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Sulfation of the Tumor Cell Surface Sialomucin of the 13762 Rat Mammary Adenocarcinoma

Steven R. Hull and Kermit L. Carraway

Department of Anatomy and Cell Biology, University of Miami School of Medicine, Miami, Florida 33101

ASGP-1, the major cell surface sialomucin of the 13762 ascites rat mammary adenocarcinoma, is at least 0.5% of the total ascites cell protein and has sulfate on 20% of its O-linked oligosaccharide chains. We have used this system to investigate the O-glycosylation pathway in these cells and to determine the temporal relationship between sulfation and sialylation. The two major sulfated oligosaccharides (S-1 and S-2) were isolated as their oligosaccharitols by alkaline borohydride elimination, anion exchange HPLC, and ion-suppression HPLC. From structural analyses S-1 is proposed to be a branched, sulfated trisaccharide $^{-}O_4$S-GlcNAcβ1,6-(Galβ1,3)-GalNAc and S-2 its sialylated derivative $^{-}O_4$S-GlcNAcβ1,6-(NeuAcα2,3-Galβ1,3)-GalNac. Pulse labeling with sulfate indicated that sulfation occurred primarily on a form of ASGP-1 intermediate in size between immature and mature sialomucin. Pulse-chase analyses showed that the intermediate could be chased into mature ASGP-1. The concomitant conversion of S-1 into S-2 had a half-time of less than 5 min. Monensin treatment of the tumor cells led to a 95% inhibition of sulfation with the accumulation of unsulfated trisaccharide GlcNAcβ1,6-(Galβ1,3)-GalNAc and sialylated derivative GlcNAcβ1,6-(NeuAcα2,3-Galβ1,3)-GalNAc. These data suggest that sulfation of ASGP-1 is an intermediate synthetic step, which competes with β-1,4-galactosylation for the trisaccharide intermediate and thus occurs in the same compartment as β-1,4-galactosylation. Moreover, sulfation precedes sialylation, but the two are rapidly successive kinetic events in the oligosaccharide assembly of ASGP-1.

Key words: Golgi, sialylation, glycoprotein, oligosaccharide, O-glycosylation

Sialomucins, glycoproteins containing large amounts of O-linked oligosaccharides, are prominent components at the cell surfaces of a number of tumors [1–6]. Their presence has been implicated in masking recognition of tumor cell surface antigens by host immune effector mechanisms [7,8] and correlated with metastatic potential of selected, variably metastatic clones of the 13762 NF rat mammary adenocarcinoma [9]. Moreover, cell surface sialomucins may provide useful markers for the

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diagnosis and therapy of human tumors, since many of the monoclonal antibodies which recognize tumor-associated carcinoma antigens appear to be directed against mucin epitopes [10–17].

Sulfate is a constituent of many secreted mucins [18]. Some studies have suggested an implication in the pathogenesis of cystic fibrosis [19]. Much less is known about the sulfation of tumor cell surface sialomucins. Ascites sublines of the 13762 rat mammary tumor show different amounts of sulfation of their predominant sialomucin [20,21], but the physiological significance of these differences is unknown. Recently, Yamori et al. [22] have shown a decreased amount of sulfated mucin associated with colon tumors and their metastases compared to normal colonic tissues. The distribution (secreted or cell surface) of this mucin and its site of sulfation have not been characterized.

We have been using 13762 ascites tumor cells to characterize the structures [4,23] and biosynthesis [6,24] of tumor cell surface mucins. ASGP-1, the sialomucin of these cells, comprises more than 0.5% of the total cell protein [4]. Different 13762 sublines have substantially different oligosaccharide compositions [21,23], but most of the oligosaccharides are derivatives of the tetrasaccharide Galβ1,4-GlcNAcβ1,6-(Galβ1,3)-GalNAc to which sialic acid, fucose, or α-galactose have been added [23]. In the MAT-B1 subline about 20% of the oligosaccharides are sulfated. Compositional analyses of these sulfated oligosaccharides suggest that they have a trisaccharide rather than tetrasaccharide core [20].

Biosynthesis of ASGP-1 occurs in at least three steps. During or shortly after translation a small fraction of serine or threonine residues is glycosylated to yield an immature form of the glycoprotein [24]. The immature form is converted to a larger species with a half-time of about 30 min (premature form), which is more slowly converted to a mature form [24]. Initiation of oligosaccharide formation by addition of GalNAc to polypeptide occurs at all three stages of biosynthesis [24–26]. Elongation of most of the chains to tetrasaccharide occurs during the intermediate stage. The final step in the formation of tetrasaccharide, β-1,4-galactosylation, can be inhibited by monensin without inhibiting sialylation [25]. Thus, β-1,4-galactosylation and sialylation appear to occur in different compartments.

Termination of the ASGP-1 oligosaccharides must occur by sulfation or sialylation or both. The present study was undertaken to define these termination steps and their relationship to the maturation step of the pathway. The results of structural and biosynthetic studies indicate that sulfation occurs in the same compartment with β-1,4-galactosylation and precedes sialylation.

MATERIALS AND METHODS

Materials

D-[6-^{3}H]glucosamine (25–40 Ci/mmol) and [^{35}S]H_2SO_4 were products of ICN. [^{3}H]Threonine was from Amersham. PNA, Triton X-100, Sepharose CL-2B, and Sephadex G-25 were obtained from Sigma. RPMI Select-Amine kit was from Gibco. Electronic-grade HCl gas was from Liquid Carbonics (Miami). Lithium dodecyl sulfate (LDS) was a product of Boehringer Mannheim. Spherisorb-SAX (5 μm) and Spherisorb-NH_2 (5 μm) were from Chromtec (Riviera Beach, FL). Lichrosorb Si-60 (5 μm) was from E. Merck. BioGel P6, Ag50 (H^+) and Ag1 (acetate) were from BioRad. Monensin and *Vibrio cholera* sialidase were from Calbiochem.

Cells and Cell Labeling

The MAT-B1 subline of the 13762 rat mammary adenocarcinoma was maintained by weekly passage as described previously [27]. After removal from the peritoneal cavity, cells were washed four times in cold Dulbecco's phosphate-buffered saline, pH 7.4, without calcium and resuspended in RPMI 1640 medium with 10% dialyzed calf serum, 5 mM pyruvate, and 2.5 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) at a concentration of 1×10^7 cells/ml. For pulse-chase experiments using sulfate and/or amino acid, cells were preincubated 30 min at 37°C in RPMI lacking $MgSO_4$ and/or the appropriate amino acid. For pulse-chase experiments using radiolabeled sugars, the cells were preincubated in basic medium containing 10% of the normal amount of glucose. At the end of this depletion step the cells were centrifuged and resuspended in 0.2–1.0 ml of the same medium containing the required isotope (2–10 mCi/ml sulfate; 0.5–2.5 mCi/ml [^{3}H]threonine or [^{3}H]glucosamine). After pulse labeling for 1–15 min the cells were washed rapidly in 10 volumes of phosphate-buffered saline (PBS) containing 5 mM unlabeled sulfate, amino acid, and/or sugar and then lysed or chased in complete RPMI containing 5 mM unlabeled precursor. For preparative equilibrium labeling, cells were suspended at a density of 5×10^6 cells/ml and incubated for 3–4 h in complete RPMI media lacking sulfate and the appropriate amino acid or sugar.

Preparation of ASGP-1

Cell lysates from pulse/chase or equilibrium labeling experiments were prepared as described previously [24]. Briefly, cells were lysed in boiling 2% LDS, sheared with a 25-gauge needle, and diluted 1:5 in Triton buffer (190 mM NaCl, 50 mM Tris, pH 7.4, 6 mM EDTA, 2.5% w/v Triton X-100). After centrifuging at 40,000 rpm in an SW 50.1 rotor for 40 min at 4°C, soluble PNA was added to the supernatant to precipitate ASGP-1 [24,25] and the mixture was incubated overnight at 4°C on a rotary mixer. The resulting precipitate was washed three times in Triton buffer.

Preparation of Sulfated Oligosaccharitols of ASGP-1

Following alkaline borohydride elimination [23], samples were lyophilized, desalted sequentially over Sephadex G-25 (1×40 cm) and Ag50(H^+) (1×5 cm), and evaporated from methanol under nitrogen to remove borate salts. The desalted oligosaccharitols were solubilized in 0.1 M pyridine acetate, pH 6.0, and chromatographed on a column of BioGel P6 (1×100 cm) in the same buffer at 4°C.

Anion-Exchange HPLC of Sulfated Oligosaccharitols

Anion-exchange HPLC of desalted oligosaccharitols was performed on an LDC Milton Roy HPLC system by using either a Spherisorb-SAX (5 μm) column (0.46×25 cm) or a Spherisorb-NH_2 (5μm) column (0.46×25 cm). Separations were achieved (unless noted otherwise in figure legends) by using a linear gradient formed by proportioning solvent A (2.5 mM KH_2PO_4, pH 4.0) and solvent B (125 mM KH_2PO_4, pH 4.0) as follows: T_0, 100% A; T_{24}, 100% B. A flow rate of 1 ml/min was used and 0.3- or 0.5-min fractions were collected. The column was routinely calibrated with neutral and sialylated oligosaccharitols as previously described [23].

Ion-Suppression HPLC of Sulfated Oligosaccharitols

Ion-suppression HPLC of sulfated oligosaccharitols was accomplished by using a Spherisorb-NH_2 (5 μm) column (0.46 × 25 cm). Separations were effected with a delayed linear gradient formed by proportioning solvent A (80% acetonitrile, 3% acetic acid-triethylamine, pH 5.5) and solvent B (3% acetic acid-triethylamine, pH 5.5) as follows: T_0, 85:15 (A:B); T_{12}, 85:15 (A:B); T_{22}, 20:80 (A:B). The flow rate was 1.0 ml/min; 0.3-min fractions were collected.

Periodate Treatment of Sulfated Oligosaccharitols

The procedure for mild periodate treatment was performed as described previously [23]. Oligosaccharitols were treated with 50 mM sodium periodate at 4°C by using 50 mM sodium acetate buffer, pH 4.5, for 18–24 h. After destruction of excess periodate with ethylene glycol, the samples were analyzed by anion-exchange HPLC.

Enzyme Digestions of Sulfated Oligosaccharitols

Oligosaccharitols were digested with 100 mU jack bean β-hexosaminidase (V labs, Covington, LA) or 10 mU *V. cholera* sialidase in 100–200 μl of 50 mM sodium citrate, pH 4.5, 0.02% sodium azide for 18–24 h at 37°C. The digestion mixture was heated for 3 min at 100°C, desalted, and analyzed by anion-exchange and/or ion-suppression HPLC.

Monensin Treatment of Cells

Cells (1×10^8) were treated with 1×10^{-6} M monensin for 30 min, resuspended in media with 2.0 mCi [^{35}S]SO_4, and incubated for 2 more h in the presence of 1×10^{-6} M monensin. After the incubation cells were lysed and sulfate incorporation into ASGP-1 was quantitatively compared to a control incubation performed in the absence of monensin by recovery of sulfate radiolabel in PNA precipitates.

Gel Filtration Chromatography of ASGP-1 in 0.1% LDS

PNA-precipitated ASGP-1 was solubilized in electrophoresis sample buffer (50 mM Tris, 1 mM EDTA, 20 mM dithiothreitol, 30% glycerol, 1% bromphenol blue) containing 1% LDS and applied to a Sepharose CL-2B column (1 × 40 cm) which had been equilibrated in 0.1% LDS, 10 mM Tris (pH 8.0), 0.02% sodium azide. Fractions (40 drops) were collected and appropriate aliquots were analyzed for radioactivity.

RESULTS

Identification and Separation of Sulfated Oligosaccharides of MAT-B1 ASGP-1

The first stage in describing the sulfation of the ASGP-1 oligosaccharides is the identification and characterization of the major sulfated oligosaccharides. BioGel P6 gel filtration of the oligosaccharitols obtained from [^{35}S]SO_4-radiolabeled MAT-B1 ASGP-1 by alkaline borohydride elimination reveals two major sulfated species (Fig. 1). The larger (S-2) elutes slightly slower than the previously described monosialylated tetrasaccharitol Galβ1,4-GlcNAcβ1,6-(NeuNAcα2,3-Galβ1,3)-GalNAcOH,

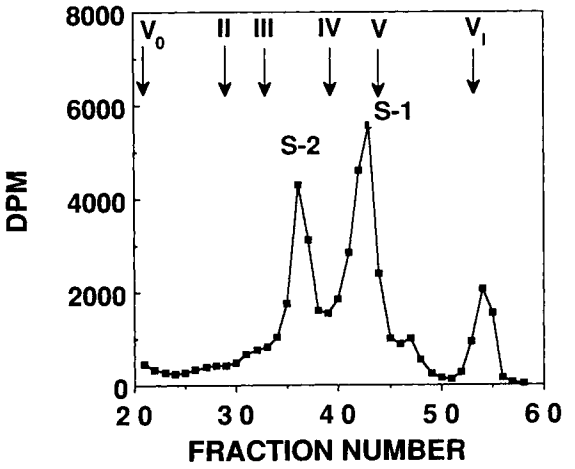

Fig. 1. BioGel separation of two major sulfated oligosaccharitols of MAT-B1 ASGP-1. Oligosaccharitols were obtained from [^{35}S]SO_4-labeled ASGP-1 by alkaline β-elimination as described and analyzed on a column (1.0 × 100 cm) of BioGel P6 (200–400 mesh). Fractions (1.2 ml) were collected and 25 μl of each fraction was analyzed for radioactivity. The column was calibrated with the following standards:V_0, intact ASGP-1; II, disialylated tetrasaccharitol NeuAcα2,3-Galβ1,4-GlcNAcβ1,6-(NeuAcα2,3-Galβ1,3)-GalNAcOH; III, monosialylated tetrasaccharitol Galβ1,4-GlcNAcβ1,6-(NeuAcα2,3-Galβ1,3)-GalNAcOH; IV, tetrasaccharitol Galβ1,4-GlcNAcβ1,6-(Galβ1,3)-GalNAcOH; and V, disaccharitol Galβ1,3-GalNAcOH.

while the smaller (S-1) elutes slightly slower than the tetrasaccharitol Galβ1,4-GlcNAcβ1,6-(Galβ1,3)-GalNAcOH [23]. Previous compositional analyses [20] indicated that S-1 and S-2 contain equal amounts of galactose, glucosamine, and galactosamine but that S-2 also contains sialic acid. Thus, S-1 must be a sulfated trisaccharide and S-2 a sulfated, sialylated trisaccharide. Although our first studies suggested that the latter contained two sialic acids [20], later analyses [23] and the present studies indicate the presence of only one. Previous methylation analyses and periodate studies [23] also indicated that all of the ASGP-1 oligosaccharides are derivatives of the group 3 core GlcNAcβ1,6-(Galβ1,3)-GalNAc [28].

Structural Determination of S-1

S-1 elutes from anion-exchange HPLC as a monoanionic species (Fig. 2), indicating that it is a monosulfated species. Analysis of S-1 by ion-suppression HPLC [29], which separates oligosaccharides by size and hydrogen-bonding ability, provided further evidence that this oligosaccharitol is a monosulfated trisaccharitol (Fig. 3A), since it elutes between the neutral trisaccharitols and tetrasaccharitols previously characterized from ASGP-1. Moreover, desulfation of S-1 under a variety of conditions yields as the largest product a trisaccharitol, which is identical by gel filtration and HPLC to the branched trisaccharitol GlcNAcβ1,6-(Galβ1,3)-GalNAcOH obtained by β-galactosidase treatment of Galβ1,4-GlcNAcβ1,6(Galβ1,3)-GalNAcOH [23]. Figure 4 shows the results of hydrolysis of S-1 with methanolic HCl. These conditions cause some glycoside hydrolysis as well as desulfation, resulting in products smaller than the trisaccharitol. The results indicate that desulfated S-1 is the branched trisaccharitol GlcNAcβ1,6-(Galβ1,3)-GalNAcOH.

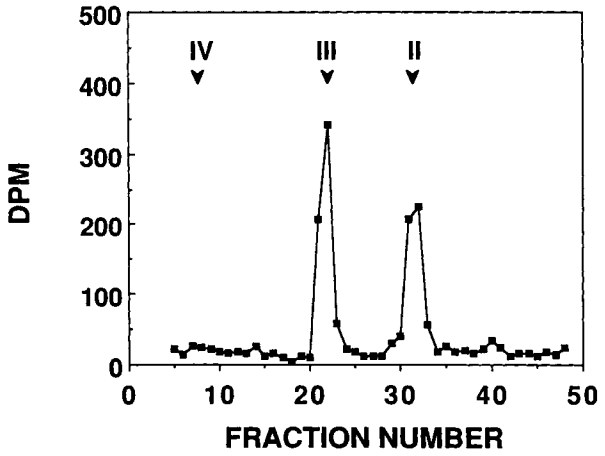

Fig. 2. Separation of sulfated oligosaccharitols by anion-exchange HPLC. [^{35}S]SO_4-labeled oligosaccharitols obtained by β-elimination of ASGP-1 were desalted and analyzed by anion-exchange HPLC on a column (0.45 × 25 cm) of Spherisorb-NH_2 (5 μm) eluted with a gradient over 24 min from 2.5 mM KH_2PO_4, pH 4.0, to 125 mM KH_2PO_4, pH 4.0. Fractions (0.5 min) were collected at a flow rate of 1.0 ml/min. The column was calibrated with standards described in the legend to Figure 1.

We have previously shown that mild periodate treatment of the branched oligosaccharitols from ASGP-1 cleaves the galactosaminitol and permits separation of the two branches [23]. The location of the sulfate on S-1 was determined by mild periodate treatment of S-1 radiolabeled with 6-[^{3}H]glucosamine. Anion exchange HPLC showed that 84% of the glucosamine label of the periodate products eluted as a monoanionic species (Fig. 5). Since both glucosamine and galactosamine of the branched oligosaccharide are labeled in the 6-position, the sulfate must be predominantly, if not exclusively, associated with the 1,6-glucosamine branch rather than the 1,3-galactose branch of the trisaccharide.

Further evidence for the location of the sulfate on glucosamine was obtained by β-hexosaminidase treatment of S-1 and desulfated S-1. Glucosamine was released from S-1 only when it was desulfated prior to hexosaminidase digestion (data not shown).

We propose that the structure of S-1 is the following:

S-1 $^{-}O_4S$-GlcNAcβ1,6-(Galβ1,3)-GalNAcOH

Determination of the Structure of S-2

The behavior of S-2 on anion-exchange HPLC indicates that it is a dianionic oligosaccharide (Fig. 2). On ion-suppression HPLC S-2 elutes as a species larger than a branched, neutral pentasaccharide (Fig. 3B), which is consistent with the compositional data suggesting that it is a branched trisaccharide substituted with sialic acid and sulfate [20]. After treatment of S-2 with sialidase the resulting oligosaccharide eluted on anion-exchange HPLC as a monoanionic species (Fig. 6). Moreover, S-1 and sialidase-treated S-2 coeluted when cochromatographed on ion-suppression HPLC (Fig. 7). Since the HPLC conditions employed have been shown to resolve structural isomers [30], these data indicate that S-2 is the sialylated product of S-1.

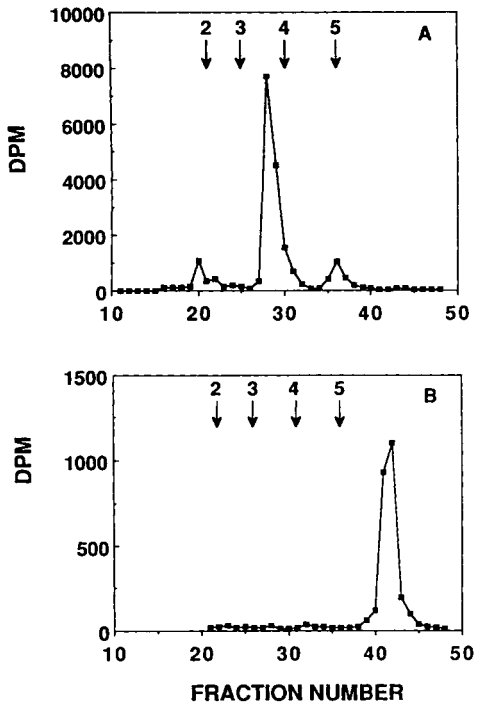

Fig. 3. Separation of sulfated oligosaccharides by ion-suppression HPLC. [^{35}S]SO_4-labeled S-1 (**A**) and S-2 (**B**) isolated by anion-exchange HPLC as in Figure 2 were analyzed by ion-suppression HPLC on a column (0.45 × 25 cm) of Spherisorb-NH_2 (5 μm). The flow rate was 1.0 ml/min and 0.3-min fractions were collected. The column was calibrated with the following standards: 2, disaccharitol, Galβ1,3-GalNAcOH; 3, trisaccharitol GlcNAcβ1,6-(Galβ1,3)-GalNAcOH; 4, tetrasaccharitol Galβ1,4-GlcNAcβ1,6-(Gal-β1,3)-GalNAcOH; and 5, pentasaccharitol, Galα1,3-Galβ1,4-GlcNAc-β1,6-(Galβ1,3)-GalNAcOH.

When [6-^{3}H]glucosamine-labeled S-1 was treated with mild periodate, 66% of the label eluted as a monoanionic species by anion exchange HPLC (Fig. 8). Moreover, all of the sulfate label eluted as a monoanionic species and coeluted with the monoanionic glucosamine label. Since these periodate cleavage conditions cleave only the galactosaminitol and the tail of the sialic acid (positions 8 and 9), the sulfate and sialic acid must be on different branches of the oligosaccharide. If they were in the same branch, the label would be contained in a dianionic species after periodate treatment. Since the studies above show sulfate associated with glucosamine in the trisaccharide, the sialic acid must be associated with galactose of the 1,3-branch.

Since all sialic acids on ASGP-1 oligosaccharides are located on the 3-position of galactose [23], we propose that the structure of S-2 is the following:

S-2 $^{-}O_4$S-GlcNAcβ1,6-(NeuAcα2,3-Galβ1,3)-GalNAcOH

Kinetics of the Sulfation of ASGP-1

The maturation of ASGP-1 can be analyzed by pulse-chase labeling with threonine followed by PNA precipitation [24,25]. The specificity of the PNA precipi-

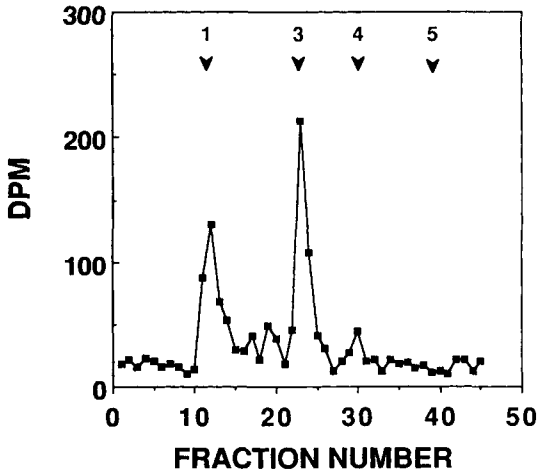

Fig. 4. Desulfation of S-1 with methanolic HCl. S-1, purified by ion-suppression HPLC as described in Figure 3, was treated with 0.5 N anhydrous methanolic HCl at room temperature for 4 h, neutralized with NaOH, desalted with Ag50/Ag1 mixed bed resin, and analyzed by HPLC on a column (0.46 × 25 cm) of 5-μm silica gel modified with diaminobutane [23]. The column was calibrated with S-1 and GalNAcOH (arrow 1); trisaccharitol GlcNAcβ1,6-(Galβ1,3)-GalNAcOH, 3; tetrasaccharitol, 4; and pentasaccharitol, 5.

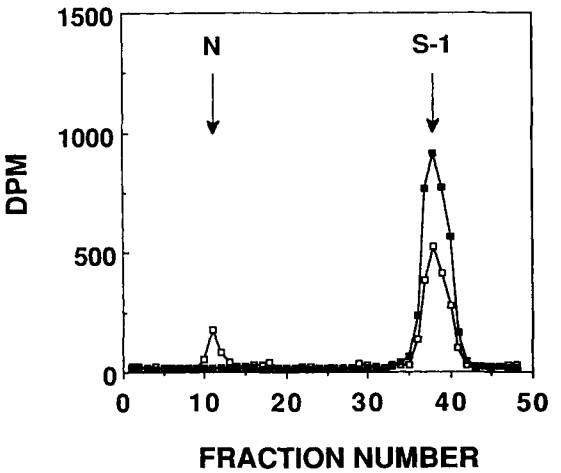

Fig. 5. Effect of mild periodate treatment on S-1. S-1 labeled with [^{3}H]glucosamine (□) and [^{35}S]sulfate (■) was treated with periodate under mild conditions and analyzed by anion-exchange HPLC as described. The column was calibrated with S-1. The small neutral fraction (N) of glucosamine (16%) was observed with previous periodate studies on Galβ1,4-GlcNAcβ1,6-(NeuAcα2,3-Gal-β1,3)-GalNAcOH [23]. Its source is unknown.

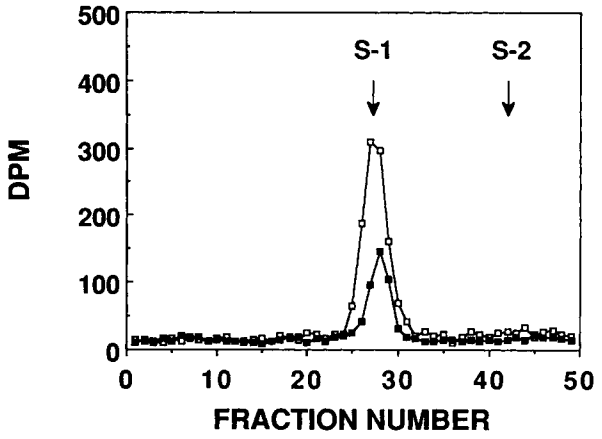

Fig. 6. Anion exchange HPLC analysis of sialidase-treated S-2. S-2, labeled with [^{3}H]glucosamine (□) and [^{35}S]SO_4 (■) was purified by ion-suppression HPLC as noted in Figure 3 and treated with sialidase as described. The products were analyzed by anion-exchange HPLC by using a column (0.45 x 25 cm) of Spherisorb-SAX (5 μm) eluted with a linear gradient over 40 min from 15 mM Na_2PO_4, pH 5.2, to 400 mM Na_2PO_4, pH 5.2. Flow rate was 0.5 ml/min and 0.5-min fractions were collected. The column was calibrated with S-1 and S-2 treated similarly except without enzyme.

tation of ASGP-1 has been characterized extensively, as described previously [24,25]. Results of the pulse-chase studies indicate that an immature form of ASGP-1 is chased into a premature form with a half-time of about 30 min. The premature form is then more slowly converted to a mature form [25]. To determine whether sulfation occurs on the immature, premature, or mature form, aliquots of cells were labeled for 10 min with either [^{35}S]SO_4 or [^{3}H]threonine or [^{3}H]glucosamine and lysed with

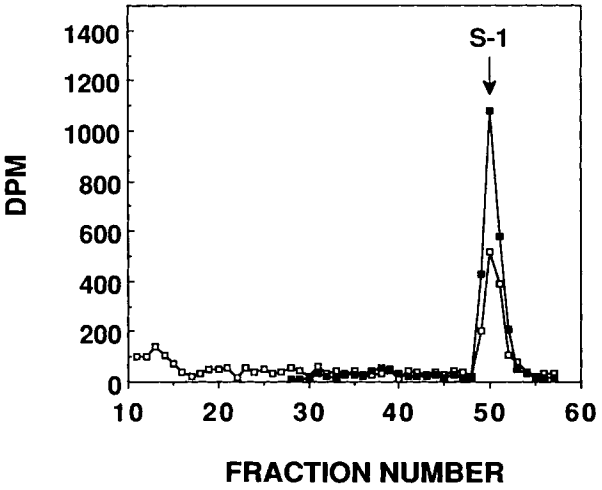

Fig. 7. Ion-suppression HPLC analysis of sialidase-treated S-2. Glucosamine-labeled S-2 (□) was treated with sialidase and cochromatographed with sulfate-labeled S-1 (■) by ion-suppression HPLC as described in Figure 3, except that the column was eluted isocratically with A:B (75:25).

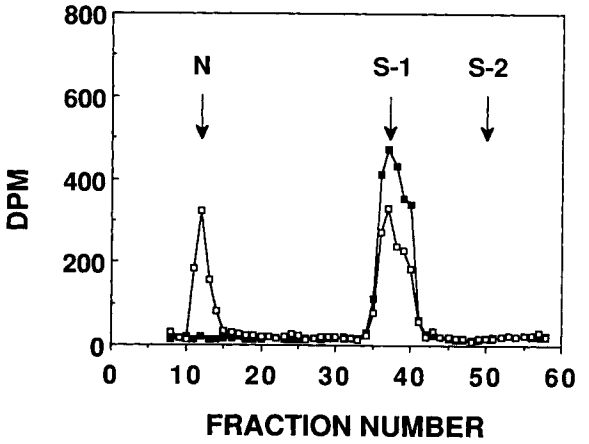

Fig. 8. Effect of mild periodate treatment on S-2. S-2, labeled with glucosamine (□) and sulfate (■), was treated with periodate and analyzed by anion-exchange HPLC as described in Figure 5. The HPLC column was calibrated with neutral oligosaccharide (N), S-1 and S-2. The neutral glucosamine peak results from the neutral material observed in Figure 4 and glucosamine label from the 9-position of the sialic acid, which is released by periodate.

detergent. The cell lysates were precipitated with PNA, and the precipitates were examined by Sepharose CL-2B chromatography. In Figure 9A the chromatographic profile shows that the majority of the sulfate label elutes as a species with a K_{av} of 0.5. The mature (glucosamine label) and immature (threonine label) forms of the glycoprotein are larger and smaller, respectively, than the sulfated form. This size of the sulfated species corresponds to the premature form previously described [25] and suggests that sulfation is an intermediate event in the maturation of ASGP-1. Reducing the pulse time to 2 min did not decrease the size of the sulfate-labeled species, indicating that the immature form is not significantly sulfated (data not shown). Pulse-chase analysis (Fig. 10) of sulfate-labeled cells indicated that the intermediate-sized sulfated species could be chased into mature ASGP-1.

Effect of Monensin on Sulfation of ASGP-1

Recently we showed that monensin retards maturation of ASGP-1 and reduces its mature size by blocking addition of galactose to the trisaccharide GlcNAcβ1,6-(Galβ1,3)-GalNAc by β-1,4-galactosyltransferase [25]. The structures described above for the sulfated oligosaccharides suggest that sulfation may also prevent β-1,4-galactose addition. Thus sulfation probably occurs at the same locus in the biosynthetic pathway as the β-1,4-galactosylation. If so, one might expect sulfation to be inhibited by monensin. To test this hypothesis, cells were preincubated with monensin and then radiolabeled with sulfate for 2 h. Analysis of the PNA precipitates of cell lysates indicated that monensin inhibited incorporation of sulfate into ASGP-1 by 95%. As shown previously, monensin-treated cells accumulated the trisaccharide GlcNAcβ1,6(Galβ1,3)-GalNAc and sialylated trisaccharide GlcNAcβ1,6-(NeuAcα2,3-Galβ1,3)-GalNAc [25].

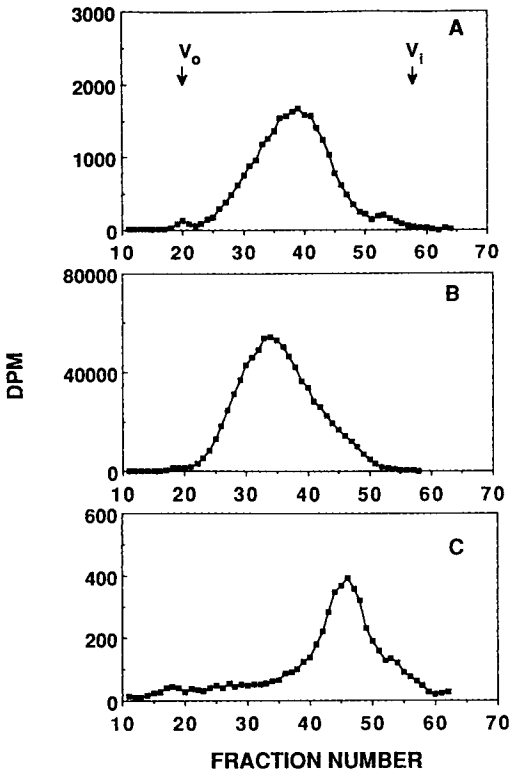

Fig. 9. Sepharose CL-2B chromatography of sulfate pulse-labeled ASGP-1. Cells were pulse labeled with $[^{35}S]SO_4$ (**A**), [^{3}H]glucosamine (**B**), or [^{3}H]threonine (**C**), and lysed, and the lysates were precipitated with PNA. The precipitates were solubilized and chromatographed over a column (1 × 40 cm) of Sepharose CL-2B eluted with 0.1% LDS, 10 mM Tris, 0.02% NaN_3. Fractions (0.7 ml) were collected and 40 μl of selected fractions was analyzed for radioactivity. The column was calibrated as follows: V_o, aggregated ASGP-1; V_i, bromophenol blue.

Pulse-Chase Analysis of Sulfated Oligosaccharides

The structures of the sulfated oligosaccharides and the effects of monensin suggest that oligosaccharides are sulfated on glucosamine, which blocks subsequent galactosylation and sialylation on the 1,6-branch. Transfer of ASGP-1 to a subsequent compartment then occurs to permit sialylation on the 1,3-branch. If this model is correct, sulfation should precede sialylation, and S-1 should be chased into S-2 in a pulse-chase analysis. When cells were pulsed with sulfate label for 1 min, little labeling of S-2 was observed (Fig. 11A). However, a subsequent chase with unlabeled sulfate rapidly converted the labeled S-1 to its equilibrium mixture with S-2 (Fig. 11B,C). The half-time for this conversion is less than 5 min.

DISCUSSION

The structures and biosynthesis of the ASGP-1 sulfated oligosaccharides can best be understood by considering a working model for the biosynthetic scheme, as

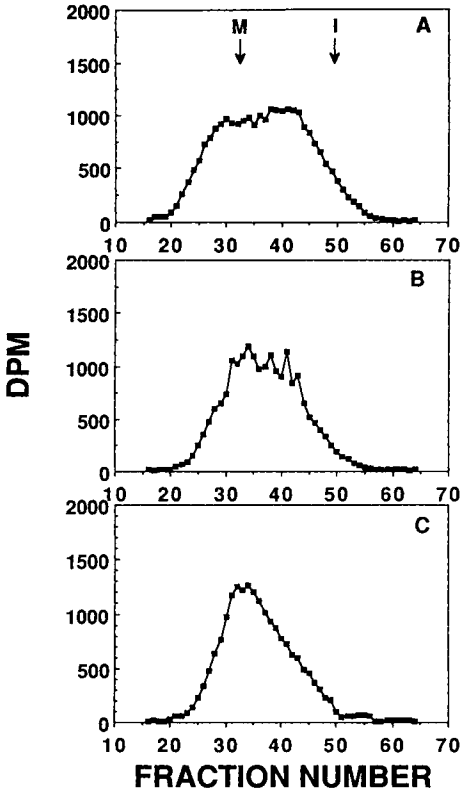

Fig. 10. Pulse-chase analysis of sulfation of ASGP-1. Cells were pulse-labeled for 15 min with [^{35}S]SO_4 and chased for 0 (**A**), 30 (**B**), or 60 (**C**) min. After cell lysis and PNA precipitation the samples were analyzed by Sepharose CL-2B chromatography. Elution positions of mature (M) and immature (I) ASGP-1 were determined as in Figure 9.

proposed in Figure 12. This is a modification of a scheme presented previously [25]. In this scheme we propose that ASGP-1 is first observed as a lightly glycosylated immature form, which contains small amounts of neutral oligosaccharides (mono, di, tri and tetra), represented by the disaccharide in Figure 12. Transfer of the immature form to a second compartment is proposed to result in a burst of glycosylation which initiates numerous new oligosaccharides and elongates them primarily to trisaccharides. A second transfer moves the glycoprotein to the compartment containing β-1,4-galactosyltransferase and the sulfate transferase. These two enzymes compete for the trisaccharide substrate. Trisaccharide substituted on the glucosamine with sulfate is blocked from further additions on the 1,6-branch. Addition of sialic acid to the 1,3-branch yields the sulfated, sialylated trisaccharide. Trisaccharide substituted on the glucosamine with β-1,4-galactose to form Galβ1,4-GlcNAcβ1,6(Galβ1,3)-GalNAc can be further substituted with α-galactose, fucose, or sialic acid to yield neutral or sialylated pentasaccharides and hexasaccharides as described previously [23].

Both the structural analyses of the sulfated oligosaccharides and the monensin effects support this model. The ability of monensin to block the actions of both β-

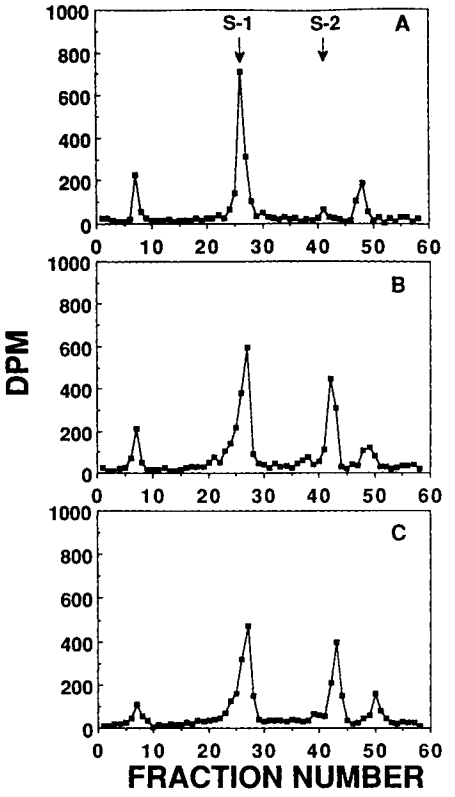

Fig. 11. Pulse-chase sulfate labeling of S-1 and S-2. Cells were pulsed-labeled with [^{35}S]SO_4 for 1 min, chased for 0 (**A**), 5 (**B**), and 10 (**C**) min with cold sulfate, and lysed, and the lysates were precipitated with PNA. Oligosaccharitols were released from the precipitates and analyzed by anion-exchange HPLC.

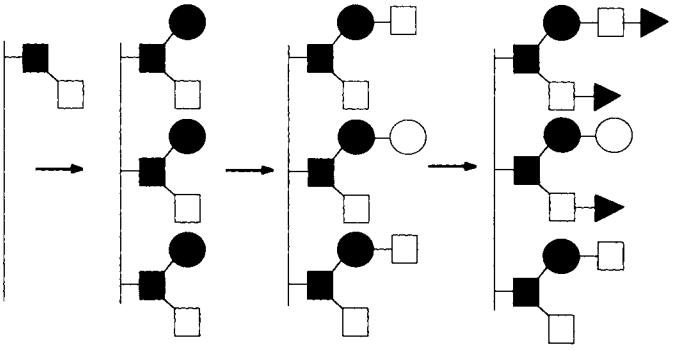

Fig. 12. Biosynthetic scheme for ASGP-1 oligosaccharides. ■, GalNAc; ●, GlcNAc; □, Gal; ○, sulfate; ▶, sialic acid.

1,4-galactosyltransferase and sulfate transferase indicates that they are in the same compartment and competing for the same substrate. The fact that sialyltransferase is not affected suggests that it is primarily in another compartment. We suspect that a single sialyltransferase performs the transfers on the 1,3- and 1,6-branches, but this has not been established. A surprising feature of our studies is the rapidity of the sialylation of S-1 ($t_{1/2} < 5$ min). These results suggest that the passage through the intermediate stages of the glycosylation (trisaccharide to sialylated species) must be rapid. This rapid transit is consistent with the failure to observe significant amounts of intermediate forms of ASGP-1 with threonine pulse-chase labeling studies [25]. Since the half-time for maturation of ASGP-1 is quite long (>4 h), it must reside in some sialylating or postsialylating compartment for some time.

The results summarized above suggest that ASGP-1 passes rapidly through the compartment containing the β-1,4-galactosyltransferase. Since complex N-linked oligosaccharides contain a similar β-1,4-galactose, it will be interesting to determine whether the complex oligosaccharides are similarly rapidly passed through this compartment. These results also provide biochemical evidence that much of the β-1,4-galactosyltransferase and sulfate transferase of the O-linked pathway in these cells is in a separate compartment from the sialyltransferase(s). Such findings support recent immunocytochemical studies showing β-1,4-galactosyltransferase and sialyltransferase(s) in morphologically distinct compartments [31].

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