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Enhanced 3-O-sulfation of galactose in Asn-linked glycans and *Maackia amurenesis* lectin binding in a new Chinese hamster ovary cell line

Xiaomei Bai, Jillian R. Brown, Ajit Varki, and Jeffrey D. Esko[1]

Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, 9500 Gilman Drive, University of California, San Diego, La Jolla, CA 92093-0687, USA

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We report the characterization of two Chinese hamster ovary cell lines that produce large amounts of sulfated N-linked oligosaccharides. Clones 26 and 489 were derived by stable transfection of the glycosaminoglycan-deficient cell mutant pgsA-745 with a cDNA library prepared from wild-type cells. Peptide:N-glycanase F released nearly all of the sulfate label, indicating that sulfation had occurred selectively on the Asn-linked glycans. Hydrazinolysis followed by nitrous acid treatment at pH 4 and borohydride reduction yielded reduced sulfated disaccharides that comigrated with standard Gal3SO$_4$β1-4anhydromannitol. The disaccharides were resistant to periodate oxidation but became sensitive after the sulfate group was removed by methanolysis, indicating that the sulfate was located at C3 of the galactose residues. *Maackia amurensis* lectin bound to the sulfated glycopeptides on the cell surface and in free form, even after sialidase treatment. This finding indicates that the lectin requires only a charged group at C3 of the galactose unit and not an intact sialic acid. Growth of cells with chlorate restored sialidase sensitivity to lectin binding, indicating that sulfation and sialylation occurred largely at the same sites. The enhanced sulfation was due to elevated sulfotransferase activity that catalyzed transfer of sulfate from phosphoadenosine-5′-phosphosulfate to Galβ1-4(3)GlcNAcβ-O-naphthalenemethanol.

Key words: sulfated N-linked oligosaccharides/ sulfotransferase/CHO cell mutants/stable transfection/ MAA-lectin binding

Introduction

The major sulfated glycans in vertebrates generally consist of glycosaminoglycans, such as chondroitin sulfate and heparan sulfate, which contain hundreds of sulfate residues covalently attached to the chains. Sulfated glycans on glycoproteins have been reported as well, in the form of Man4SO$_4$ (Yamashita *et al.*, 1983), Man6SO$_4$ (Freeze and Wolgast, 1986), GlcNAc6SO$_4$ (Edge and Spiro, 1984; Roux *et al.*, 1988; Spiro and Bhoyroo, 1988; de Waard *et al.*, 1991; Noguchi *et al.*, 1992; Sampath *et al.*, 1992; Hokke *et al.*, 1993; Shilatifard *et al.*, 1993; Hemmerich *et al.*, 1994; Hemmerich and Rosen, 1994; Crommie and Rosen, 1995; Lo-Guidice *et al.*, 1995), Gal3SO$_4$ (Spiro and Bhoyroo, 1988; de Waard *et al.*, 1991; Lo-Guidice *et al.*, 1995, 1994; Kamerling *et al.*, 1988; Hard *et al.*, 1992), Gal6SO$_4$ (Hemmerich *et al.*, 1994, 1995; Hemmerich and Rosen, 1994; Brown *et al.*, 1994; Fukuta *et al.*, 1997; Shailubhai *et al.*, 1999), GalNAc4SO$_4$ (Green and Baenziger, 1988a,b; Bergwerff *et al.*, 1995), and GlcA3SO$_4$ (Chou *et al.*, 1986, 1987; Margolis and Margolis, 1993; Schachner and Martini, 1995). Although their relative abundance is low compared to the sulfated glycosaminoglycans, glycans containing these sulfated sugars can have specific and potent biological properties. For example, the recognition of peripheral GalNAc4SO$_4$ residues by receptors on hepatic reticuloendothelial cells facilitates rapid clearance of lutropin and thyrotropin from the circulation (Fiete *et al.*, 1991). The sulfated, sialylated oligosaccharides on O-linked glycoproteins expressed on high endothelial venules facilitate high-affinity binding to L-selectin on leukocytes, thus enabling the rolling and eventual extravasation of the cells into lymph nodes (Vestweber and Blanks, 1999). The potent activities encoded in these glycans depend on specific protein–carbohydrate interactions in which the sulfate group plays an essential role in determining the affinity and specificity of binding.

The various sulfation reactions that give rise to the sulfated glycans are catalyzed by distinct sulfotransferases located in the Golgi. Several of the glycoprotein-specific sulfotransferases have been purified and characterized (Kato and Spiro, 1989; Hooper *et al.*, 1995; Spiro *et al.*, 1996; Spiro and Bhoyroo, 1998), and cDNAs for a Gal 6-O-sulfotransferases (Fukuta *et al.*, 1997), GlcNAc 6-sulfotransferases (Uchimura *et al.*, 1998a,b; Lee *et al.*, 1999; Bistrup *et al.*, 1999), and a GlcA 3-O-sulfotransferase (Ong *et al.*, 1998; Bakker *et al.*, 1997) have been cloned. Like other Golgi transferases, all of the enzymes appear to be type II transmembrane glycoproteins and show a high degree of specificity for their oligosaccharide substrates. By analogy to the sulfotransferases involved in glycosaminoglycan assembly, it seems likely that each enzyme is part of a multigene family whose members differ in distribution and developmental expression (Hashimoto *et al.*, 1992; Eriksson *et al.*, 1994; Orellana *et al.*, 1994; Bakker *et al.*, 1997; Honke *et al.*, 1997, Kobayashi *et al.*, 1997, 1999; Habuchi *et al.*, 1998; Ong *et al.*, 1998; Aikawa and Esko, 1999; Shworak *et al.*, 1999; Aikawa *et al.*, 2001).

3-O-sulfation of Gal residues on glycoproteins was first described on thyroglobulins from various species (Spiro and Bhoyroo, 1988; de Waard *et al.*, 1991). The 3-O-sulfotrans-

[1]To whom correspondence should be addressed

ferase activity responsible for its assembly exhibits selectivity for the terminal disaccharide, Galβ1-4GlcNAc-, independently of the underlying oligosaccharide or glycoprotein (Kato and Spiro, 1989). Because α2-3 sialyltransferases, α1-2 fucosyltransferases, and α1-3 Gal transferase act on identical substrates, the four enzymes could potentially compete with each other and convey different chemical and possibly biological properties to the modified chains. Here, we report the activation of a glycoprotein Gal 3-O-sulfotransferase in two Chinese hamster ovary (CHO) cell lines by introduction of a wild-type cDNA library into a strain devoid of glycosaminoglycans (Esko *et al.*, 1985). As expected, sulfation results in decreased sialylation. Surprisingly, a heretofore unappreciated reactivity of *Maackia amurensis* lectin (MAL) for 3-O-sulfated Gal-terminated oligosaccharides was demonstrated.

Results and discussion

Enhanced sulfation of macromolecules in clone 26 and 489

In CHO cells, glycosaminoglycans dominate the sulfated oligosaccharide fraction, accounting for >95% of $^{35}SO_4$ incorporation into all macromolecules. Thus, in the CHO cell mutant, pgsA-745, sulfation is greatly reduced due to a defect in xylosyltransferase, which initiates glycosaminoglycan biosynthesis (Esko *et al.*, 1985). Using this strain, we thought that it might be possible to clone the missing enzyme or for a sulfotransferase that acts on other oligosaccharides by introducing a cDNA library from wild-type CHO cells into the mutant and screening for cells that incorporated normal amounts of $^{35}SO_4$. To analyze a large number of cells, individual colonies generated from transfected cells were replica plated onto discs of polyester cloth (see *Materials and methods*). The replicated colonies on the discs were then screened semi-quantitatively by autoradiographic measurement of $^{35}SO_4$ incorporation into acid-precipitable macromolecules. An occasional colony appeared that incorporated much higher levels of $^{35}SO_4$ than did the surrounding parental colonies (data not shown). Initial studies showed that one of these colonies regained the capacity to make glycosaminoglycans (data not shown). Two of the clones (clone 26 and 489) did not and were selected for further study. Their phenotypes, which are described below, were stable for many generations.

Analysis of the ^{35}S-labeled macromolecules produced by these two lines yielded surprising results. As expected from the autoradiographic data, the amount of ^{35}S-labeled macromolecules that precipitated with trichloroacetic acid was five- to sevenfold higher than in the parental line, pgsA-745 (Table I). Few of the ^{35}S-counts were recovered in the glycolipid fraction in the clones and parental cells (Table I), although CHO cells are thought to make a small amount of sulfatide (Murphy-Ullrich *et al.*, 1988). Treating clone 26 and 489 cells with trypsin released much of the ^{35}S-counts, consistent with the idea that the enhanced incorporation had most likely occurred in glycoproteins or proteoglycans expressed on the cell surface (Table I). However, when the cells were analyzed by the glycan isolation procedure (GIP) (Norgard-Sumnicht *et al.*, 2000) the ^{35}S-counts unexpectedly did not fractionate like glycosaminoglycans. This was confirmed using a slightly modified procedure for isolating the glycosaminoglycan chains (Table I). Instead, the radioactivity appeared in the glycopeptide fraction predicted to contain N- and O-linked glycans ($GP_{1–100}$ and $GP_{300–1000}$ in the GIP protocol). When samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), multiple ^{35}S-labeled proteins on the gels were observed in the transfectants. When samples from wild-type CHO cells were electrophoresed, the broad diffuse bands typical of proteoglycans obscured the gels. Only very faint bands were present in pgsA-745 cells (data not shown). Treatment with peptide:N-glycosidase F (PNGase F) caused the appearance of multiple low molecular mass sulfated oligosaccharide bands at the expense of the glycoprotein bands.

Analysis of the sulfated N-glycans

The idea that the sulfated material was due to enhanced sulfation of Asn-linked chains was confirmed by gel filtration chromatography of the ^{35}S-labeled glycoproteins and glycans liberated by PNGase F (Figure 1). Enzymatic digestion resulted in a clear shift in the elution position of >95% of the ^{35}S-labeled material from glycoproteins in the void fraction to free glycans at a more included position. Very little effect was observed in either wild-type or the parental line (pgsA-745).

When the PNGase F–released glycans were solvolyzed (see *Materials and methods*), 87% of the ^{35}S-counts were liberated as free sulfate, suggesting the presence of ester-linked sulfate groups. Roux *et al.* (1988) have reported that the CHO glycosaminoglycan-deficient mutant pgsB-761 (defective in

Table I. Sulfation of macromolecules (c.p.m./μg protein)

Strains	Total macromolecules[a]	Glycosaminoglycans[b]	Trypsin-releasable[c]	Glycolipids[d]
Wild-type	2400 ± 900	2400 ± 140	4100 ± 1800	200 ± 30
pgsA-745	220 ± 50	70 ± 30	350 ± 4	100 ± 20
Clone 26	1500 ± 300	100 ± 40	1200 ± 40	140 ± 10
Clone 489	1000 ± 130	120 ± 50	800 ± 190	170 ± 20

Wild-type, glycosaminoglycan-deficient pgsA-745, clone 26 and clone 489 cells were labeled with $^{35}SO_4$. Portions of the cells were treated under various conditions to quantitate the distribution of counts amongst various macromolecular fractions (see *Materials and methods*).

[a] ^{35}S-counts precipitated with 10% TCA.

[b] ^{35}S-counts eluted from DEAE-Sepharose between 0.3 and 1 M NaCl (Bame and Esko, 1989).

[c] ^{35}S-counts released from cells by mild trypsin treatment.

[d] ^{35}S-counts extracted with $CHCl_4$ and methanol (Murphy-Ullrich *et al.*, 1988).

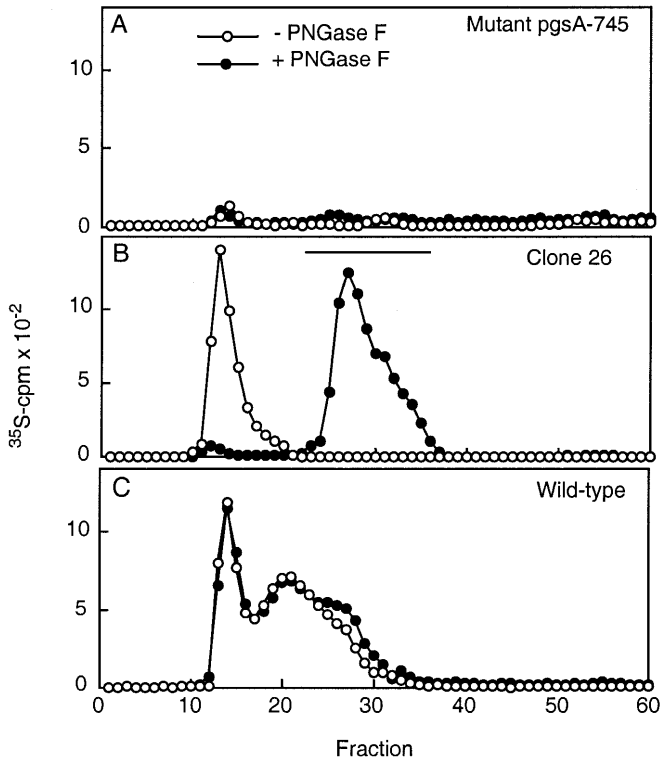

Fig. 1. Sephacryl S-200 chromatography of PNGase F–treated samples demonstrates the accumulation of sulfated N-glycans. The parent pgsA-745, clone 26, and wild-type cells were metabolically labeled with $^{35}SO_4$, boiled in SDS, and treated with (filled circles) or without (open circles) PNGase F. Samples were then chromatographed on a Sephacryl S-200 column (see *Materials and methods*). The bar in panel **B** indicates the fractions that were pooled for further analysis. (**A**) Parental cells, pgsA-745. (**B**) Clone 26. (**C**) wild-type CHO cells.

galactosyltransferase I; Esko *et al.*, 1987), had small amounts of Neu5Acα2-3Galβ1-4GlcNAc6SO_4 in N-glycans. To test if the transfectant made more of this sulfated oligosaccharide, the free chains were treated with various endo- and exoglycosidases and analyzed by chromatography on Sephadex G-50. Keratanases I (endo-β-galactosidase, requires [Galβ1-4GlcNAc6SO_4]$_n$ for activity) and keratanase II (endo-β-glucosaminidase, requires [GlcNAc6SO_4β1-3Gal(±6SO_4)]$_n$ for activity) did not liberate any of the counts as sulfated disaccharides, but the enzymes degraded [^{35}S]keratan sulfate isolated from Madin-Darby canine kidney (MDCK) cells (Toma *et al.*, 1996). Similarly, sequential treatment with *Arthrobacter ureafaciens* neuraminidase, exo-β-galactosidases from different sources, and β-hexosaminidase A under the conditions known to cleave GlcNAc6SO_4 residues liberated less than 10% of the ^{35}S-counts (Roux *et al.*, 1988). Together, these findings suggested that at most only a small proportion of sulfate groups were located at C6 of GlcNAc residues.

To locate the position of the sulfate groups, N-glycans biosynthetically labeled with $^{35}SO_4$, [6-^{3}H]Gal, and [6-^{3}H]GlcN were isolated as above and treated with hydrazine and nitrous acid at high pH (Spiro *et al.*, 1996). These conditions result in N-deacetylation of GlcNAc and GalNAc residues, cleavage of the hexosaminic bond, and deaminative ring contraction at the reducing end to form anhydromannose (aMan) or anhydrotalose, respectively. Reduction with $NaBH_4$ then yields anhydromannitol (aManol) or anhydrotalitol, thus stabilizing the released oligosaccharides against further degradation. Based on the known structure of the chains in CHO cells, one would expect the labeled cleavage products to consist of sulfated, sialylated, or unmodified Gal-aManol units, and possibly sulfated anhydrotalitol if the cells contained terminal GalNAc residues. Gel filtration chromatography of the sample showed that ~50% of the ^{35}S-labeled material was cleaved under these conditions, yielding a major peak (Figure 2C, fractions 26–31, 42% of counts) near the V_t of the column (fraction 35). Attempts to further improve the yield were unsuccessful, raising the possibility that some of the sulfate may have been located on the mannose rich core of the chains (e.g., Man4SO_4; Yamashita *et al.*, 1983). However, the yield of [^{3}H]Gal-labeled fragments from labeled glycans was also ~50%, suggesting that the low yield of cleavage products was due to incomplete deacetylation, deamination, or cleavage of the chains. The [^{3}H]Gal- and [^{3}H]GlcN-containing products separated into three peaks, one of which coincided with the major ^{35}S-labeled material (Figures 2A and 2B, 10% of counts). This suggested that ~24% (10%/0.42) of the disaccharides contained sulfate groups. The elution position of the last peak was ahead of the location where standard aMan6SO_4 eluted (V_t), suggesting that the transfectant did not produce

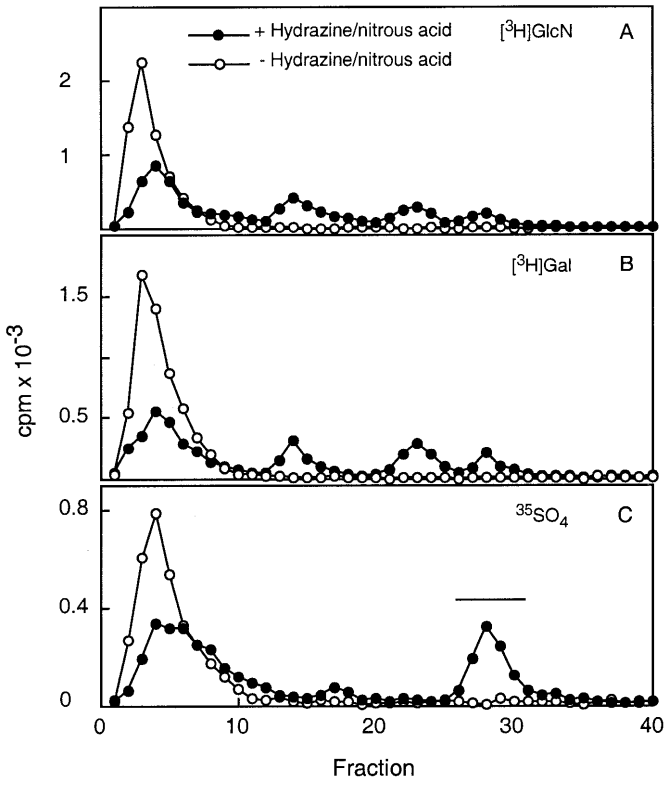

Fig. 2. Hydrazine/nitrous acid releases sulfated fragments containing Gal and GlcNAc. The N-glycan preparation from clone 26 (cf. Figure 1) was treated with (filled circles) or without (open circles) hydrazine/nitrous acid and fractionated on a Bio-Gel P4 column. (**A**) [^{3}H]GlcN-labeled sample. (**B**) [^{3}H]Gal-labeled sample. (**C**) $^{35}SO_4$-labeled sample. The bar indicates the fractions that were pooled for further analysis. The V_t was at fraction 34.

terminal GalNAc4SO_4 residues like those found on pituitary glycohormones (Green and Baenziger, 1988a). The material in fractions 22–24 was sensitive to β-galactosidase, consistent with the structure Galβ1-4aManol. The material in fractions 13–15 was sensitive to neuraminidase, suggesting the structure Siaα2-3Galβ1-4aManol.

When the ^{35}S-labeled disaccharide was applied to a QAE-Sephadex column, all of the counts eluted between 20–70 mM NaCl, which suggested that they had a –1 charge (Roux *et al.*, 1988). Neuraminidase treatment had no effect on the elution position (Figure 3), suggesting that sulfation and sialylation were mutually exclusive, at least with respect to individual disaccharide units. As shown below, sialylation and sulfation could occur on the same N-glycan, but apparently on different arms of multiantennary chains. In wild-type CHO cell N-glycans, sialylation occurs exclusively in α2-3 linkages to Gal residues (Lee *et al.*, 1989). Thus, the lack of any sulfated, sialylated disaccharide liberated by hydrazinolysis/nitrous acid treatment (and the insensitivity of the glycans to hexosaminidase and keratanases) suggested that the sulfate group might be located at C3 of Gal residues.

Terminal Gal3SO_4 has been found in calf, human, and porcine thyroglobulins (Spiro and Bhoyroo, 1988; Kamerling *et al.*, 1988; de Waard *et al.*, 1991; Baumeister and Herzog, 1988), Tamm-Horsfall glycoprotein (Hard *et al.*, 1992) and in viral glycoproteins grown in MDCK, MDBK, BHK, and LLC-Pk1 cells, but not in other cell lines (Karaivanova and Spiro, 1998). Evidence for its presence in clone 26 was obtained by thin-layer chromatography (TLC) of the [^{35}S]disaccharides liberated by hydrazinolysis/nitrous acid treatment, which showed that the major component migrated about halfway up the plate (R_f = 0.48), exactly in the position as standard [^{3}H]Gal3SO_4β1-4aManol (Figure 4). Sulfated [^{3}H]Gal-labeled and [^{3}H]GlcN-labeled disaccharides from clone 26 had the same R_f values. Under these conditions a neutral disaccharide standard ([^{3}H]Galβ1-4aManol) had an R_f value of 0.53 and a monosulfated [^{35}S]disaccharide prepared from MDCK cell keratan sulfate (presumably Galβ1-4aManol6SO_4) had an R_f value of 0.21.

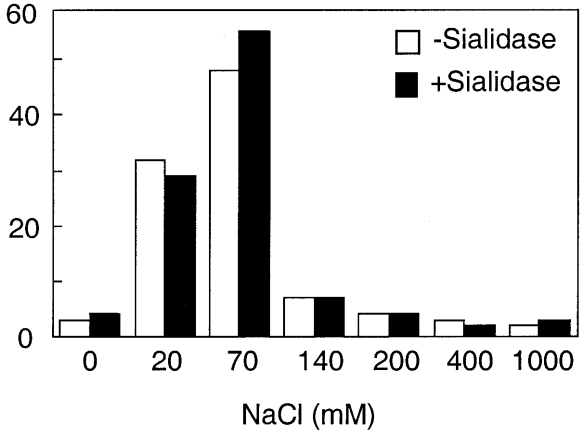

Fig. 3. The ^{35}S-disaccharides have a –1 charge and are resistant to sialidase. ^{35}S-disaccharides isolated in Figure 2 were treated with (solid bars) or without (open bars) sialidase (see *Materials and methods*). Samples were then applied to a column of QAE-Sephadex and eluted stepwise with increasing concentrations of NaCl in 2 mM Tris base.

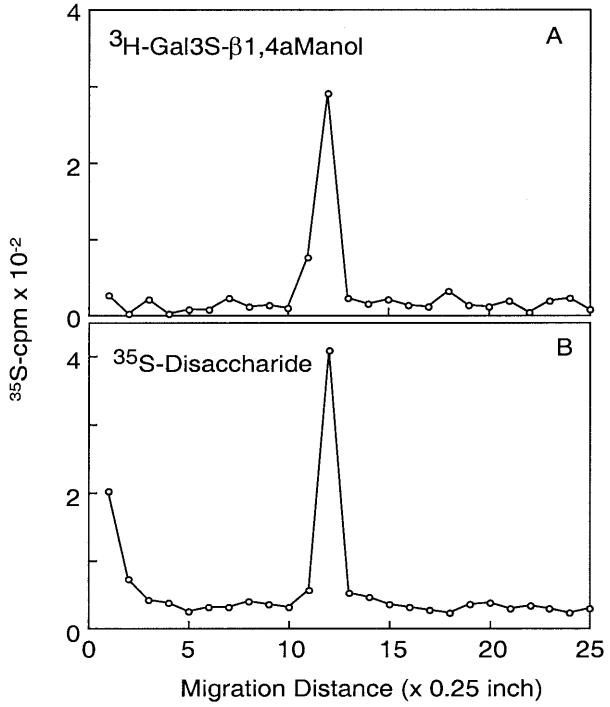

Fig. 4. TLC shows that the ^{35}S-disaccharides comigrated with Gal3SO_4β1-4aManol. Disaccharides liberated from the N-glycans by hydrazine/nitrous acid treatment and containing a –1 charge were analyzed by ascending cellulose TLC using pyridine/ethyl acetate/water/acetic acid (5:5:3:1, v/v/v/v) (see *Materials and methods*). One-quarter-inch strips were cut from the plate, and radioactive material was eluted with water and counted by liquid scintillation spectrometry. (**A**) Standard [^{3}H]Gal3SO_4β1-4aManol. (**B**) disaccharide sample from clone 26.

Oxidation with periodate followed by mild acid hydrolysis will cleave oligosaccharides at sugar residues containing vicinal hydroxyl groups. As expected, the standard disaccharide [^{3}H]Gal3SO_4β1-4aManol was resistant to oxidation (Figure 5A), whereas standard [6-^{3}H]Gal was virtually destroyed under these conditions (data not shown). Treatment of the [^{3}H]disaccharide from clone 26 did not result in loss of counts or a shift in mobility after TLC (Figure 5B). Because Gal6SO_4β1-4aManol, Gal4SO_4β1-4aManol, and Galβ1-4aManol6SO_4 would have been sensitive due to the presence of vicinal hydroxyl groups on the Gal residues, the resistance of the sulfated disaccharide in clone 26 suggested that it most likely had the structure Gal3SO_4β1-4GlcNAc. Consistent with this interpretation, methanolysis of the sample released the sulfate group based on its elution from QAE-Sephadex (Figure 5C, inset), and rendered the disaccharide sensitive to periodate (Figure 5C).

Distribution of the 3-O-sulfated Gal residue

The location of the sulfate group at C3 would tentatively explain why sulfation and α2-3 sialylation would be mutually exclusive. Previous studies have shown that sialylation and sulfation can occur on different antennae of the same N-glycan chain. In thyroglobulin, sulfation occurs preferentially on the α1-6 branch of biantennary chains and α2-6 sialylation occurred on the α1-3 branch (Spiro and Bhoyroo, 1988; Kamerling *et al.*, 1988; de Waard *et al.*, 1991). However, studies of the human Tamm-Horsfall glycoprotein indicate that sulfation can occur on either arm (Hard *et al.*, 1992), although

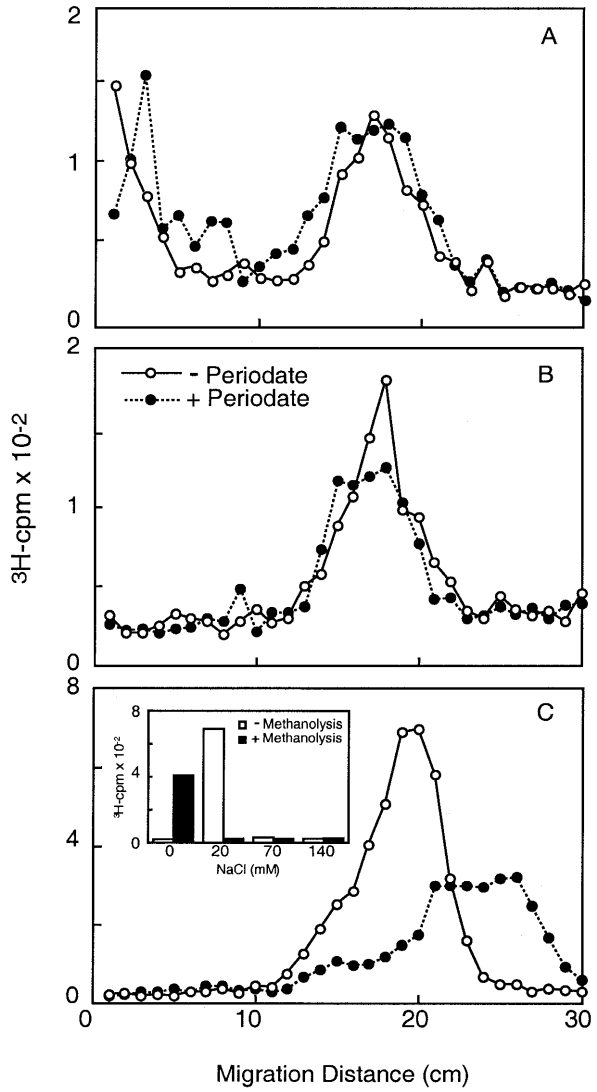

Fig. 5. Periodate treatment does not oxidize the sulfated disaccharide. (**A**) Standard [^{3}H]Gal3SO$_4$ β1-4aManol. (**B**) [^{3}H]Gal disaccharides containing a –1 charge. (**C**) [^{3}H]Gal disaccharides after methanolysis. Samples were treated with (filled circles) or without (open circles) periodate (see *Materials and methods*) and separated by descending chromatography on Whatman No. 3MM in a solvent of pyridine/ethyl acetate/water/acetic acid (5:5:3:1, v/v/v/v). The paper was dried; 1-cm strips were cut, extracted in water, and counted by liquid scintillation counting. The inset in **C** is a chromatogram of the disaccharide on QAE-Sephadex, showing that methanolysis converted the −1 charged disaccharide to a neutral species.

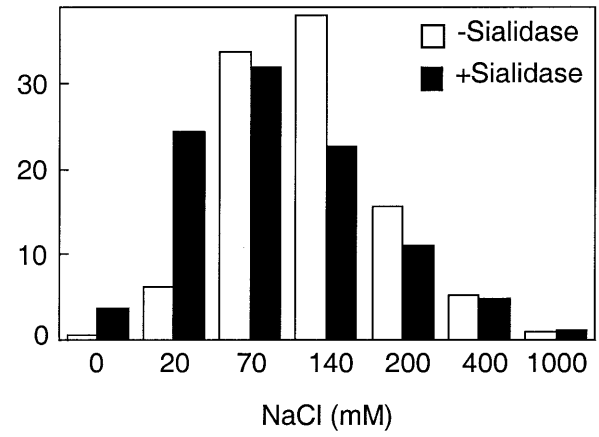

Fig. 6. Intact N-glycans carry a mixture of sulfate and sialic acid residues. ^{35}S-labeled N-glycans released by PNGase F from clone 26 were treated with (solid bars) or without (open bars) sialidase (see *Materials and methods*). Samples were then step-eluted from a QAE-Sephadex column with different concentrations of NaCl, and radioactivity in each fraction was monitored by liquid scintillation spectrometry.

the overall extent of modification was low in this protein. To examine the distribution of sulfate and sialic acid residues in the N-glycans from clone 26, intact ^{35}S-labeled chains were subjected to anion-exchange chromatography. As shown in Figure 6, the chains constitute a heterogeneous population of molecules with one to four negative charges. Treatment of the samples with neuraminidase resulted in loss of material from fractions eluting with 140–200 mM salt (three to four negative charges) and accumulation of material eluting at 20 mM NaCl (one negative charge). Only a small amount of material eluted in the neutral fraction (no salt), indicating that sialylation and sulfation occurred on the same chains and that some of them were actually multisulfated. Similar results were obtained after treatment with mild acid, which cleaves sialic acid but not sulfates (data not shown). Treatment of [^{3}H]GlcN-labeled N-glycans from wild-type cells under the same conditions showed that ~90% of bound material was converted to neutral species by neuraminidase treatment (data not shown), consistent with the idea that most if not all of the charge was due to sialic acids.

These observations suggested that the N-glycan chains were hybrid structures containing Gal-terminated antennae that were either 3-O-sulfated or 3-O-sialylated. Because these reactions compete for the same substrate, we wondered if sulfation reduced the overall degree of sialylation. To examine this question, oligosaccharides were prepared from the cells by PNGase treatment and analyzed by affinity chromatography on a column of immobilized MAL lectin (a mixture of MAL-I and -II, Figure 7). Most of the ^{35}S-labeled oligosaccharides isolated from pgsA-745 and clone 26 cells passed through the column like the [^{14}C]glucose standard, but much of the material from clone 26 was retarded by one or two fractions (Figure 7A). A small amount of material from the transfectant bound tightly, eluting with 0.1 M lactose. Binding of both the retarded and tightly bound material was resistant to sialidase treatment but sensitive to methanolysis, which removed the sulfate groups. None of the ^{35}S-labeled material from the parental line bound with comparable characteristics. These findings suggested that MAL will bind to oligosaccharides terminating with 3-O-sulfated galactose units.

To confirm these findings, cells were examined by staining with MAL-I and -II and flow cytometry. In initial experiments we used a mixture of MAL-I and -II, but binding to the cells was insensitive to sialidase treatment, possibly due to the reactivity of MAL-I with N-acetyllactosamine units (Wang and Cummings, 1988; Sata *et al.*, 1989; Knibbs *et al.*, 1991; Konami *et al.*, 1994; Kaku *et al.*, 1993; Imberty *et al.*, 2000). When purified MAL-II was used instead, binding once again occurred to both wild-type and clone 26 cells, but the staining of the transfectant was actually somewhat greater than in parental cells (compare untreated samples in Figures 8A and

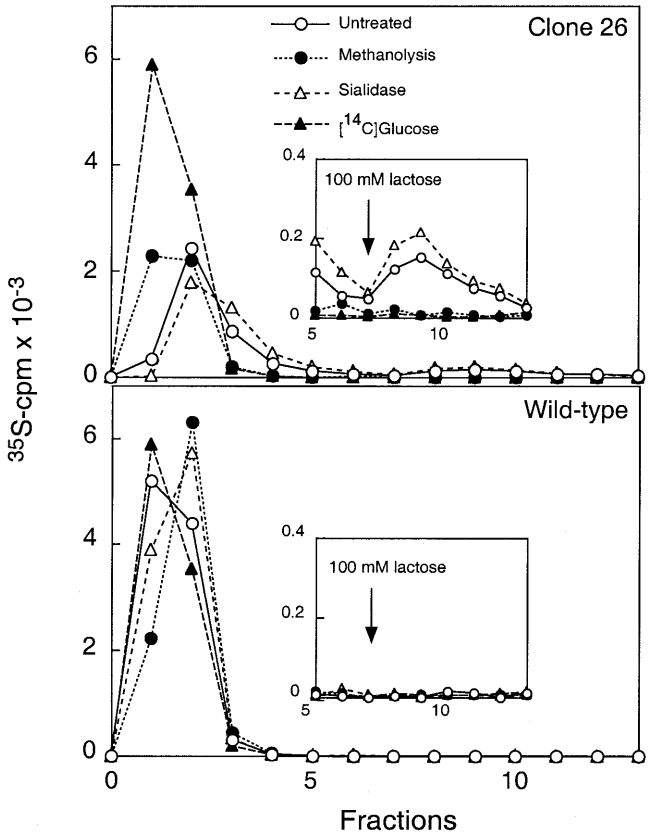

Fig. 7. Affinity chromatography of glycopeptides on MAL-agarose. ^{35}S-labeled N-glycans were released by PNGase F from wild-type and clone 26 cells and analyzed by chromatography on a column of MAL-I and -II lectins conjugated to agarose. Samples were loaded and washed with buffer, and strongly bound material was eluted with 0.1 M lactose at fraction 7. The insets expand the ordinate scale for fractions 5–13.

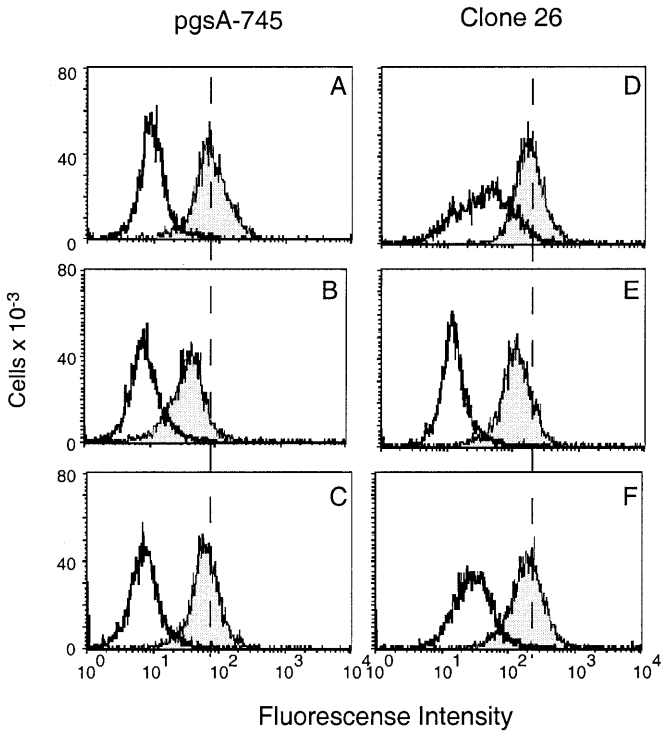

Fig. 8. MAL-II binds to both sulfated and sialylated chains. Binding of biotinylated lectin to cells was measured by fluorescein-avidin-DCS staining and flow cytometry before (shaded) or after (unshaded) treatment with sialidase (see *Materials and methods*). (**A, B, C**) PgsA-745. (**D, E, F**) Clone 26. **A** and **D**, cells were incubated in sulfate-free growth medium without chlorate; **B** and **E**, cells were cultured in presence of 20 mM sodium chlorate to inhibit sulfation; **C** and **F**, cells were cultured in presence of 20 mM sodium chlorate and 300 μM Na_2SO_4.

8D). Binding to the parent was completely lost upon neuraminidase treatment (Figure 8A), reducing the level to the value obtained in the absence of lectin (data not shown). In contrast, binding to clone 26 cells was substantially resistant to neuraminidase (Figure 8D), suggesting that clone 26 contained less terminal α2-3 sialic acid and that the lectin apparently bound equally well or better to the sulfated termini. This idea was confirmed by growing clone 26 and pgsA-745 cells with chlorate, an inhibitor of phosphoadenyl 5′-phosphosulfate (PAPS) formation and macromolecular sulfation. As shown in Figures 8B and 8E, chlorate had a minor effect on the cells, causing a slight decrease in binding. After chlorate treatment, however, clone 26 cells showed greatly enhanced sensitivity to neuraminidase, behaving much like the parental line (Figure 7B). The addition of sulfate substantially reversed the effect of chlorate (Figures 8C and 8F). These findings demonstrated that MAL-II recognizes 3-O-sulfated and 3-O-sialylated termini equally well. MAL apparently requires only a charged group at C3 of galactose, consistent with other studies showing that the side chain of the sialic acid and substitution of the amino group with acetate or glycolate appears of little significance for binding (Knibbs *et al.*, 1991; Imberty *et al.*, 2000).

Assay of O-sulfotransferase activity

The presence of 3-O-sulfation of Gal residues in N-glycans has not been previously described in CHO cells (Karaivanova and Spiro, 1998). However, the fact that the cDNA library that gave rise to clone 26 was from wild-type CHO cells suggested that the gene for the sulfotransferase was probably expressed at low levels in the parental lines. The high level of expression in clone 26 may reflect the activity of the strong immediate-early cytomegalovirus (CMV) promoter in the plasmid either driving expression of the transfected cDNA or an endogenous locus in close proximity to the integration site for the plasmid. Although we have not yet distinguished which of these possibilities is correct, both explanations suggested that clone 26 should contain much higher levels of Gal 3-O-sulfotransferase activity. Kato and Spiro (1989) have characterized a sulfotransferase from thyroid responsible for the 3-O-sulfation of terminal β-linked galactose residues in N-linked glycans. Assays with various native and desialylated/desulfated glycopeptides indicated that the enzyme acted on complex-type N-glycans with free terminal Gal. The same enzyme sulfated free N-acetyllactosamine and its ethyl glycoside, showing that it had no absolute requirement for recognition of a protein or the underlying glycan.

Using these conditions as a guide, we established an assay for the sulfotransferase with synthetic Galβ1-4GlcNAcβ-O-naphthalenemethanol as acceptor. Using this analog facilitated product isolation because the hydrophobic aglycone bound strongly to C18 reversed-phase resins, whereas the [^{35}S]PAPS donor did not. As shown in Figure 9, extracts from clone 26 and 489 transferred ^{35}S-counts to products, whereas extracts from wild-type and pgsA-745 cells had very low to negligible activity. The products of the *in vitro* reaction and the oligosaccharides derived from the transfectant were subjected to acid hydrolysis, which yielded $t_{1/2}$ values of 55–80 min for the release of the sulfate groups. These values fall within the range expected for sulfate esters located at a secondary alcohol in an equatorial position (Rees, 1963), consistent with the idea that the sulfate group transferred *in vitro* was at the same position as that found in the oligosaccharides isolated from the cells.

The product of the reaction and the substrate were also treated with mild periodate and separated by TLC (Figure 10). The substrate was sensitive to oxidation, yielding products that migrated further on the plate. In contrast, the sulfated product generated *in vitro* was resistant to oxidation. These findings are consistent with the sulfate being located at C3 of the terminal Gal, because its location at the other positions would not impart resistance to periodate.

Interestingly, the isomeric disaccharide Galβ1-3GlcNAcβ-O-naphthalenemethanol had comparable activity, which contrasts earlier data showing that free type I disaccharide was less active (Kato and Spiro, 1989). Whether this difference was due to the presence of the aglycone, concentration effects, or different sources of enzyme is unclear. 3-O-sulfation of Gal in type I repeats (Galβ1-3GlcNAc) has not been described previously, possibly due to the absence of this Gal transferase in many tissues. Wild-type CHO cells do not make the type 1 structures based on methylation analysis, exoglycosidase treatments, and a lack of expression of Lewis a antigen (Galβ1-3(Fucα1-4)GlcNAc) after expression of FucT-III (R. Cummings, personal communication). Because cDNAs encoding several Galβ1-3 transferases have recently become available, it should now be possible to express them in clone 26 cells and test if Galβ1-3GlcNAc- units can act as substrates for 3-O-sulfation (Hennet *et al.*, 1998; Amado *et al.*, 1998; Isshiki *et al.*, 1999).

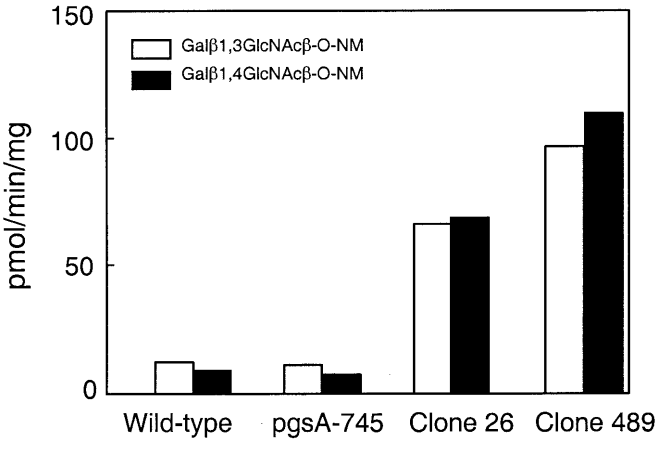

Fig. 9. Galactose:O-sulfotransferase activity is elevated in the transfectants. Galactose:O-sulfotransferase activity in cell lysates was measured by the transfer of ^{35}S-counts from [^{35}S]PAPS to Galβ1-3GlcNAcβ-O-NM (open bars) and Galβ1-4GlcNAcβ-O-NM (filled bars). The reaction products were separated by C-18 reverse-phase chromatography (see *Materials and methods*). Each bar represents the average of two or more assays, and the error in the measurement was <10%.

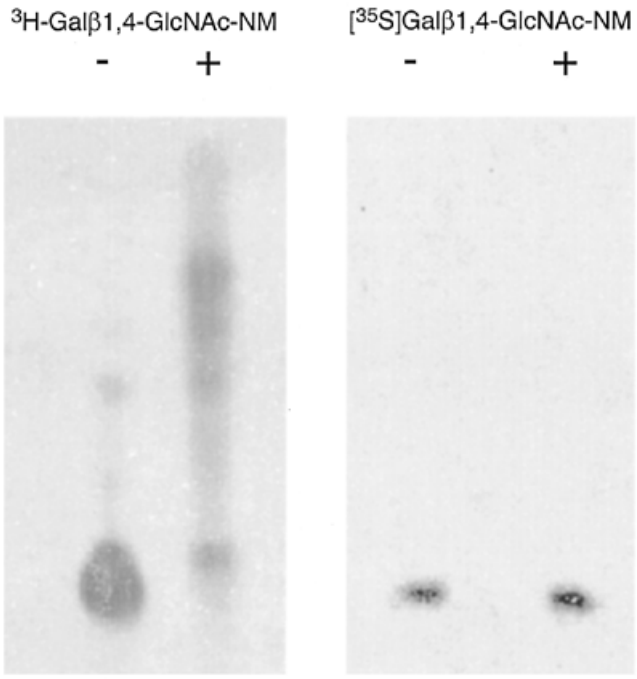

Fig. 10. TLC of disaccharides. [6-^{3}H]Galβ1,4GlcNAcβ-O-NM and [^{35}S]Galβ1,4GlcNAcβ-O-NM were analyzed by TLC before (–) and after (+) periodate oxidation (see *Materials and methods*). The position of the disaccharides and the oxidation products was determined by autoradiography.

Clones 26 and 489 should facilitate cloning of the cDNA encoding Gal 3-O-sulfotransferase. Initial attempts using the polymerase chain reaction (PCR) and probes that hybridize to the flanking sequence in the plasmid used to create the original cDNA library have not yielded a full-length clone with the expected properties of a sulfotransferase. This observation is consistent with the idea that the enhanced enzyme activity may be due to activation of an endogenous locus by juxtaposition of the strong immediate early CMV promoter in the plasmid. Nevertheless, the high level of expression of the enzyme activity suggests that it might be possible to isolate the cDNA by subtractive techniques or by hybridization to DNA arrays. A Gal 3-O-sulfotransferase involved in sulfatide synthesis was cloned (Honke *et al.*, 1997), but this enzyme is selective for a glycolipid substrate (Gal-ceramide). As shown in Table I, the activated sulfotransferase in clone 26 does not result in enhanced synthesis of sulfatide in CHO cells, although the parental cells produce a small amount of sulfated glycolipid (Murphy-Ullrich *et al.*, 1988). Another 3-O-sulfotransferase that can act on both type I and type II Gal-GlcNAc repeats was recently reported (Honke *et al.*, 2001), but hybridization of

mRNA and PCR analysis did not detect this transcript in the transfectants (data not shown). Thus, the Gal 3-O-sulfotransferase amplified in clones 26 and 489 appears to be unique and may act only on N-linked glycans versus other types of oligosaccharides (also see Chandrasekaran *et al.*, 1997, 1999).

Clones 26 and 489 also present interesting sulfated glycans at the cell surface that might act as ligands for mammalian lectins. Studies of synthetic analogs of carbohydrate ligands for selectins have shown that they can have either sialic acid at C3 of the terminal Gal residues or sulfate (Chandrasekaran *et al.*, 1997; Koenig *et al.*, 1997; Ng and Weis, 1997; Sanders *et al.*, 1999; Galustian *et al.*, 1999). Although the natural ligands are thought to be mucin-type oligosaccharides (i.e., O-linked glycans) containing sialylated or sulfated Lewis-type termini (Galβ1-3/4(Fucα1-4/3)GlcNAc-), N-glycans containing similar terminal structures may be active as well if present at sufficient density. The availability of clone 26 now makes it possible to test this and related hypotheses.

Materials and methods

Cell culture

CHO cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61, Rockville, MD). Mutant pgsA-745 is a mutant defective in xylosyltransferase and glycosaminoglycan biosynthesis (Esko *et al.*, 1985, 1987). The cells were grown under an atmosphere of 5% CO_2 in air and 100% relative humidity in Ham's F-12 growth medium (Gibco) supplemented with 7.5% (v/v) fetal bovine serum (FBS; Hyclone Laboratories), 100 μg/ml of streptomycin sulfate, and 100 Units/ml of penicillin G. Sulfate-free medium was prepared from individual components (Ham, 1965), substituting chloride salts for sulfate and using dialyzed FBS (Hyclone). Low-glucose medium contained 1 mM instead of 10 mM glucose. MDCK cells (ATCC CCL 34) were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS.

Screening technique

Mutant pgsA-745 cells were cotransfected with a wild-type CHO cell cDNA library prepared in pCDNA I (Invitrogen) and pMAMneo (Clontech) at a plasmid ratio of ~5:1. Stable transfectants were selected with 0.4 mg/ml of geneticin (Gibco G418, corrected for activity). Resistant colonies were replica plated onto discs of polyester cloth as described previously (Esko, 1989) and metabolically labeled with 10 μCi/ml of $^{35}SO_4$ (25–40 Ci/mg, DuPont NEN) in sulfate-free Ham's F12 medium for 4 h. The colonies were fixed with 10% trichloroacetic acid (TCA), washed with 2% TCA, and exposed to X-ray film. Colonies that exhibited an enhanced level of incorporation of label by autoradiography were picked from the original master dishes and subcloned by a second round of replica plating. Two clones identified in this way (clones 26 and 489) had virtually identical properties and may have been siblings.

Metabolic labeling and lysis of cells

Cells were metabolically labeled for 12–72 h with 10 μCi/ml of $H_2{}^{35}SO_4$ in sulfate-free Ham's F12 medium or 10 μCi/ml of [6-^{3}H]glucosamine HCl and 10 μCi/ml of [6-^{3}H]galactose (25–40 Ci/mg, DuPont NEN) in low-glucose medium for 12 h. After removing the spent medium, the cell layer was washed three times with phosphate buffered saline (PBS), scraped into 1 ml of PBS (Dulbecco and Vogt, 1954), and centrifuged at $1000 \times g$ for 10 min. Cell pellets were resuspended in a lysis buffer of 50 mM Tris–HCl, pH 7.5, 1% SDS, and 0.1 M 2-mercaptoethanol and boiled for 10 min (Roux *et al.*, 1988).

Quantitation of sulfated macromolecules

A portion of $^{35}SO_4$-labeled cells was treated with 10% TCA, centrifuged, washed with 2% TCA, and counted by liquid scintillation spectrometry as a measure of sulfated macromolecules. Another portion of cells was treated with 0.125% trypsin for 5 min and centrifuged, and an aliquot of the supernatant was counted as a measure of trypsin-sensitive, cell surface glycoproteins and proteoglycans. A third portion of cells treated with protease and the glycosaminoglycan fraction was isolated by anion-exchange chromatography (Bame and Esko, 1989). A fourth portion of cells was extracted with chloroform:methanol:water (2:3:1, v/v/v), and the lipid fraction was analyzed by silicic acid chromatography to assess the amount of ^{35}S-counts in glycolipids (Murphy-Ullrich *et al.*, 1988).

Samples were also analyzed by SDS–PAGE before and after treatment for 24 h at 37°C with 10 mU *Flavobacterium meningospecticum* PNGase F (Boehringer Mannheim) in 20 mM Tris–HCl buffer, pH 7.5, containing 0.1% SDS, 50 mM ethylenediamine tetraacetic acid (EDTA), and 20 mM 2-mecaptoethanol. Samples were boiled under reducing conditions and electrophoresed through a 10% gel. After electrophoresis, the gel was dried and exposed to X-ray film.

Isolation of Asn-linked N-glycans

Cells were radiolabeled with $^{35}SO_4$ as described above, 100 μCi/ml of D-[6-^{3}H]glucosamine HCl (40 Ci/nmol, DuPont NEN), or 100 μCi/ml of [6-3H]galactose in low-glucose medium. Radioactive N-glycans were isolated using a modification of the procedure described by Roux *et al.* (1988). Briefly, an aliquot of radiolabeled cells was dissolved in SDS and treated with PNGase F as described above, boiled for 10 min, and centrifuged to remove a small amount of precipitated protein. The supernatant containing the released glycans was applied to Sephacryl S-200 column (1.5 × 50 cm) in a buffer of 20 mM Tris–HCl, pH 6.0, containing 0.2% SDS. The column was eluted at a flow rate of 6 ml/h, and 1-ml fractions were collected. Radioactive material eluting in the included volume was pooled and saturated KCl was added (1:100, v/v) to precipitate the SDS. After overnight incubation at 4°C, the sample was centrifuged at $5000 \times g$ for 30 min, and the supernatant was lyophilized and desalted by passing through a PD-10 column (Pharmacia). The sample was lyophilized again and resuspended in appropriate buffer for further analysis.

To separate neutral and charged glycans, samples were dissolved in 2 mM Tris-base and applied to a 0.5-ml column of QAE-Sephadex (Sigma) prepared in a disposable pipette tip. The column was first washed with 2 mM Tris base (10 ml) and then eluted (2.5 ml each) sequentially with buffer containing 20 mM, 70 mM, 140 mM, 200 mM, 400 mM, and 1000 mM NaCl as described (Roux *et al.*, 1988). The presence of labeled oligosaccharides was monitored by following the radioactivity in each fraction.

Enzymatic and chemical analysis

Purified N-glycans were treated for 24 h at 37°C with the following enzymes:

1. 20 mU of *A. ureafaciens* neuraminidase (Oxford Glycosystems) in 100 µl of 100 mM sodium acetate buffer, pH 6.0, containing 4 mM calcium acetate;
2. 20 mU of jack bean or bovine testicular β-galactosidases (Oxford Glycosystems) in a buffer of 50 mM sodium acetate, pH 4, before and after treatment with neuraminidase or 10 mM HCl at 100°C for 30 min to chemically desialylated the chains;
3. 10 U human placental β-hexosaminidase A (kindly provided by H. Freeze, Burnham Institute, La Jolla, CA). Samples were first chemically desialylated, lyophilized, resuspended in 100 µl of 50 mM sodium formate buffer, pH 4.5, and incubated with β-galactosidase. An aliquot (15 µl) was mixed with 100 mM sodium formate buffer (185 µl), pH 3.5, and β-hexosaminidase A was added. These conditions remove GlcNAc-6SO_4 residues from the termini of N-glycans (Roux *et al.*, 1988);
4. 10 mU of *Flavobacterium heparinum* heparin lyase II (Seikagaku) in 50 mM sodium phosphate buffer, pH 7.6, containing 0.1 M NaCl and 1 mM $CaCl_2$;
5. 10 mU of *Proteus vulgaris* chondroitinase ABC (Seikagaku) in 50 mM Tris–HCl buffer, pH 8.0, containing 50 mM sodium acetate;
6. 20 mU of *Pseudomonas* keratinase I (keratan sulfate endo-β-galactosidase) in a buffer of 50 mM Tris–HCl, pH 7.4; or
7. 1 mU of *Bacillus* keratanase II (keratan sulfate endo-β-N-acetylglucosaminidase, Seikagaku) in a buffer of 10 mM sodium acetate, pH 6.5.

Enzymes were inactivated by heating the samples in a water bath at 100°C for 2 min. The material was applied to a Sephadex G-50 column (29 × 1 cm) and eluted with 0.5 M pyridinium acetate, pH 5.0 (6 ml/h), to separate intact chains from cleaved oligosaccharides. The eluate was collected (0.5-ml fractions) and counted by liquid scintillation. To remove the sulfate group, samples were solvolyzed at 100°C for 7 h in 0.3 ml of dimethyl sulfoxide reagent (90% dimethylsulfoxide/10% methanol, v/v) titrated with HCl to pH 4 (Nagasawa *et al.*, 1977). In some experiments the material was analyzed by anion-exchange chromatography on a column of QAE-Sephadex as described above.

Hydrazine/nitrous acid treatment

Deamination of radiolabeled glycans to oligosaccharides was achieved by N-deacetylation of GlcNAc residues followed by nitrous acid cleavage at high pH (5). Briefly, ^{35}S-, [^3H]GlcN- and [^3H]Gal-labeled N-glycans were deacetylated by treatment at 96°C for 16 h with 100% anhydrous hydrazine. Excess hydrazine was removed by repeated evaporation of the sample to dryness from toluene followed by desalting on a PD-10 column. After lyophilization, the sample was treated at room temperature for 2 h with nitrous acid at pH 4.0 prepared with 0.2 M $NaNO_2$ in 0.4 N acetic acid. The cleaved oligosaccharides were reduced with 30 mM $NaBH_4$ at room temperature for 2 h. After decomposition of the $NaBH_4$ with acetic acid, the sample was repeatedly evaporated to dryness from methanol/acetic acid. Samples were dissolved in 0.5 ml of 0.5 M pyridinium acetate, pH 5.0, and chromatographed on a column of Bio-Gel P4 (Bio-Rad, 1.0 × 100 cm) in the same buffer (4 ml/h). Fractions (1 ml) were collected, and an aliquot was taken for liquid scintillation counting. Peak fractions were pooled as indicated in the figures, concentrated by evaporation, and desalted by chromatography on a 100-mg (3-ml) column of Hypersep PGC Pk30 (Hypersil, UK) as described (Packer *et al.*, 1998). The column was prewashed with three bed volumes of 80% acetonitrile in 0.1% trifluoracetic acid (TFA) (v/v) followed by three volumes of water. After loading the sample, the column was washed with water to remove salts. The column was washed with 25% (v/v) acetonitrile to elute neutral oligosaccharides and then with 25% (v/v) acetonitrile containing 0.05% TFA (v/v) to elute charged oligosaccharides. These samples were dried under high vacuum.

In some experiments, the radiolabeled oligosaccharides liberated by hydrazine/nitrous acid treatment were fractionated by anion exchange chromatography (Spiro and Bhoyroo, 1988). Samples were chromatographed on a 0.8 × 5 cm column of AG-1 X2 (200–400 mesh, acetate) by step elution with different buffers. Neutral products were eluted with 35 ml of water (fraction 1); sialic acid–containing products were eluted with 45 ml of 1 M formic acid (fraction 2); monosulfated components with 35 ml of 0.7 M pyridine acetate, pH 5 (fraction 3); and disulfated saccharides with 35 ml of 2 M pyridine acetate, pH 5. The samples were lyophilized for further analysis.

TLC

The ^{35}S- and ^3H-labeled oligosaccharides in fraction 3 described above (monosulfated components) and disaccharides separated by Bio-gel P4 chromatography were analyzed by cellulose TLC (0.1 mm thickness, plastic sheets, Merck) (Spiro and Bhoyroo, 1988). Standards of [^3H]Galβ1-4aManol, [^3H]Gal3Sβ1-4aManol, [^3H]Galβ1-4aManol6S were kindly provided by R.G. Spiro (Harvard University, Cambridge, MA). Ascending chromatography was performed in a solvent of pyridine/ethyl acetate/water/acetic acid (5:5:3:1, v/v/v/v) using a wick of Whatman No. 3MM paper clamped to the top of the plate during the chromatography. After 22 h, the plate was dried and cut into 1/4-inch strips. The radiolabeled material was extracted with water and counted by liquid scintillation.

Periodate oxidation

Samples were oxidized at 4°C for 16 h in the dark in 100 µl of 0.1 M sodium metaperiodate in 0.04 M sodium acetate buffer, pH 4.5 (Spiro and Bhoyroo, 1988). The reaction was stopped by the addition of 100 µl of 0.2 M glycerol at room temperature. After 1 h, the samples were adjusted to pH 10 with sodium borate buffer and reduced with 30 mM $NaBH_4$ at room temperature for 2 h. After removal of excess borohydride, samples were analyzed by descending paper chromatography on Whatman No. 3MM as described above. A standard sample of [6-^3H]galactose showed >95% loss of counts, indicating that the reaction was nearly quantitative. Some samples were subjected to methanolysis prior to periodate oxidation to remove the sulfate ester (Wing *et al.*, 1992). Briefly, desalted glycans were resuspended in 50 mM HCl in methanol and incubated in a sealed tube at room temperature for 15 h. The sample was then dried under vacuum. The extent of desulfation

was checked by QAE-Sephadex chromatography as described above.

MAL affinity chromatography.

MAL agarose affinity chromatography was performed as described (Wang and Cummings, 1988). MAL lectin (a mixture of MAL-I and -II) immobilized on agarose beads (EY Laboratories) was poured into pipette tips to make small columns (~0.2 ml), and pre-equilibrated with a buffer of 20 mm KH_2PO_4, pH 7.4, 150 mM NaCl, and 0.02% sodium azide. The samples were loaded on the column and eluted with 10 bed volumes of the equilibration buffer, followed by 10 bed volumes of buffer containing of 100 mM lactose. Fractions (1.5 bed volumes) were collected and monitored by liquid scintillation counting.

Galactose:O-sulfotransferase assay.

The activity of PAPS:galactose O-sulfotransferase was measured in CHO cell homogenates essentially as described by Kato and Spiro (1989). Cells were grown to confluence, rinsed three times with cold PBS, and detached with a rubber policeman in 50 µl of buffer containing 0.25 M sucrose, 50 mM Tris acetate buffer (pH 7.4), 1% (w/v) Triton X-100, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Total cell protein was estimated by the Bio-Rad protein assay kit using bovine serum albumin as standard. The cells were homogenized by brief sonication and the solution was clarified by centrifugation at 12,000 × *g* for 15 min. The sulfate donor, [^{35}S]PAPS, was prepared using yeast homogenates as described by Renosto and Segel (1977) and Robbins (1962).

The standard reaction mixture contained 50 mM Tris acetate, pH 7.0, 0.1% (v/v) Triton X-100, 20 mM manganese acetate, 100 mM NaF, 10 mM EDTA, 2 mM ATP, 50 µM [^{35}S]PAPS (~0.2 Ci/mmol), 7.5 mM of Galβ1-4GlcNAcβ-O-naphthalenemethanol or Galβ1-3GlcNAcβ-O-naphthalenemethanol (Sarkar *et al.*, 2000), and 50–100 µg of cell protein in a final volume of 25 µl. The mixture was incubated for 60 min at 37°C, and the reaction was stopped by adding 1 ml of 0.5 M NaCl. Next, the samples were applied to a 100-mg Sep-Pak Vac RC C18 Cartridges (Waters), which were prewashed with 100% methanol, water, and 0.5 M NaCl. After sample application, unincorporated radioactive material were removed by washing the column with 0.5 M NaCl (2.5 ml) and water (25 ml). Product was eluted with 40% methanol in water (2.5 ml) and counted by liquid scintillation spectrometry.

The *in vitro* product was further characterized by acid hydrolysis and periodate oxidation. The samples was first separated from unreacted substrate by sequential chromatography on QAE and C18 resins. One portion was subjected to acid hydrolysis to determine the rate of sulfate loss (Rees, 1963). Another portion was subjected to periodate oxidation, followed by TLC on aluminum-backed silica gel 60 high-performance TLC plates (Merck) in a solvent of ethyl-acetate: glacial acetic acid:methanol:H_2O (10:3:3:2, v/v/v/v). The plate was dried and subjected to autoradiography. The disaccharide standard, [^{3}H]Galβ1,4GlcNAc-naphthalenemethanol, was synthesized enzymatically by galactosylation of GlcNAc-naphthalenemethanol using UDP-[^{3}H]Glc (8.4 Ci/mmol, NEN Life Sciences Products) together with crude bacterial lysate containing a GalT/Epimerase fusion protein (pcw:galE-ltgB) as the enzyme source (Blixt *et al.*, 2001).

Lectin binding

PgsA-745 and clone 26 cells were grown in sulfate-free medium with and without 20 mM sodium chlorate. One set of plates contained both sulfate and chlorate. After 3 days, cells were detached in buffer containing 5 mM EDTA, 20 mM NaH_2PO_4, pH 7.4, and 150 mM NaCl. The cells were washed three times with phosphate buffer, and 1–5 × 10^6 cells were digested with 20 mU of *A. ureafaciens* neuraminidase for 1 h at room temperature in 50 mM HEPES buffer, pH 6.9, containing 2 mM $CaCl_2$ and 0.15 M NaCl. The cells were washed three times with PBS and incubated for 30 min at 4°C with 100 µl of 10 µg/ml biotinylated MAL-I or -II (Vector Laboratory) in PBS. After three washes, the cells were resuspended in 0.2 ml of buffer containing 5 mg/ml of fluorescein-avidin-DCS (Vector Laboratory). The cells were incubated for 20 min at 4°C in the dark, washed, and resuspended in cold PBS. Flow cytometry analysis was done on a FACS Star (Becton-Dickinson).

Acknowledgments

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Abbreviations

aMan, anhydromannose; aManol, anhydromannitol; CHO, Chinese hamster ovary; CMV, cytomegalovirus; EDTA, ethylenediamine tetraacetic acid; FBS, fetal bovine serum; GIP, glycan isolation procedure; MAL, *Maackia amurensis* lectin; MDCK, Madin-Darby canine kidney; NM, β-naphthalenemethanol; PAGE, polyacrylamide gel electrophoresis; PAPS, phosphoadenyl 5′-phosphosulfate; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PNGase F, peptide:N-glycosidase F; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid; TFA, trifluoracetic acid; TLC, thin-layer chromatography.

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