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Complex-Type N-Linked Oligosaccharides of gp120 from Human Immunodeficiency Virus Type 1 Contain Sulfated *N*-Acetylglucosamine

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The major envelope glycoproteins gp120 and gp41 of human immunodeficiency virus type 1, the causative agent for human AIDS, contain numerous N-linked oligosaccharides. We report here our discovery that *N*-acetylglucosamine residues within the complex-type N-linked oligosaccharides of both gp120 and its precursor, gp160, are sulfated. When human Molt-3 cells persistently infected with human T-cell leukemia virus III_B were metabolically radiolabeled with $^{35}SO_4$, gp160, gp120, and to some extent gp41 were radiolabeled. The $^{35}SO_4$-labeled oligosaccharides were quantitatively released by *N*-glycanase treatment and were bound by immobilized *Ricinus communis* agglutinin I, a lectin that binds to terminal β-galactosyl residues. The kinetics of release of sulfate upon acid hydrolysis from $^{35}SO_4$-labeled gp120 indicate that sulfation occurs in a primary sulfate ester linkage. Methylation analysis of total glycopeptides from Molt-3 cells metabolically radiolabeled with [^{3}H]glucosamine demonstrates that sulfation occurs at the C-6 position of *N*-acetylglucosamine. Fragmentation of the gp120-derived $^{35}SO_4$-labeled glycopeptides by treatment with hydrazine and nitrous acid and subsequent reduction generated galactosyl-anhydromannitol-6-$^{35}SO_4$, which is the expected reaction product from GlcNAc-6-sulfate within a sulfated lactosamine moiety. Charge analysis of the [^{3}H]galactose- and [^{3}H]glucosamine-labeled glycopeptides from gp120 and gp160 indicates that approximately 14% of the complex-type N-linked oligosaccharides are sulfated.

Human immunodeficiency virus type 1 (HIV-1), the retrovirus responsible for AIDS, contains two major envelope glycoproteins, designated gp120 and gp41, that arise by proteolytic cleavage of a precursor, gp160 (5, 26, 47, 58). The glycosylation of Asn residues within gp160 is essential for its maturation and for eventual production of infective virus (33). The peptide sequence of the envelope glycoproteins indicates that gp120 and gp41 contain, respectively, approximately 24 and 3 N-glycosylation consensus sites (Asn-X-Ser/Thr) (47). Site-specific glycosylation studies of recombinant forms of these glycoproteins and sensitivity of the native glycoproteins to deglycosylation treatments indicate that most of the consensus sites are N glycosylated (32).

Structural features of N-linked oligosaccharides of the viral envelope glycoprotein gp120 have been reported for some recombinant forms expressed in Chinese hamster ovary cells (32, 38) as well as for those synthesized by persistently infected cultured human H9 T-lymphoblastoid cells (16, 37) and by persistently infected human Molt-3 cells (35). The studies demonstrated that both high-mannose- and complex-type N-linked oligosaccharides occur on gp120 from all mammalian sources. Although there is little evidence at present that the oligosaccharides of gp120 are directly involved in interactions of the virus with target cells, some oligosaccharides containing sulfate residues have anti-HIV activity (1, 3, 21, 28, 31, 41, 49, 56). These studies stimulated us to investigate the oligosaccharide structures of the envelope glycoproteins in a human T-cell line, Molt-3, persistently infected with HIV-1 strain HTLV-III_B. During our analysis of the oligosaccharides of metabolically radiolabeled envelope glycoproteins produced by these cells, we observed that $^{35}SO_4$ was incorporated into gp160, gp120, and gp41. In this report, we demonstrate that some of the complex-type N-linked oligosaccharides in the envelope glycoproteins of HIV-1 synthesized by persistently infected Molt-3 cells contain sulfate linked to the C-6 position of outer-chain *N*-acetylglucosamine residues. The presence of sulfated oligosaccharides on HIV-1 is intriguing in light of studies by others demonstrating that sulfated oligosaccharides have antiviral activity.

MATERIALS AND METHODS

Materials. *Ricinus communis* agglutinin I (RCA-I)–agarose was purchased from Vector Laboratories, and concanavalin A (ConA)-Sepharose was obtained from Pharmacia. Lactose, α-methylmannoside, α-methylglucoside, sodium acetate, QAE-Sephadex, dimethyl sulfoxide, β-galactosidase, and sodium nitrite were purchased from Sigma Chemical Co. Pronase (grade B) was obtained from Calbiochem, recombinant *N*-glycanase was obtained from Genzyme, and *Arthrobacter ureafaciens* neuraminidase was purchased from Boehringer Mannheim. Keratan sulfate was obtained from Seikagaku-kojyo Co. Ltd. CMP-[U-^{14}C]*N*-acetylneuraminic acid

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(1.8 mCi/mmol), D-[6-^{3}H]glucosamine (27 Ci/mmol), D-[6-^{3}H]galactose (25 Ci/mmol), and $Na_2{}^{35}SO_4$ (250 to 1,000 mCi/mmol) were obtained from New England Nuclear. [U-^{14}C]*N*-acetylneuraminic acid was prepared by neuraminidase digestion of CMP-[U-^{14}C]*N*-acetylneuraminic acid.

Maintenance of cultured Molt-3 cells and infection with HTLV-III$_B$. Molt-3 cells persistently infected with and producing HTLV-III$_B$ were obtained from M. Essex (Harvard University School of Public Health, Boston, Mass.) and cultivated in RPMI 1640 medium supplemented with 20% fetal calf serum.

Metabolic radiolabeling and isolation of viral glycoproteins. Cells (5×10^6) in 5 ml of medium (RPMI 1640) were radiolabeled with either [^{3}H]glucosamine or [^{3}H]galactose at a concentration of 1 mCi/ml for 24 h. Cells (5×10^7) in 15 ml of minimal essential medium (Joklik modification) were radiolabeled with $^{35}SO_4$ at a concentration of 0.83 mCi/ml for 20 h. The cells were collected by centrifugation, washed in phosphate-buffered saline, and resuspended at 25°C in radio immunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 7.4] containing 0.5% aprotinin, 1% Nonidet P-40, 2 mM EDTA, 0.15 M NaCl, and 1% deoxycholate). Free virus was pelleted by centrifugation for 1 h at 14,000 rpm in a microcentrifuge and resuspended in 0.5 ml of RIPA buffer. To the supernatant, 1/10 volume of 10× RIPA buffer was added. The viral glycoproteins were obtained by immunoprecipitation with a high-titer serum from an AIDS patient as described previously (35). Immunoprecipitated and radiolabeled glycoproteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions and were visualized by fluorography.

***N*-Glycanase digestion of the glycoproteins.** The radiolabeled bands corresponding to gp120 were excised from gels and were swollen in a 15-ml conical tube with 250 μl of 50 mM sodium phosphate buffer (pH 7.4). The buffer was changed three times in a period of 2 h to equilibrate the pH of the gel slice. One unit of *N*-glycanase was added to the conical tube containing the swollen gel in 250 μl of fresh buffer. The *N*-glycanase digestion was carried out at 37°C for 24 h in a toluene atmosphere and was repeated three times at 24-h intervals by the addition of 1-U portions of enzyme.

Lectin affinity chromatography. Radiolabeled oligosaccharides released by the *N*-glycanase digestion were fractionated on ConA-Sepharose and RCA-I–agarose as described previously (34).

Treatment of the released oligosaccharides with neuraminidase. Oligosaccharides were dried under reduced pressure and were suspended in 49 μl of sodium acetate buffer (pH 4.5), 1 μl of *A. ureafaciens* neuraminidase (10 mU/μl) was added, and the mixture was incubated at 37°C for 16 h (8).

Mild acid treatment. Oligosaccharides were dried under reduced pressure in a screw-cap tube, 0.01 N HCl (0.5 ml) was added, and the mixture was heated at 100°C for 30 min (8). The reaction was stopped by the addition of 0.5 ml of 0.01 N NaOH, and the sample was desalted on a Sephadex G-25 column (1 by 50 cm) in 7% *n*-propanol.

Preparation of [^{3}H]galactose-labeled oligosaccharides and [^{3}H]galactose- and [^{3}H]glucosamine-labeled glycopeptides. Following SDS-PAGE and fluorography, gp120 was identified by its mobility, and the region containing the gp120 was excised from the gel. To prepare oligosaccharides from gp120, the excised gel slice was treated with *N*-glycanase as described above, and the radiolabeled oligosaccharides were isolated in the void volume following chromatography on a column of Sephadex G-25 (1 by 50 cm) in 7% *n*-propanol. Alternatively, the gel slice was treated with pronase to prepare radiolabeled glycopeptides (8).

QAE-Sephadex anion-exchange chromatography. QAE-Sephadex was swollen in 2 mM pyridine acetate buffer (pH 5.5), and columns were made with 0.25 ml of resin poured into a Pasteur pipette plugged with glass wool. The [^{3}H]galactose-labeled oligosaccharides not bound by ConA-Sepharose were either treated or not treated with *A. ureafaciens* neuraminidase or were desialylated by using mild acid as described above and then applied to a column of QAE-Sephadex. Bound oligosaccharides were eluted by a step gradient of 6 ml each of 20, 70, 140, 250, and 1,000 mM pyridine acetate, collecting 1-ml fractions.

Desulfation of oligosaccharides. Sulfate was removed from oligosaccharides by a chemical procedure (51). After lyophilization, 300 μl of dimethyl sulfoxide (pH 4.5) containing 10% methanol was added to the radiolabeled oligosaccharides released by the *N*-glycanase digestion. This mixture was heated at 100°C for 6 h in an argon atmosphere, and the reaction was terminated by the addition of 0.25 ml of 0.01 N NH_4OH. The reaction mixture was chromatographed on a column of Sephadex G-25 to separate the oligosaccharides from the reaction components. The oligosaccharides in the fractions containing the void volume were pooled, dried under reduced pressure, and then dissolved in 1 ml of 2 mM pyridine acetate before application to QAE-Sephadex.

Digestion of the glycopeptides with β-galactosidase. Glycopeptides and oligosaccharides were treated with mild acid to remove sialic acid and were chemically desulfated. To this dried material was added 50 μl of sodium citrate buffer (50 mM, pH 4.6) and 20 mU of jack bean β-galactosidase, and the mixture was incubated at 37°C for 48 h (29). The treated material was boiled for 5 min and then desalted by chromatography on a column of Sephadex G-25 in 7% *n*-propanol. When [^{3}H]galactose-labeled glycopeptides were treated with β-galactosidase, the released radioactivity was recovered in the included volume of the Sephadex G-25 column and analyzed by chromatography on a Dionex high-pressure liquid chromatography system, using a PA-1 column (4 by 250 mm).

Kinetics of acid hydrolysis of $^{35}SO_4$-labeled glycopeptides. $^{35}SO_4$-labeled glycopeptides derived by pronase digestion both from gp120 and from the total cellular glycoproteins were incubated with 0.6 ml of 0.25 N HCl at 100°C to release sulfate (14, 46). Portions of the reaction mixtures (0.1 ml) were removed at specified times (0, 0.5, 1, 2, 3, and 4 h), and Ba^{2+} was added to precipitate the released radiolabeled sulfate. The radioactivity remaining in the supernatant was measured by liquid scintillation counting, and a graph of the percentage of intact sulfate ester versus time was generated. From this graph, the $t_{1/2}$ of hydrolysis was determined by extrapolation.

Methylation analysis of the oligosaccharides. The [^{3}H]galactose- and [^{3}H]glucosamine-labeled glycopeptides were methylated, hydrolyzed, reduced, acetylated, and analyzed by condensational gas chromatography as described previously (7).

Hydrazine and nitrous acid treatment. The $^{35}SO_4$-labeled glycopeptides derived from gp120 and from total Molt-3 cellular glycoproteins were desialylated by mild acid treatment, de-N-acetylated by hydrazine treatment, fragmented by nitrous acid treatment, and then reduced with $NaBH_4$. The method followed was that described previously (10, 51) except that after reduction, the charged oligosaccharides were separated by chromatography on a column of QAE-Sephadex. The resulting oligosaccharides that eluted with 20

mM pyridine acetate were further separated by descending paper chromatography.

Descending paper chromatography. Oligosaccharides were separated by using either solvent system A (*n*-butyl alcohol–acetic acid–1 M NH_4OH [3:2:1]) for 30 h (51) or solvent system B (pyridine-ethyl acetate-acetic acid-water [5:5:1:3]) for 16 h. The standard Galβ1-4-anhydromannitol-6-SO_4 was prepared from keratan sulfate by hydrazine and nitrous acid treatment and reduction with NaB^3H_4 as described previously (10). The distribution of radioactivity on the chromatograms resulting from treatment of the $^{35}SO_4$-labeled oligosaccharides was measured by liquid scintillation counting of 1-cm sections of the paper as previously described (8).

RESULTS

Persistently infected Molt-3 cells incorporate $^{35}SO_4$ into viral glycoproteins. Molt-3 cells persistently infected with HTLV-III_B were metabolically radiolabeled with $^{35}SO_4$, as well as with [^{3}H]glucosamine and [^{3}H]galactose, and the viral glycoproteins were isolated by immunoprecipitation, separated by SDS-PAGE, and visualized by fluorography. Immunoprecipitations were performed on cells isolated by centrifugation, total culture medium, and virus isolated from the culture medium. As shown in Fig. 1A, significant radioactivity from $^{35}SO_4$ was incorporated into both gp160 and gp120. (In some experiments, we have observed incorporation of $^{35}SO_4$ into gp41, although the labeling seems to be less and more variable.) In a control experiment using uninfected Molt-3 cells (Fig. 1B), we demonstrated that no radiolabeled glycoproteins were immunoprecipitated. Consistent with our previous observations, gp41, gp120, and gp160 could be metabolically radiolabeled with [^{3}H]glucosamine and [^{3}H]galactose (Fig. 1C). These observations led us to examine the possibility that the oligosaccharides of gp160 and gp120 might be sulfated. Because of the small amount of radiolabel incorporated into gp41, it was not chemically characterized further.

$^{35}SO_4$ incorporated in gp120 and gp160 is contained in N-linked oligosaccharides. To determine whether the $^{35}SO_4$ was present in oligosaccharides, the gel bands containing $^{35}SO_4$-labeled gp160 and gp120 were excised and treated with *N*-glycanase as described in Materials and Methods. In both cases radioactivity was released, indicating that the sulfate was contained in N-linked oligosaccharides (data not shown). Approximately 80 to 90% of the $^{35}SO_4$ contained within gp120 was released by the *N*-glycanase treatment, similar to the amount released by pronase digestion. To rule out the possibility that *N*-glycanase contained contaminating protease activity that caused release of peptides rather than oligosaccharides, we treated [^{35}S]cysteine-labeled gp160 with *N*-glycanase under the same conditions. No [^{35}S]cysteine-labeled material was released, indicating that *N*-glycanase had no significant proteolytic activity, and thus the radiolabeled sulfate was likely to be contained in sulfated oligosaccharides.

Radioactivity incorporated in gp120 is contained in complex-type N-linked oligosaccharides. To determine whether the sulfate was incorporated into high-mannose- or complex-type oligosaccharides, a portion of the *N*-glycanase-released $^{35}SO_4$-labeled oligosaccharides from gp120 was applied to a column of ConA-Sepharose. This lectin interacts with high affinity with high-mannose/hybrid-type Asn-linked oligosaccharides, interacts less strongly with biantennary complex-type Asn-linked oligosaccharides, and does not interact appreciably with either tri- and tetra-antennary or bisected

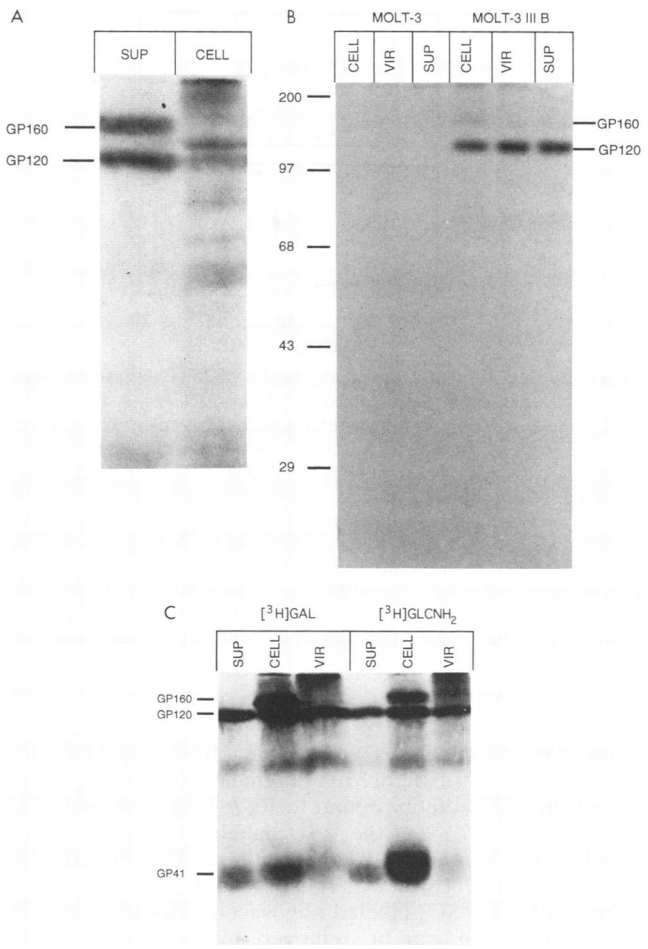

FIG. 1. Electrophoresis of immunoprecipitated viral proteins from the metabolically radiolabeled HTLV-III_B-infected Molt-3 cells. Persistently infected Molt-3 cells were metabolically radiolabeled with either $^{35}SO_4$, [^{3}H]galactose, or [^{3}H]glucosamine as described in Materials and Methods. Immunoprecipitations were performed with human antiserum on cells isolated by centrifugation (CELL), total culture media (SUP), and virus isolated from the culture media (VIR) and were subjected to SDS-PAGE under reducing conditions. The radiolabeled viral proteins gp160, gp120, and gp41 were visualized by fluorography, and their positions are indicated by lines. (A) Immunoprecipitation of gp120 and gp160 from persistently infected Molt-3 cells metabolically radiolabeled with $^{35}SO_4$; (B) control immunoprecipitation of gp120 and gp160 from uninfected Molt-3 cells (left-hand three lanes) and persistently infected Molt-3 cells (right-hand three lanes) (sizes are indicated in kilodaltons); (C) immunoprecipitation of gp41, gp120, and gp160 from persistently infected Molt-3 cells metabolically radiolabeled with [^{3}H]galactose or [^{3}H]glucosamine.

biantennary complex-type Asn oligosaccharides (34). More than 95% of the *N*-glycanase-released oligosaccharides were not bound by ConA-Sepharose (data not shown). These results indicate that high-mannose-type N-linked oligosaccharides are not sulfated and that the sulfate residues occur within complex-type N-linked oligosaccharides.

To further characterize the type of oligosaccharides containing sulfate residues, the oligosaccharides derived from $^{35}SO_4$-labeled gp120 were applied to a column of RCA-I–agarose before and after neuraminidase digestion. This immobilized lectin binds with high affinity to complex-type

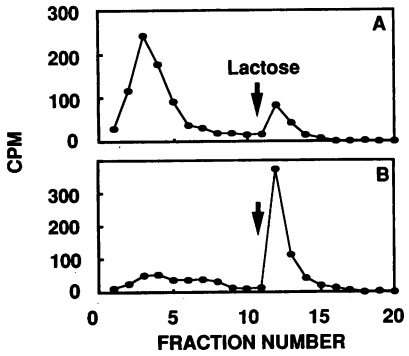

FIG. 2. Lectin affinity chromatography of *N*-glycanase-released $^{35}SO_4$-labeled oligosaccharides from gp120 on RCA-I–agarose. The band corresponding to $^{35}SO_4$-labeled gp120 derived from metabolically radiolabeled infected Molt-3 cells was excised from an SDS-polyacrylamide gel and subjected to *N*-glycanase digestion as described in Materials and Methods. After desalting, a portion of the released oligosaccharides was treated with neuraminidase. The oligosaccharides were applied to a column of RCA-I–agarose before (A) and after (B) neuraminidase digestion. The bound oligosaccharides were eluted with 200 mM lactose as indicated by the arrows. Portions of the fractions were subjected to liquid scintillation counting, and the profile of radioactivity is shown for each column.

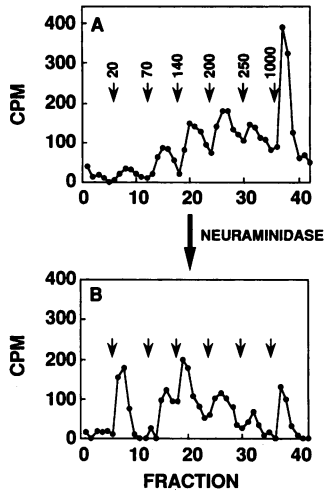

FIG. 3. Anion-exchange chromatography of $^{35}SO_4$-labeled gp120 on QAE-Sephadex. The region containing the $^{35}SO_4$-labeled gp120 derived from metabolically radiolabeled infected Molt-3 cells was excised from an SDS-polyacrylamide gel and subjected to *N*-glycanase digestion. The released complex-type N-linked oligosaccharides were isolated by chromatography on ConA-Sepharose, then desalted, and applied to a column of QAE-Sephadex before (A) or after (B) desialylation by neuraminidase treatment as described in Materials and Methods. The arrows indicate sequential elution with a step gradient of pyridine acetate (20, 70, 140, 200, 250, and 1,000 mM).

N-linked oligosaccharides with terminal β1-4-linked galactosyl residues (2, 29). Before neuraminidase treatment, about 20% of the radioactivity bound to RCA-I–agarose, whereas after treatment, 72% of the $^{35}SO_4$-labeled oligosaccharides bound to the immobilized lectin and could be eluted with 200 mM lactose (Fig. 2). These results indicate that a majority of the $^{35}SO_4$-labeled oligosaccharides terminate in sialic acid which can be removed by neuraminidase to expose a terminal β1-4-linked galactosyl residue. The combination of sensitivity of the $^{35}SO_4$-labeled oligosaccharides to *N*-glycanase and the properties of their interactions with immobilized lectins demonstrate that the $^{35}SO_4$ is incorporated into the complex N-linked-type oligosaccharide(s) of gp120. However, we addressed the possibility that some of the $^{35}SO_4$-labeled material might also be present in a glycosaminoglycan derivative. The $^{35}SO_4$-labeled oligosaccharides were treated for 24 h with chondroitinases A, B, and C, keratanase, or endo-β-galactosidase, and the treated material was analyzed by descending paper chromatography (data not shown). No radiolabeled material was released by these enzyme treatments, supporting the conclusion that the $^{35}SO_4$-labeled material is not a glycosaminoglycan but is derived from complex-type N-linked oligosaccharides.

To assess the distribution of negative charges on the anionic oligosaccharides of gp120, the $^{35}SO_4$-labeled oligosaccharides were subjected to anion-exchange chromatography on a column of QAE-Sephadex (Fig. 3) before and after desialylation by neuraminidase treatment as described in Materials and Methods. In this anion-exchange chromatography, oligosaccharides with one, two, three, four, and five negative charges are eluted by 20, 70, 140, 200, and 250 mM NaCl, respectively, and oligosaccharides containing more than five negative charges are eluted by 1 M NaCl (57). As shown in Fig. 3A, before neuraminidase treatment, the $^{35}SO_4$-radiolabeled oligosaccharides were found as species containing one (<2%), two (10%), three (16%), four (23%), five (18%), or more than five (31%) negative charges. After desialylation with neuraminidase, the profile of charged species shifted toward lesser charged, but not uncharged, species. These species contained one (17%), two (15%), three (26%), four (18%), five (9%), and more than five (15%) negative charges. These data suggest that the glycopeptides from gp120 contain negative charges contributed both by sialic acid and by sulfate and that some glycopeptides contain multiple sulfate residues.

Sulfation occurs on outer sugar residues of the N-linked oligosaccharides [^{3}H]galactose-labeled gp120. We next sought to determine approximately what percentage of the oligosaccharides were sulfated. The [^{3}H]galactose-labeled glycopeptides were isolated by chromatography on ConA-Sepharose, and unbound glycopeptides (representing more than 95% of the applied radioactivity) were desalted on a column of Sephadex G-25. To determine the proportion of neutral and negatively charged oligosaccharides, a portion of these glycopeptides was subjected to anion-exchange chromatography on QAE-Sephadex before (Fig. 4A) and after (Fig. 4B) mild acid hydrolysis. Rather than using neuraminidase treatment to remove sialic acid residues, we employed mild acid hydrolysis because the *A. ureafaciens* neuraminidase may not work efficiently on certain modified sialic acids and may work with differential rates, depending on the sialic acid linkage. Treatment with mild acid results in the complete release of sialic acid residues, but sulfate esters are relatively resistant to such mild acid treatment. Seventy-six percent of the [^{3}H]galactose-labeled radioactivity was bound by QAE-Sephadex, demonstrating that those glycopeptides were negatively charged (Fig. 4A). After the mild acid treatment to remove sialic acid, 14% of the radioactivity was recovered in residually charged material that bound to QAE (Fig. 4B). These results suggest that approximately 14% of the N-linked oligosaccharides of the complex type might contain

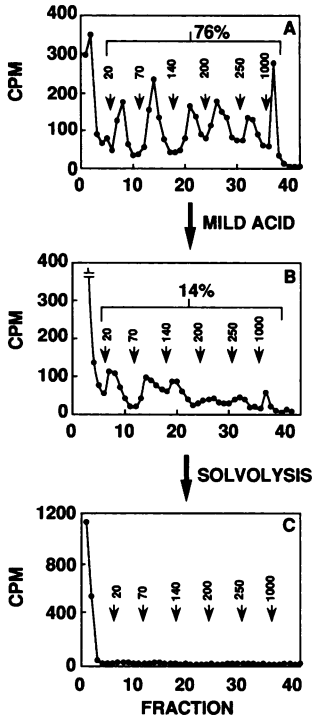

FIG. 4. Analysis of [^{3}H]galactose-labeled gp120 glycopeptides by anion-exchange chromatography on QAE-Sephadex before and after desialylation and desulfation. The region containing the [^{3}H]galactose-labeled gp120 derived from metabolically radiolabeled infected Molt-3 cells was excised from an SDS-polyacrylamide gel and subjected to pronase digestion. The glycopeptides with complex-type N-linked oligosaccharides, which were not bound by ConA-Sepharose, were desalted, and a portion was applied to a column of QAE-Sephadex (A). Another portion of the complex-type N-linked oligosaccharides was desialylated by treatment with mild acid and then applied to a column of QAE-Sephadex (B), where 14% of the total radioactivity was bound by the column. A portion of the mild acid-treated glycopeptides was desulfated by solvolysis and then applied to QAE-Sephadex (C). The arrows from left to right in each chromatogram indicate sequential elution with a step gradient of pyridine acetate (20, 70, 140, 200, 250, and 1,000 mM).

sulfated moieties. To assess whether the residual negative charge on the desialylated glycopeptides was due to sulfate residues, the glycopeptides were subjected to solvolysis to effect complete desulfation, and the desalted glycopeptides were then reapplied to QAE-Sephadex. As shown in Fig. 4C, more than 95% of the radioactivity was recovered as a neutral species that did not bind to the anion-exchange resin. Since chemical desulfation resulted in conversion of the residually charged glycopeptides to neutral species, these results demonstrate that about 14% of the glycopeptides are sulfated.

In studies of sulfated glycoproteins in other systems, it has been shown that sulfation of complex-type N-linked oligosaccharides can occur on inner GlcNAc residues in the core chitobiosyl sequence and also on Gal and GlcNAc residues in the outer-branch lactosamine sequences. We therefore wanted to determine the location of sulfate residues in gp120 produced by Molt-3 cells. We reasoned that if all sulfation occurred in the inner core of the glycopeptides, then β-galactosidase treatment should release 100% of the [^{3}H]galactose label in penultimate galactose residues after desialylation. We found, however, that β-galactosidase treatment of the desialylated glycopeptides released only 75% of the radioactivity. The released galactose was separated from the residual glycopeptides by size exclusion chromatography on Sephadex G-25 (data not shown). This released radioactivity was contained exclusively in galactose, as evidenced by composition analysis of the radioactivity contained in the Sephadex G-25 included volume (data not shown). We considered the possibility that the inability of β-galactosidase to release all of the galactose radioactivity might be due to the presence of sulfated GlcNAc residues in the penultimate position to the galactose. Indeed, when the glycopeptides were desulfated by solvolysis prior to treatment with β-galactosidase, we observed that more than 95% of the radiolabeled galactose could be released (data not shown).

Thus, this sequential treatment of the [^{3}H]galactose-labeled glycopeptides with β-galactosidase before and after desulfation resulted altogether in the release of more than 95% of the galactose in the glycopeptides. Taken together, the results of exoglycosidase sensitivity of the [^{3}H]galactose-labeled glycopeptides before and after solvolysis suggest that sulfate is present on monosaccharide residues in the outer chains of the complex-type N-linked oligosaccharides. These data, however, cannot distinguish whether sulfation occurs on Gal and/or GlcNAc residues. This issue was addressed by the following experiments.

Hydrolysis kinetics indicates the presence of a primary sulfate ester linkage to galactose or glucosamine on gp120 and cellular glycoproteins. Sulfate esters differ in their sensitivity to acid hydrolysis based on their spatial orientation, and thus it is possible to characterize the type of linkage by analyzing the hydrolysis kinetics as a function of time (23, 46). Portions of the $^{35}SO_4$-labeled glycopeptides derived from gp120 and from total $^{35}SO_4$-labeled-glycopeptides of Molt-3 cells were hydrolyzed with 0.25 N HCl, samples were removed at various time points, and released sulfate was precipitated with Ba^{2+} (14, 23, 46). The percentage of sulfate ester remaining was plotted logarithmically as a function of time (Fig. 5). Our data indicate a $t_{1/2}$ of 137 min for sulfate release from total Molt-3 cell glycoproteins (Fig. 5A) and a $t_{1/2}$ of 140 min for release of sulfate from gp120 glycopeptides (Fig. 5B). It has been demonstrated previously that the $t_{1/2}$ values for hydrolysis of sulfate esters in 0.25 N HCl at 100°C are 10 to 40 min for the axial orientation, 40 to 87 min for the equatorial orientation, and 93 to 144 min for the primary sulfate ester (14, 23, 46). Therefore, our results indicate that sulfate esters occur as a single class of primary sulfate esters linked to a monosaccharide(s) within the complex-type N-linked oligosaccharides of gp120 and other glycoproteins synthesized by Molt-3 cells.

Methylation analysis demonstrates that the C-6 position of glucosamine residues is sulfated. We next sought to determine the specific sugar residue(s) to which primary sulfate residues are linked within the oligosaccharides of gp120, using the technique of methylation analysis. Because relatively large amounts of material are required for methylation analysis, we performed methylation on the total complex-type N-linked oligosaccharides from Molt-3 cells rather than from gp120 itself. All studies performed, including those described above, indicate that sulfation of the glycopeptides in gp120 produced by the persistently infected Molt-3 cells is reflective of the sulfation of nonviral glycopeptides produced by these cells. The total cellular [^{3}H]galactose- and [^{3}H]glucosamine-labeled glycopeptides were desialylated by mild acid treatment, and the residually charged species were obtained by chromatography on QAE-Sephadex. A portion

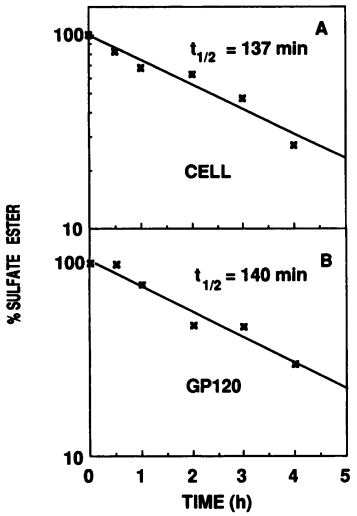

FIG. 5. Hydrolysis kinetics of sulfate esters of the $^{35}SO_4$-labeled glycopeptides derived from gp120 and from Molt-3 cellular glycoproteins. The $^{35}SO_4$-labeled glycopeptides from gp120 and from Molt-3 cellular glycoproteins were obtained by pronase digestion. These glycopeptides were hydrolyzed, the released sulfate was precipitated with Ba^{2+} as described in Materials and Methods, and the amount of radiolabeled sulfate precipitated was quantified in a scintillation counter. The percentage of intact sulfate ester was determined and plotted with respect to time of hydrolysis. (A) Molt-3 cellular glycopeptides; (B) gp120 glycopeptides.

of the charged glycopeptides was desulfated, and analysis by QAE-Sephadex chromatography confirmed that they were converted to neutral species after solvolysis (data not shown). Methylation analysis of the charged species indicated the presence of 3,4,6-tri-*O*-methyl-*N*-methyl-1,5,-di-*O*-acetylglucosaminitol (8%), 3,6-di-*O*-methyl-*N*-methyl-1,4,5-tri-*O*-acetylglucosaminitol (76%), and 3-*O*-methyl-*N*-methyl-1,4,5,6-tri-*O*-acetylglucosaminitol (16%) (Fig. 6A) and of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol (16%), 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol (65%), and 2,3,4-tri-*O*-methyl-1,5,6-tri-*O*-acetylgalactitol (19%) (Fig. 6B). There was no significant change in the methylation pattern of galactose residues before and after solvolysis (compare Fig. 6B with Fig. 6D). However, after chemical desulfation, there was little or no detectable 3-*O*-methyl-*N*-methyl-1,4,5,6-tri-*O*-acetylglucosaminitol (<2%) (Fig. 6C). We repeated the methylation analysis shown in Fig. 6 on two other occasions and obtained similar results (data not shown). These data demonstrate that sulfation occurs on the C-6 position of approximately 16% of the *N*-acetylglucosamine residues within glycopeptides produced by Molt-3 cells and further demonstrate that sulfation does not occur on galactose residues. This result is consistent with the rate of hydrolysis of the sulfate ester within Molt-3 cellular glycopeptides, as described above, which indicated that sulfate occurs mainly in a primary ester linkage.

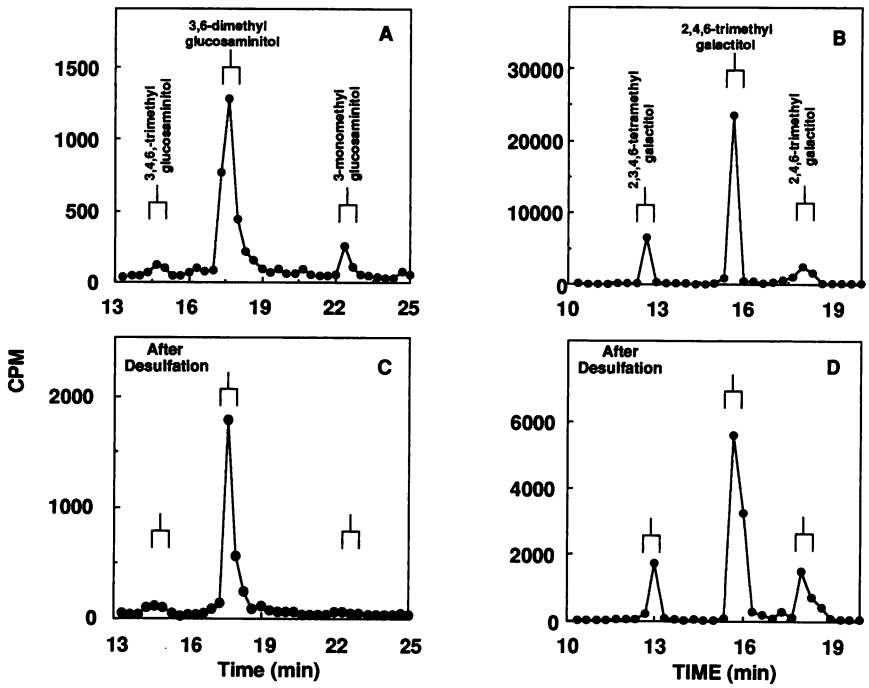

FIG. 6. Methylation analysis of [^{3}H]galactose- and [^{3}H]glucosamine-labeled Molt-3 cellular glycopeptides. Negatively charged [^{3}H]galactose- and [^{3}H]glucosamine-labeled glycopeptides derived from Molt-3 total cellular glycoproteins were obtained after desialylation as described in Results and were methylated before (A and B) and after (C and D) desulfation by solvolysis. The methylated species were separated by condensational gas chromatography, and the profiles of radioactivity measured by scintillation counting of fractions collected at 20-s intervals during the chromatography are shown. (A and C) [^{3}H]glucosamine-labeled glycopeptides; (B and D) [^{3}H]galactose-labeled glycopeptides. The elution positions of authentic partially methylated, peracetylated sugar derivatives derived from monosaccharides with the indicated linkages are indicated by brackets within each panel with the following designations: 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetylgalactitol (2,3,4,6-tetramethylgalactitol), 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol (2,4,6-trimethylgalactitol), 2,3,4-tri-*O*-methyl 1,5,6-tri-*O*-acetylgalactitol (2,3,4-trimethylgalactitol), 3,4,6-tri-*O*-methyl-*N*-methyl-1,5-di-*O*-acetylglucosaminitol (3,4,6-trimethylglucosaminitol), 3,6-di-*O*-methyl-*N*-methyl-1,4,5-tri-*O*-acetylglucosaminitol (3,6-dimethylglucosaminitol), and 3-*O*-methyl-*N*-methyl-1,4,5,6-tri-*O*-acetylglucosaminitol (3-monomethylglucosaminitol).

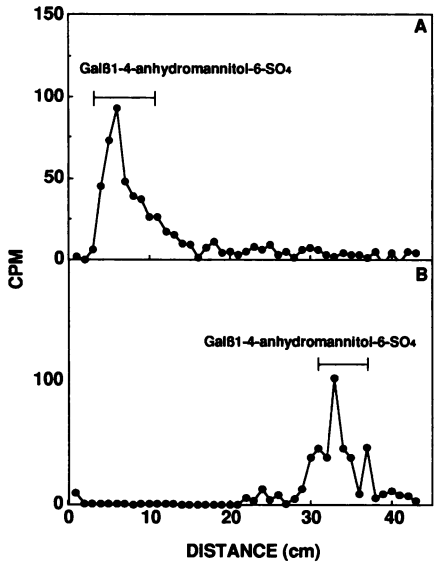

FIG. 7. Descending paper chromatograms of the oligosaccharide derivatives produced by hydrazine/nitrous acid treatment of $^{35}SO_4$-labeled gp120. $^{35}SO_4$-labeled glycopeptides derived from gp120 were desialylated by mild acid treatment, de-N-acetylated and fragmented with hydrazine and nitrous acid, and then reduced with $NaBH_4$ as described in Materials and Methods. The resultant charged oligosaccharides were isolated by chromatography on columns of QAE-Sephadex and then separated by descending paper chromatography in solvent system A (see Materials and Methods) for 16 h or in solvent system B for 30 h. The position of elution of authentic Galβ1-4-anhydromannitol-6-SO_4 derived from keratan sulfate is indicated by the horizontal line in each panel.

Hydrazine/nitrous acid fragmentation of the $^{35}SO_4$-labeled gp120 indicates the presence of C-6 sulfated glucosamine. From the results presented above, we reasoned that sulfate residues within the glycopeptides of gp120 and the total cellular glycopeptides might occur on GlcNAc residues within the lactosamine sequence Gal-GlcNAc that is common to complex-type N-linked oligosaccharides. It has been shown that hydrazine and nitrous acid treatment of oligosaccharides results in cleavage of GlcNAc residues at the reducing side and conversion of GlcNAc to anhydromannose (10, 51). Hydrazine and nitrous acid fragmentation of 6-sulfated GlcNAc residues with the lactosamine sequence should result in the production of the derivative Gal-β1-4-anhydromannose-6-SO_4 (or Gal-β1-4-anhydromannitol-6-SO_4 after reduction with $NaBH_4$). Glycopeptides derived from $^{35}SO_4$-labeled gp120 were desialylated, treated with hydrazine to de-N acetylate, and then fragmented by treatment with nitrous acid followed by reduction with $NaBH_4$ as described in Materials and Methods. To determine the nature of the charged fragments, the reaction products were applied to QAE-Sephadex. The majority of the radioactivity appeared as a single charged species eluting with 20 mM pyridine acetate (data not shown). This species was then characterized by descending paper chromatography using two different solvent systems (Fig. 7). The radiolabeled material recovered by this procedure had an R_f value similar to that of the authentic standard Galβ1-4-anhydromannitol-6-SO_4. The $^{35}SO_4$-labeled oligosaccharides from Molt-3 cells were also fragmented by hydrazine and nitrous acid and then reduced with $NaBH_4$, and upon analysis by descending paper chromatography they generated fragments with chromatographic mobilities similar to those derived from gp120 (data not shown).

We conclude from these results that the oligosaccharides of gp120 in HIV-1 produced by Molt-3 cells contain sulfate at the C-6 position of GlcNAc residues within *N*-acetyllactosamine sequences. This conclusion is supported by (i) similarities in the rates of hydrolysis of sulfate esters in cellular and viral glycopeptides, (ii) similarities in fragments produced by hydrazine/nitrous acid treatment of $^{35}SO_4$-labeled oligosaccharides from cellular and viral glycopeptides, (iii) demonstration by methylation analysis that all detectable sulfate esters within the cellular glycopeptides residue at the C-6 positions of GlcNAc residues, and (iv) demonstration that sulfate esters within gp120 glycopeptides residue on complex-type N-linked oligosaccharides containing *N*-acetyllactosamine sequences.

DISCUSSION

A number of studies in recent years have focused on the glycosylation of gp160, gp120, and gp41 of HIV. Many of these studies have been conducted with recombinant forms of the envelope glycoproteins produced in nonlymphocytic and nonhuman systems, such as Chinese hamster ovary cells (11, 32, 39). Only a few studies have been performed with envelope glycoproteins derived from human T-cell lines, and these have used the H9 lymphoblastoid cell line (16, 37, 53). In general, these studies have revealed that virtually all of the 24 consensus N-linked glycosylation sites are utilized and that the N-linked oligosaccharides are a mixture of high-mannose-, hybrid-, and complex-type with the general carbohydrate composition of sialic acid, galactose, fucose, mannose, and *N*-acetylglucosamine. In addition, recent studies indicate that the Molt-3 cell-derived HIV gp160/120 contains *N*-acetylgalactosamine and Ser/Thr-linked oligosaccharides (35).

Our current studies on the persistently infected Molt-3 cells indicate that complex N-linked oligosaccharides of gp120/160 contain sulfate at the C-6 position of some *N*-acetylglucosaminnyl residues in the nonreducing end of the oligosaccharides. The proposed structure for this modification of the N-linked oligosaccharide is as follows:

$$\begin{array}{c} SO_3^- \\ | \ 6 \\ \text{NeuAc}\alpha2\text{-}3/6\text{Gal}\beta1\text{-}4\text{GlcNAc}\beta1\text{-} \end{array}$$

This conclusion is supported by multiple lines of evidence, including (i) the metabolic incorporation of $^{35}SO_4$ into *N*-glycanase-releasable oligosaccharides, (ii) the binding of $^{35}SO_4$-labeled glycopeptides to immobilized RCA-I and enhancement of binding upon treatment of glycopeptides with neuraminidase, (iii) the kinetics of hydrolysis of the sulfate ester, (iv) the production of Galβ1-4-anhydromannitol-6-$^{35}SO_4$ following hydrazine and nitrous acid fragmentation, and (v) methylation analysis of [^{3}H]glucosamine-labeled oligosaccharides. Our studies also indicate that the persistently infected Molt-3 cells add sulfate to *N*-acetylglucosamine in many of their cellular oligosaccharides in addition to the virus-specific ones. Although we did not analyze the sulfate residues on gp41 because of limitation of materials, this glycoprotein was radiolabeled, and it is likely that it is sulfated similarly to gp120/160. However, since gp41 contains considerably fewer N-linked oligosaccharides than

does gp120/160, it is understandable that it is less radiolabeled.

There have been a number of studies demonstrating a relationship between the activity of the virus and sulfated carbohydrate residues. It has been reported that sulfated carbohydrates inhibit HIV-induced cytopathic effects in various $CD4^+$ lymphocytic cell lines, inhibit syncytium formation, and inhibit gp120 binding to cells (1, 3, 21, 28, 31, 41, 48, 56). In addition, gp120 itself is reported to have lectin-like activity and may bind to glycoprotein oligosaccharides (15) and sulfated galactocerebroside (sulfatide) (20). It remains to be determined, however, whether and to what extent sulfated carbohydrates might in some way be directly involved in vivo in virus adhesion and pathogenesis and whether the observed carbohydrate binding by gp120 is pertinent to the interactions of the virus with cells.

Although the direct identification of sulfated carbohydrates in gp120/160 of HIV-1 is a novel finding, the general occurrence of sulfate in glycoprotein oligosaccharides is not uncommon. *N*-Acetylglucosamine-6-sulfate has been found in a number of different glycoproteins and cell types, including chicken adipose lipoprotein lipase (23), *Dictyostelium discoideum* lysosomal enzymes (30), bovine pulmonary artery endothelial cells (48), and human and bovine thyroglobulin (51). Other types of sulfated GlcNAc and sulfation of monosaccharides other than GlcNAc in glycoprotein oligosaccharides have been described (4, 6, 13, 14, 19, 22, 43, 52, 55, 59, 60). Interestingly, the envelope glycoproteins of several other viruses, including herpes simplex virus type 1 (24), vesicular stomatitis virus (25), the paramyxovirus simian virus 5 (44), influenza virus (40), and others (42), have been reported to contain sulfate.

Sulfated carbohydrates are attracting increased attention largely because of two recently highlighted systems in which sulfated carbohydrates are expressed uniquely in animal glycoconjugates. Pituitary glycoproteins contain terminal GalNAc-4-sulfate units in complex-type N-linked oligosaccharides (18, 50). Recently, Fiete et al. (12) have shown that hepatic reticuloendothelial cells contain a receptor which specifically recognizes this sulfate modification of pituitary glycoproteins and facilitates their clearance. A sulfated, sialylated, and fucosylated carbohydrate ligand in a 50-kDa glycoprotein expressed by high endothelial venules appears to be a critical determinant for leukocyte attachment (27). Thus, it may be forecast that sulfated glycoconjugates, though poorly understood in terms of structure and function, may be important components of a number of protein and carbohydrate recognition systems.

Our findings demonstrate that not only are the HIV envelope glycoprotein oligosaccharides sulfated, but sulfation occurs as well on the nonviral (cellular) glycopeptides produced by the Molt-3 cell line. It is tempting to speculate from this observation that sulfation of *N*-acetylglucosamine residues occurs in many different glycoproteins and furthermore that some normal T-cells might generate sulfated glycoconjugates similar to those found here. Whether these sulfated oligosaccharides serve important functions in cellular recognition and adhesion as seen for glycoconjugates in many other systems is not known. Alternatively, sulfation might be important for protein targeting or protein association with other proteins, as seen in lysosomal enzyme biosynthesis wherein phosphorylated mannose residues are required for correct lysosomal enzyme targeting to lysosomes (9). The possibility that sulfated oligosaccharides in HIV envelope glycoproteins might be important in viral production or viral interactions with potential host cells is currently being explored.

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