4$ in 0.05 M NaOH for 16 h at 45°C by the Carlson method (2) or with 1 M $NaBH_4$ in 0.1 M NaOH for 48 h at 37°C by the Spiro method (31). At the end of treatment, samples were acidified with acetic acid and then evaporated four times with methanol to remove methylborates.

Strong acid hydrolysis of glycopeptides and oligosaccharides. Glycopeptides were desalted on a Bio-Gel P-2 column (2 by 30 cm) equilibrated with water. Oligosaccharides of higher MW released by mild alkaline borohydride treatment were also desalted with the same column, whereas the oligosaccharides eluted near the V_t of the Bio-Gel P-10 column were deionized by coupled columns of Dowex 50 (H^+ form) and Dowex 1 (formate form). Since mild alkaline borohydride treatment caused a partial deacetylation of the carbohydrate chains, the samples, before passing over the coupled columns, were re-N-acetylated as previously described (32). Under these conditions, the recovery of labeled oligosaccharides from deionizing chromatography was near 90%. The samples were then hydrolyzed in 4 N HCl for 4 h at 100°C in a final volume of 0.5 ml. At the end of this period, the samples were evaporated under reduced pressure.

TLC. Silica gel plates 60 from Merck were used for thin-layer chromatography (TLC). The solvent used was ethanol–pyridine–1-butanol–acetic acid–water (100:10:10:3:30), to which 1% (wt/vol) potassium tetraborate was added (solvent A). The plates were developed twice with this solvent. Radioactive monosaccharides were detected by autoradiography after the plates were sprayed with En^{3}Hance (New England Nuclear Corp., Boston, Mass.). Quantitation of each monosaccharide was obtained by liquid scintillation counting after scraping off the gel corresponding to radioactive spots. Standard compounds for TLC were as follows. [^{14}C]Glcn and [^{14}C]galactosamine (GalN) were from The Radiochemical Centre, Amersham, England. Labeled GalNAc-ol was prepared by reduction of GalNAc with KB^3H_4 (specific activity, 6.7 mCi/mmol) as described by Crimmin (3). The cristallized compound migrated on TLC as a homogenous compound with solvent A or ethanol–25% aqueous ammonia–water (100:30:20) saturated with $K_2B_4O_7$.

Neuraminidase digestion and detection of label released as sialic acid. Oligosaccharides in 0.1 M acetate buffer (pH 5) containing 10 mM $CaCl_2$ were incubated with 0.1 U of enzyme per ml at 37°C for 24 h. At the end of this period, the samples were boiled for 3 min and applied to a DEAE-Sephacel column to separate labeled sialic acid from desialylated material. The elution position of sialic acid was detected by loading on the column unlabeled sialic acid, which was determined by the Warren method (34).

RESULTS

Separation of pronase glycopeptides on ConA-Sepharose. Previously, gC was purified by affinity chromatography on immobilized HC_1 monoclonal antibodies from HSV-1(F)-infected BHK cells labeled with [^{3}H]mannose to characterize the N-linked oligosaccharides (23). We demonstrated that di- and triantennary, but not tetra-antennary, oligosaccharides are carried by gC. In the present study, gC was purified by the same procedure from the same virus-cell system, but with [^{14}C]glucosamine as a radioactive precursor. Since GlcN is utilized for the biosynthesis of UDP-GlcNAc and UDP-GalNAc, a pulse with [^{14}C]GlcN results in the labeling of both N-linked and O-linked oligosaccharides, the former because GlcNAc is a constituent of the core portion and of branched chains, the latter because GalNAc is usually present at the reducing end of O-linked chains. [^{14}C]GlcN-labeled gC purified by affinity chromatography on HC_1-Sepharose migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a single component with an apparent MW of 120,000 (25). The same analysis showed that no labeled component with the electrophoretic mobility of gC was detectable in the unretained fractions of HC_1-Sepharose chromatography, demonstrating that the vast majority, if not all, of gC was retained by the immunoadsorbent used.

Glycopeptides obtained after exhaustive pronase digestion of GlcN-labeled gC were subjected to ConA-Sepharose chromatography. Of the radioactivity incorporated in the glycopeptides, 79% did not bind to ConA-Sepharose, whereas 19% of the radioactivity bound to the lectin-gel and was eluted with 5 mM α-methylmannoside (Fig. 1A). Under these conditions, O- and N-linked triantennary glycopeptides do not bind to ConA-Sepharose, whereas N-linked diantennary glycopeptides are eluted in the ConA-bound fraction (14). Less than 2% of radioactive glycopeptides were strongly bound to ConA-Sepharose, being eluted with 200 mM α-methylmannoside; they were not characterized further. Samples of ConA-unbound and ConA-bound fractions were subjected to strong acid hydrolysis and analyzed for radioactive amino sugar composition on TLC. Because of acid hydrolysis, GlcNAc and GalNAc were recovered as the corresponding deacetylated products, i.e., GlcN and GalN, whereas sialic acid underwent more severe degrada-

tion. For hydrolyzed ConA-bound and ConA-unbound glycopeptides, fluorography of TLC showed two radioactive products with the chromatographic mobilities of GalN and GlcN, respectively (Fig. 1B). Radioactive components with faster mobility were also visualized, which very likely represent the products of acid degradation of sialic acid, since they were not recovered when the same fractions were subjected to acid hydrolysis after sialic acid removal. The recovery of a large amount of GlcN in ConA-unbound glycopeptides is due to the presence of N-linked triantennary chains, whereas the occurrence of GalN is indicative of O-linked chains. Since GalNAc is rarely present in N-linked oligosaccharides (15, 24), it was rather surprising to find that hydrolyzed ConA-bound glycopeptides contained GalN. This might be due to the presence in the ConA-bound fraction of a glycopeptide species carrying both N-linked diantennary and O-linked chains. It has been reported (4, 18, 33) that occurrence of oligosaccharides closely spaced on the peptide backbone inhibits the action of pronase. As a consequence, glycopeptides of high MW have been described which carry more than one oligosaccharide chain.

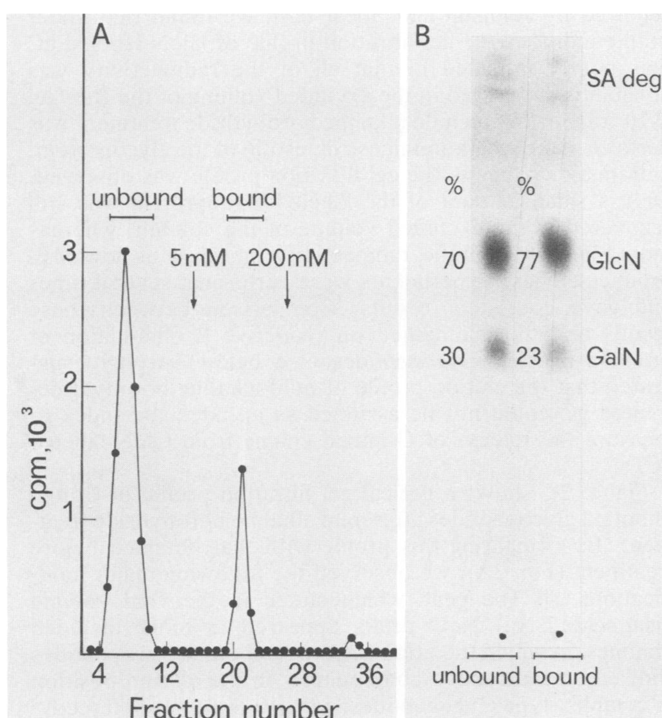

FIG. 1. Chromatography on ConA-Sepharose of radiolabeled pronase glycopeptides and their amino sugar composition. [^{14}C]GlcN-labeled gC, purified by HC_1 monoclonal antibodies immobilized to Sepharose, was digested with pronase. (A) Pronase glycopeptides were fractionated on ConA-Sepharose. The arrows indicate the change of eluate (5 and 200 mM α-methylmannoside); samples of each fraction were removed and counted for radioactivity. Fractions containing ConA-unbound and ConA-bound glycopeptides were pooled as indicated. Recovery of radioactivity from the column was 92%. (B) A sample of each pool was subjected to strong acid hydrolysis as described in the text and, after evaporation, applied to silica gel plates. TLC was developed as described (solvent A), and the labeled products were visualized by fluorography. The areas of the spots were scraped off of the plates, and radioactivity was determined by scintillation counting. Percentage distribution was calculated assuming as 100% the radioactivity recovered in the migration positions of GlcN and GalN. About 5,000 cpm of each sample was applied to the plate. Acidic degradation products of sialic acid are indicated as SAdeg.

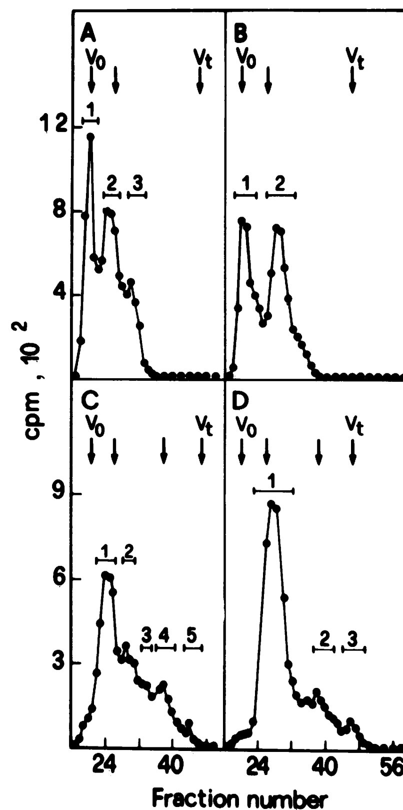

FIG. 2. Bio-Gel P-10 filtration of ConA-unbound and ConA-bound glycopeptides before and after mild alkaline borohydride degradation. (A) ConA-unbound glycopeptides and (B) ConA-bound glycopeptides shown in Fig. 1A were applied to a Bio-Gel P-10 column and monitored for radioactivity. Fractions (1 ml) were pooled as indicated by horizontal bars. The arrow after that of V_0 indicates the elution position of an N-linked complex-type glycopeptide marker. (C) ConA-unbound glycopeptides shown in Fig. 1A were subjected to mild alkaline borohydride degradation as detailed in the text. At the end of treatment, the borohydride was removed and the treated glycopeptides were chromatographed on Bio-Gel P-10 as described above. Fractions were pooled as indicated by horizontal bars. The arrows after that of V_0 indicate the elution positions of an N-linked complex glycopeptide marker and sialyllactose, respectively. (D) ConA-bound glycopeptides of Fig. 1A were subjected to mild alkaline borohydride degradation and then fractionated on Bio-Gel P-10 as described above. In all chromatograms, the recovery of radioactivity applied to the column was over 90%.

Fractionation on Bio-Gel P-10 of ConA-unbound and ConA-bound glycopeptides. Figure 2A shows the gel filtration profile of the ConA-unbound glycopeptides. A significant amount (31%) of the radioactivity was eluted in the excluded volume of the column before the elution position of a tetra-antennary glycopeptide marker, suggesting an MW for this glycopeptide species higher than that predictable for a glycopeptide carrying one triantennary chain. About 44% of the radioactivity coeluted with the N-linked complex-type glycopeptide marker, and only 16% was eluted as a species of lower MW. The three glycopeptide fractions were designated ConA-unbound 1, 2, and 3, respectively. Figure 3A shows the migration on TLC of the radioactive components obtained by strong acid hydrolysis of the three glycopeptide

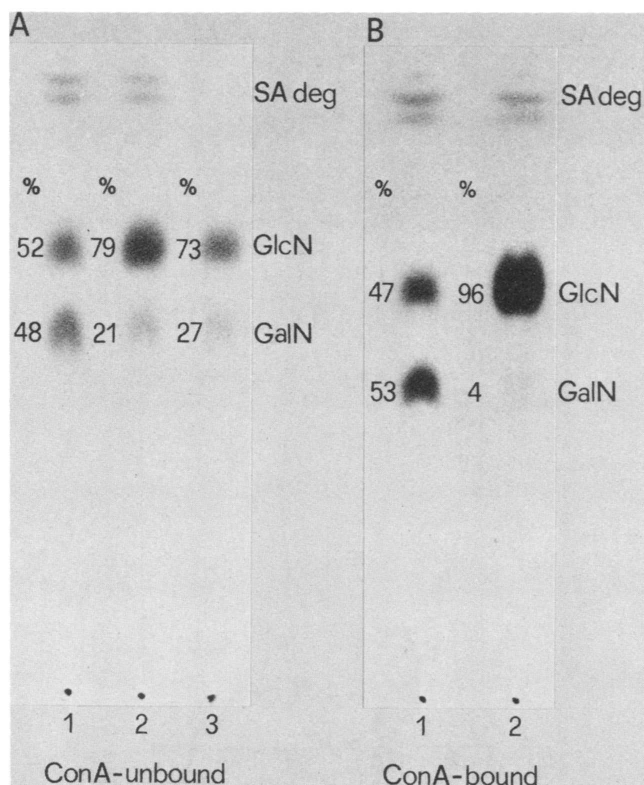

FIG. 3. TLC fluorography of strong acid hydrolysates of GlcN-labeled glycopeptides fractionated as described for Fig. 2A and B. (A) Samples of ConA-unbound glycopeptides 1, 2, and 3 shown in Fig. 2A were subjected to strong acid hydrolysis and then evaporated under reduced pressure. The hydrolyzed samples were applied to silica gel plates and chromatographed in solvent A. The percentage distribution of label in the two amino sugars was quantitated assuming as 100% the radioactivity recovered as GalN and GlcN. (B) ConA-bound glycopeptides 1 and 2 shown in Fig. 2B were analyzed as described above. About 3,000 cpm of each sample was applied to the plate. Acidic degradation products of sialic acid are indicated as SAdeg.

species. The percentage distribution of radioactive GlcN and GalN in each glycopeptide showed that GalN was present in large amount in the high-MW peak (ConA-unbound 1), although it was also detectable in the two other glycopeptides.

When the ConA-bound glycopeptides shown in Fig. 1 were chromatographed in the same Bio-Gel P-10 column (Fig. 2B), 44% of the radioactivity was eluted in the V_0 of the column, whereas the remaining radioactivity coeluted with the N-linked complex-type marker. This pattern was observed even when the ConA-bound glycopeptides were redigested with pronase, ruling out that chromatographic behavior depended on incomplete pronase digestion. Almost all of the radioactive GalN present in the ConA-bound glycopeptides was recovered in the higher-MW fraction (ConA-bound 1). In particular, in this fraction radioactivity quantitated as GalN was slightly higher than that quantitated as GlcN (Fig. 3B). The glycopeptide coeluting with the N-linked complex-type glycopeptide (ConA-bound 2) was practically devoid of GalN (Fig. 3B). The presence of both GalN and GlcN in ConA-bound 1 suggests a glycopeptide structure with multiple oligosaccharide chains O- and N-glycosidically linked to the same peptide fragment, whereas the absence of GalN in ConA-bound 2 supports the view that it contains only the N-linked diantennary chain.

Fractionation on Bio-Gel of oligosaccharides after mild alkaline borohydride treatment. ConA-unbound and ConA-bound glycopeptides were fractionated by Bio-Gel chromatography after mild alkaline borohydride treatment. Under these conditions, oligosaccharides O-glycosidically linked to hydroxyamino acids are cleaved by a mechanism designated as reductive β elimination and recovered in the reduced form; i.e., GalNAc, which usually is at the reducing end, is converted to GalNAc-ol. Two classical conditions for performing mild alkaline borohydride treatment, termed Carlson (2) and Spiro (31) degradation, have been described. The selectivity and efficiency of the two methods seem to vary depending on the structure of the glycoprotein. We checked the efficiency of the two methods by comparing the yield of the released products; the Spiro method gave more reproducible results and thus was used. In preliminary experiments, we subjected the original glycoprotein (undigested gC) to mild alkaline borohydride treatment under Carlson and Spiro conditions. In contrast to the results reported by Johnson and Spear (13), we found that under both conditions the gel filtration profile of GlcN-labeled gC was poorly modified in that all of the radioactivity was constantly recovered in the excluded volume of the Bio-Gel P-10 column. When mild alkaline borohydride treatment was performed after neuraminidase digestion of the glycoprotein, a dramatic change in the gel filtration profile was observed. Only a small amount of the labeled glycoprotein was still recovered in the excluded volume of the column, whereas more than 80% of the radioactivity emerged as low-MW products. These experiments were performed several times and gave consistent results. Comparison between these results and those obtained on reductive β elimination of pronase-digested glycopeptides (see below) strongly suggested that the elution profile of mild alkaline borohydride-treated gC could not be assumed as an adequate index to measure the release of O-linked chains from GlcN-labeled gC.

Figure 2C shows a typical gel filtration profile of ConA-unbound glycopeptides after mild alkaline borohydride treatment. By comparing this profile with that obtained before treatment (Fig. 2A), we observed the following major modifications. (i) The peak which eluted in the void volume disappeared. (ii) New peaks appeared in more included volumes, in which the original ConA-unbound glycopeptides showed no radioactive components. In the elution position of complex-type glycopeptides, a major peak was still recovered accounting for 47% of the radioactivity loaded in the column. Very likely it comprised the glycopeptide species carrying the N-linked triantennary chains, whereas the peaks generated by β elimination represented the released O-linked chains. The radioactive fractions of each peak were pooled as indicated and termed ConA-unbound treated 1, 2, 3, 4, and 5, respectively.

When ConA-bound glycopeptides subjected to mild alkaline borohydride treatment were fractionated on a Bio-Gel P-10 column, a profile of radioactive peaks was obtained that was in agreement with the view that ConA-bound fraction 1 represents a glycopeptide species carrying an O-linked oligosaccharide(s) and one N-linked diantennary chain. Indeed, the radioactive peak which emerged in the V_0 before mild alkaline borohydride treatment disappeared, and its radioactivity was partially shifted to the elution volume of diantennary glycopeptides and partially released as two lower-MW products, one coeluting with sialyllactose and the

other emerging near the most included volume of the column (Fig. 2D). Very likely they represented two O-linked chains with different structures. The fractions of each peak were pooled as indicated and designated as ConA-bound treated 1, 2, and 3, respectively.

Analysis of oligosaccharides fractionated after mild alkaline borohydride treatment. The oligosaccharide fractions separated as in Fig. 2C and D were analyzed to identify GalNAc-ol produced after mild alkaline borohydride degradation and to assess their amino sugar composition. The fractions were subjected to strong acid hydrolysis, and the migration on TLC of the radioactive products was compared with that of a standard preparation of cristalline [^{3}H]GalNAc-ol previously subjected to strong acid hydrolysis, which converts the compound to its deacetylated form. When deacetylated GalNAc-ol was subjected to TLC and developed with solvent A containing borate salt, two radioactive spots were visualized by fluorography, one migrating near the origin and the other faster than GlcN (Fig. 4). In contrast, a deacetylated preparation of *N*-acetylglucosaminitol migrated as a single product with distinguishable mobility. Labeled products with the mobilities of the two deacetylated forms of GalNAc-ol were found as major labeled components in all fractions generated by β elimination, namely ConA-unbound

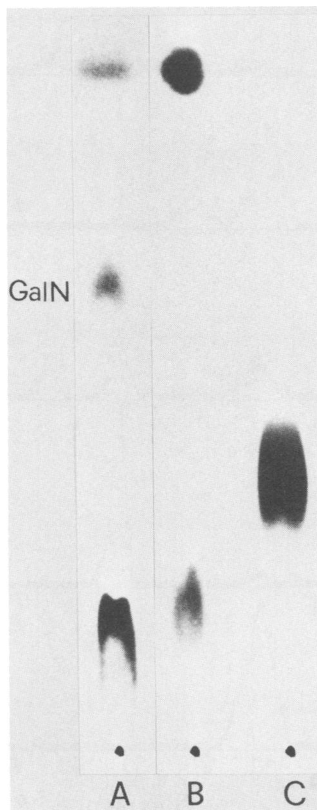

FIG. 4. Migration on silica gel plate of the strong acid hydrolysate of an O-linked oligosaccharide species generated by β elimination of ConA-unbound glycopeptides (A) in comparison with migration of deacetylated GalNAc-ol (B) and deacetylated *N*-acetylglucosaminitol (C). [^{3}H]GalNAc-ol, *N*-[^{3}H]acetylglucosaminitol, and GlcN-labeled ConA-unbound treated 5 shown in Fig. 2C were hydrolyzed in 4 N HCl for 4 h at 100°C. The samples were then subjected to TLC as described in the text (solvent A), and the labeled components were visualized by fluorography. About 3,000 cpm ofh

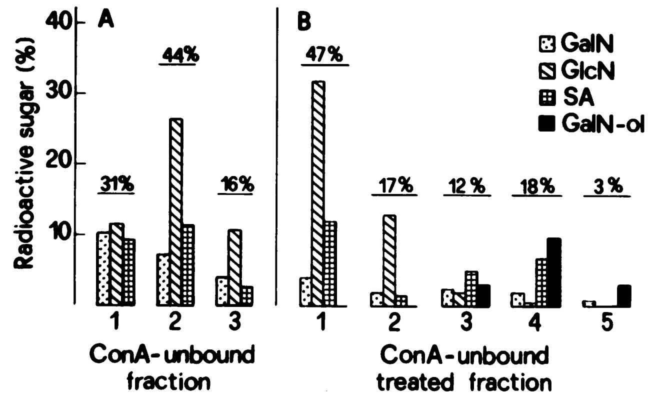

FIG. 5. Distribution of radiolabeled sugars among Bio-Gel fractions from ConA-unbound glycopeptides before and after mild alkaline borohydride treatment. (A) ConA-unbound glycopeptide fractions separated as described in the legend to Fig. 2A were subjected to strong acid hydrolysis, and the labeled sugars were separated by TLC as described in the legend to Fig. 3A. The percentage of radioactivity of each sugar was calculated assuming as 100% the total radioactivity recovered in the corresponding chromatographic lane. Each percentage was then multiplied by the fractional amount of radioactivity (indicated above horizontal bars) present in each glycopeptide fraction. The percentage assigned to sialic acid was that recovered in its degradation products (spots indicated as SAdeg in Fig. 3A). (B) ConA-unbound glycopeptides were subjected to mild alkaline borohydride treatment as described by Spiro (32). The fractions separated by Bio-Gel filtration as described in the legend to Fig. 2B were subjected to strong acid hydrolysis and analyzed by TLC as described above. The percentage distributions of radiolabeled sugars among Bio-Gel fractions were calculated as described above.

treated 3, 4, and 5 and ConA-bound treated 2 and 3, and thus they must be considered as O-linked oligosaccharides (Fig. 5B and 6B). The cleavage of O-linked oligosaccharides from the original glycopeptides was also demonstrated by a change in the relative distribution of labeled amino sugars among the residual glycopeptides. Figures 5 and 6 show the percentage distributions of labeled amino sugars among the fractions separated by Bio-Gel filtration after mild alkaline borohydride treatment (panels B) in comparison with that quantitated in untreated glycopeptides (panels A). In residual glycopeptides, i.e., ConA-unbound treated 1 and 2 and ConA-bound treated 1, the relative amount of GlcN was always increased, indicating that N-linked triantennary and diantennary chains, respectively, are the major oligosaccharides of these glycopeptide species. A small amount of GalN was still recovered in the N-linked glycopeptides, mainly in ConA-unbound treated 1 (Fig. 5B). This result may be interpreted either as the occurrence of GalNAc bound to N-linked chains or, more likely, as the persistence of O-linked chains still bound to the peptide. It was reported (18, 32) that in several cases mild alkaline borohydride treatment does not result in the complete release of O-linked chains.

Degree of sialylation of N-linked triantennary glycopeptides and O-linked oligosaccharides. To detect the extent of sialylation of O-linked oligosaccharides and N-linked triantennary glycopeptides, we subjected them to DEAE-Sephacel chromatography, which allows the separation of oligosaccharide species according to their negative charge (1, 24). The high-MW triantennary glycopeptide (ConA-unbound treated 1) could be fractionated in several peaks of increasing negative charge (Fig. 7A). This behavior confirms our previous observation (25) that triantennary glycopeptides from

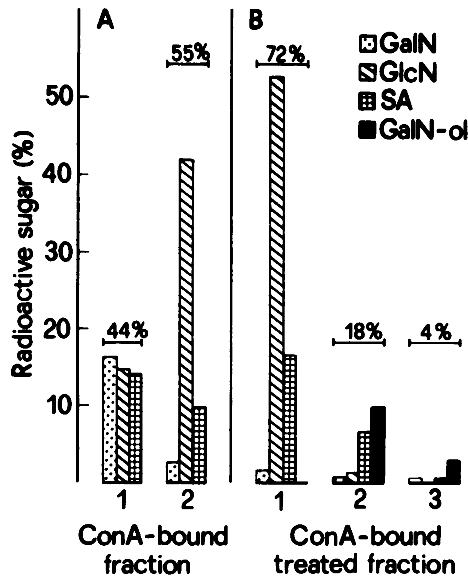

FIG. 6. Relative distribution of radiolabeled sugars among Bio-Gel fractions from ConA-bound glycopeptides before and after mild alkaline borohydride treatment. (A) ConA-bound glycopeptide fractions separated as described in the legend to Fig. 2B were subjected to strong acid hydrolysis, and the labeled sugars were separated by TLC as described in the legend to Fig. 3B. The percentage distribution of each radioactive sugar among the glycopeptides was calculated as described in the legend to Fig. 5A. (B) ConA-bound glycopeptides subjected to mild alkaline borohydride treatment were fractionated on a Bio-Gel P-10 column as shown in Fig. 2D and analyzed for labeled amino sugar composition by TLC. The percentage values were calculated as described in the legend to Fig. 5A.

gC are heterogenous with respect to the number of sialic acid residues, which range from 0 to 3. Consistent with the presence of asialylated triantennary glycopeptides, ConA-unbound treated 2 did not bind to a DEAE-Sephacel column and was recovered partially in flowthrough fractions and partially in fractions eluted at the beginning of the salt gradient (Fig. 7B). The highest-MW O-linked oligosaccharide was eluted for the large majority from a DEAE column by a salt concentration higher than that necessary to remove a monosialylated oligosaccharide such as sialyllactose (Fig. 7C). ConA-unbound treated 4 coeluted with sialyllactose, whereas the smallest O-linked oligosaccharide species emerged as a neutral component (Fig. 7D and E). These results strongly suggest that gC carries two species of sialylated O-linked oligosaccharides, with 2 and 1 sialic acid residues, respectively, and few O-linked chains devoid of sialic acid, probably originated by incomplete processing. The amounts of radioactivity recovered as sialic acid after neuraminidase digestion of O-linked sialylated chains were consistent with the degree of sialylation assigned on the basis of DEAE-Sephacel chromatographic behavior. The radioactivity released as sialic acid from the largest O-linked oligosaccharides (ConA-unbound treated 3) was almost twice that released from ConA-unbound treated 4, which coeluted with sialyllactose (data not shown).

DISCUSSION

It is apparent from the experiments reported above that gC from HSV-1-infected BHK cells contains O-linked oligosaccharides in addition to the N-linked diantennary and triantennary chains previously described (25). The presence of O-linked oligosaccharides is demonstrated by (i) the release of small oligosaccharides from large GalNAc-rich pronase glycopeptides on β elimination and (ii) the identification of GalNAc-ol in the oligosaccharides generated by mild alkaline borohydride treatment. Our results indicate that O-linked oligosaccharides occur in gC predominantly as mono- and disialylated species of low MW. Monosialylated trisaccharides and disialylated tetrasaccharides were described as major constituents of O-linked oligosaccharides in glycoprotein E1 of mouse hepatitis virus (18) and in glycoprotein gG.2 from herpes simplex virus type 2 (F. Serafini-Cessi, N. Malagolini, F. Dall'Olio, L. Pereira, and G. Campadelli-Fiume, Arch. Biochem. Biophys., in press). Johnson and Spear (13) studied the modifications in gel filtration profile of GlcN- and galactose-labeled gC after mild alkaline borohydride treatment and suggested the presence of small-MW oligosaccharides similar to the di-, tri-, and tetrasaccharides observed in submaxillary mucins, as well as the presence of large O-linked chains comparable in size and

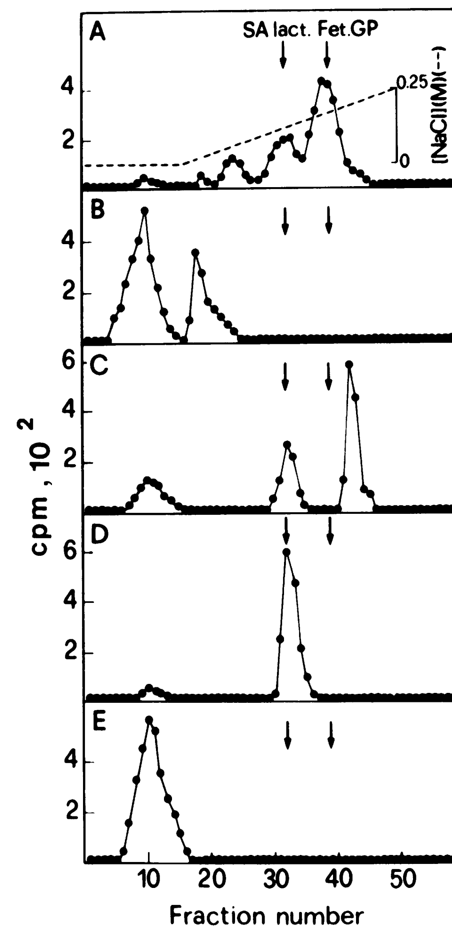

FIG. 7. DEAE-Sephacel chromatography of ConA-unbound glycopeptides fractionated by Bio-Gel filtration after mild alkaline borohydride treatment as described in the legend to Fig. 2C. The elution positions of sialyllactose (SA lact.) and triantennary fetuin glycopeptide (Fet. GP), indicated by the arrows in panel A, were determined by loading on the column unlabeled sialyllactose and unlabeled fetuin glycopeptides, which were detected by phenol-sulfuric acid test (6). (A) ConA-unbound treated 1; (B) ConA-unbound treated 2; (C) ConA-unbound treated 3; (D) ConA-unbound treated 4; (E) ConA-unbound treated 5.

composition to the large blood group substances described by Feizi et al. (7). These oligosaccharide species were rich in GlcNAc (7). Based on the present data on the elution position of the largest O-linked chain (ConA-unbound treated fraction 3 [Fig. 2C]) and the paucity in it of GlcNAc (Fig. 5B), it can be ruled out that an oligosaccharide species with the size and composition of large blood group substances occurs in gC. Our results from Bio-Gel filtration (Fig. 2C and D), ion-exchange chromatography (Fig. 7), and amino sugar analysis (Fig. 5B) of the O-linked oligosaccharides of gC indicate that the major species (ConA-unbound treated 4) behaved as a monosialylated trisaccharide with GalNAc at its reducing end. However, further studies are required to determine the chemical structure of the different size classes of O-linked chains carried by gC.

We separated from pronase-digested GlcN-labeled gC a peculiar glycopeptide, named ConA-bound 1, which carries both one N-linked diantennary chain and O-linked oligosaccharides apparently bound to a cluster of hydroxyamino acids. The exact number of O-linked oligosaccharides bound to this glycopeptide could be calculated by its contents of labeled GlcNAc and GalNAc, assuming that the specific activity of the two amino sugars was at equilibrium after the long labeling period. Since the amount of labeled GlcNAc and GalNAc was the same, four residues of GalNAc are needed to equalize the four GlcNAc residues carried by the N-linked chain (two in the chitobiosyl core and two in the branched chains). Practically all of the GalNAc of the ConA-bound glycopeptide was converted to GalNAc-ol by mild alkaline borohydride treatment, which means that all GalNAc residues are located at the reducing end of O-linked oligosaccharides. From this calculation, ConA-bound fraction 1 seems to carry four O-linked oligosaccharides and one N-linked diantennary chain on the same peptide fragment. The amino acid sequence of gC predicted from DNA sequencing studies (9) shows that two N-glycosylation sites (asparagine 42 and 74) appear to be located close to a cluster of hydroxyamino acids, suggesting that ConA-bound 1 might be generated from these portions of the peptide backbone. Because of the partial conversion of GalNAc to GalNAc-ol after β elimination of ConA-unbound fractions and the incomplete separation of the various oligosaccharide species, we do not feel that we can estimate the exact number of O-linked chains carried by ConA-unbound glycopeptides. However, since our results indicate that the majority of O-linked chains are included in the ConA-unbound fraction which, in turn, represents the major component of gC pronase glycopeptides, we suggest that the number of O-linked oligosaccharides carried by gC is much higher than that calculated to be present in the ConA-bound fraction.

Previously we reported the occurrence in HSV-1-infected BHK cells of *N*-acetylgalactosaminyltransferase, which catalyzes in vitro the addition of GalNAc to hyroxyamino acids of immature forms of HSV-1 glycoproteins, including immature gC (27). The inability of the mature forms of HSV-1 glycoproteins to accept in vitro GalNAc was related to the inadequate configuration of mature glycoproteins, which carry fully processed oligosaccharides. Current data strongly suggest that the enzyme we described does act within HSV-1-infected cells for the initial assembly of O-linked oligosaccharides of gC.

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