Sulfation of sialyl N-acetyllactosamine oligosaccharides and fetuin oligosaccharides by keratan sulfate Gal-6-sulfotransferase

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We have previously cloned keratan sulfate Gal-6-sulfotransferase (KSGal6ST), which transfers sulfate from 3′-phosphoadenosine 5′-phosphosulfate to position 6 of Gal residue of keratan sulfate. In this study, we examined whether KSGal6ST could transfer sulfate to sialyl N-acetyllactosamine oligosaccharides or fetuin oligosaccharides. KSGal6ST expressed in COS-7 cells catalyzed transfer of sulfate to NeuAcα2-3Galβ1-4GlcNAc (3′SLN), NeuAscα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (SL1L1), NeuAcα2-3Galβ1-4(6-sulfo)GlcNAcβ1-3(6-sulfo)Galβ1-4(6-sulfo)GlcNAc (SL2L4), and their desialylated derivatives except for Galβ1-4GlcNAc, but not to NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc (SLex). When the sulfated product formed from 3′SLN was degraded with neuraminidase and reduced with NaBH4, the resulting sulfated disaccharide alditol showed the same retention time in SAX-HPLC as that of [3H]Gal(6SO4)β1-4GlcNAc-ol. KSGal6ST also catalyzed sulfation of fetuin. When the sulfated oligosaccharides released from the sulfated fetuin after sequential digestion with protease and neuraminidase were subjected to a reaction sequence of hydrazinolysis, deaminative cleavage and NaBH4 reduction, the major product was co-eluted with [3H]Gal(6SO4)β1-4anhidromannitol in SAX-HPLC. These observations show that KSGal6ST is able to sulfate position 6 of Gal residue of 3′SLN and fetuin oligosaccharides. The relative rates of the sulfation of SL2L4 was much higher than the rate of the sulfation of keratan sulfate. These results suggest that KSGal6ST may function in the sulfation of sialyl N-acetyllactosamine oligosaccharide chains attached to glycoproteins.

Key words: sialyl N-acetyllactosamine/keratan sulfate/ sulfotransferase/fetuin/sialyl Lewis x

Introduction

Sulfation of sugar residues occurs not only in glycosaminoglycans but also in various oligosaccharide chains present in glycoproteins and glycolipids (Brockhausen and Kuhns, 1997). Sulfate moiety of sugar residues attached to oligosaccharide chains has been implicated in the high-affinity binding to L-selectin (Imai et al., 1993; Tsuboi et al., 1996; Galustian et al., 1997; Mitsuoka et al., 1998), the roles of HNK-1 epitope in laminin-binding, neural cell migration and outgrowth of neurons and astrocytes (Schmitz et al., 1994), regulation of circulatory half-life of glycoprotein hormones by binding to the specific receptor (Baen zig et al., 1992; Fie te et al., 1991, 1997), and progression of renal cancer (Sakakibara et al., 1989; Kobayashi et al., 1993; Honke et al., 1998). Sulfotransferases involved in the sulfation of oligosaccharides have been characterized (Hooper et al., 1995; Spiro et al., 1996; Chandrasekaran et al., 1997; Degroote et al., 1997; Bowman et al., 1988; Spiro and Bhoyroo, 1998) and some sulfotransferases were cloned (Bakker et al., 1997; Ong et al., 1998; Uchimura et al., 1998; Bistrop et al., 1999).

Although the sulfotransferases involved in the sulfation of oligosaccharides were found to share some molecular features such as being type II transmembrane protein and having putative PAPS-binding sites (Kakuta et al., 1998) with glycosaminoglycan sulfotransferases so far cloned, our knowledge about the relation of the specificity between the two sulfotransferase groups has been limited. We previously showed that chondroitin 6-sulfotransferase (C6ST), which transfers sulfate to position 6 of GalNAc residues of chondroitin (Habuchi et al., 1993), transferred sulfate to position 6 of Gal residue of keratan sulfate (Fukuta et al., 1995; Habuchi et al., 1996) and position 6 of sialyl N-acetyllactosamine oligosaccharides (Habuchi et al., 1997). A sulfotransferase which transferred sulfate to position 6 of nonreducing terminal GlcNAc residue and promoted the formation of 6-sulfo sialyl Lewis x in the transfected COS-7 cells was cloned and was found to have a significant sequence homology with C6ST (Uchimura et al., 1998). A putative sulfotransferase, NSIST, showing relatively high sequence homology to C6ST was cloned by expression cloning using mAb 3B3, which recognized a carbohydrate-containing epitope expressed on dystroglycan and other constituent of the postsynaptic membranes of Torpedo electric organ (Nastuk et al., 1998). The epitope recognized by mAb 3B3 was sensitive to the digestion with neuraminidase but not to the digestion with chondroitinase, keratanase or heparitinase (Bowe et al., 1994).

Keratan sulfate Gal-6-sulfotransferase (KSGal6ST), which catalyzes transfer of sulfate to position 6 of Gal residue of keratan sulfate, was cloned from the fetal human brain library (Fukuta et al., 1997). When the cloned cDNA was transfected in COS-7 cells, the expressed sulfotransferase transferred sulfate to position 6 of Gal residue of keratan sulfate, but not to...
were incubated with the expressed KSGal6ST, and the radio-
sulfate to sialyl paper we investigated whether KSGal6ST could transfer
N-saccharides attached to intact fetuin could be sulfated by
as observed in C6ST. We also investigated whether oligo-
saccharides were found to serve as acceptors for
sialyl Lewis x tetrasaccharide could not be detected.
Materials and methods
Structural analyses of 35S-labeled 3′SLN
To determine the position to which 35SO4 was transferred to
3′SLN, we degraded the radioactive product formed from
3′SLN with neuraminidase and reduced with NaBH4. The
disaccharide alditol thus obtained was subjected to Partisil 10-
SAX HPLC after separation with paper electrophoresis (Figure
2). The 35S-radioactivity was eluted at the position of
[3H]Gal(6SO4)β1-4GlcNAcβ1-3Galβ1-4GlcNAc(6SO4). These
results indicate that 35SO4 was transferred to Gal residue of
3′SLN, but not to GlcNAc residue.
Sensitivity of 35S-labeled products derived from L1L1 to β-
galactosidase digestion
L1L1 contained two Gal residues. To obtain the information
about the location of 35SO4 transferred to L1L1, we investi-
gated the sensitivity of the sulfated products to β-galactosidase digestion. 35S-Labeled products derived from L1L1 was mixed with nonradioactive L1L1, and digested with β-galactosidase. The mixture of 35S-labeled products and the nonradioactive oligosaccharides were applied to the Superdex 30 column before or after digestion with β-galactosidase. The eluate from the column was monitored by absorption at 210 nm and 35S-radioactivity (Figure 3). After β-galactosidase digestion of the mixture of 35S-labeled material derived from L1L1 and nonradioactive L1L1, both the absorption at 210 nm due to nonradioactive L1L1 and 35S-radioactivity were completely shifted to more retarded position (Figure 3B,D). Since β-galactosidase is unable to cleave the glycosidic bond if the galactose is sulfated, these results suggest that all of 35SO4 transferred to L1L1 was located to the reducing end side Gal residue. Such a property of KSGal6ST appears to make a clear contrast to the property of C6ST; about one-third of 35SO4 was transferred to the reducing end side Gal residue of L1L1 by C6ST.

Structural analysis of sulfated oligosaccharides released from sulfated fetuin

When 35S-labeled fetuin was digested with keratanase I, no depolymerized products were observed, while 35S-labeled keratan sulfate was completely degraded under the same conditions (Figure 4), indicating that the incorporation of 35SO4 into the polymer fraction was not due to the incorporation into the potentially contaminating keratan sulfate. We also confirmed that 35S-labeled fetuin was not degraded with chondroitinase ABC digestion (data not shown). After Actinase E digestion, 35S-labeled oligosaccharides with a size of about pentadecasaccharide were released (Figure 5A). When the pentadecasaccharide fraction was further digested with neuraminidase, oligosaccharides with a size of about undecasaccharide were formed (Figure 5B). The undecasaccharide fraction was subjected to the sequential reaction of deacetylation, deaminative cleavage and NaBH4 reduction, and the final products were separated with paper chromatography (Figure 6A). About 45% of the total radioactivity was migrated to the position of [3H]Galβ1-4ManR(6SO4) and [3H]Gal(6SO4)β1-4AManR. 35S-Radioactivity remaining at the paper origin in Figure 6A may be due to the larger oligosaccharides, and was not examined further. The fractions indicated by a horizontal bar in Figure 6A was applied to a Partisil 10-SAX column (Figure 6B). Major 35S-radioactivity was eluted at the position of [3H]Gal(6SO4)β1-4AManR, but no radioactive peak

### Table I. Incorporation of 35SO4 into sialyl N-acetyllactosamine oligosaccharides, fetuin, and keratan sulfate

<table>
<thead>
<tr>
<th>Acceptors</th>
<th>Relative incorporation of 35SO4 %</th>
<th>K_m (mM)</th>
<th>V_max (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LNS</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3’SLN</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLex</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1L1</td>
<td>7.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL1L1</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2L4</td>
<td>48</td>
<td>10.4</td>
<td>85</td>
</tr>
<tr>
<td>SL2L4</td>
<td>250</td>
<td>0.65</td>
<td>20</td>
</tr>
<tr>
<td>Fetuin</td>
<td>8.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratan sulfate</td>
<td>100</td>
<td>0.38 b</td>
<td>6.1</td>
</tr>
</tbody>
</table>

*Not detected.

Expressed as the concentration of repeating disaccharide units.

![Fig. 3. β-Galactosidase digestion of 35S-labeled L1L1. 35S-Labeled L1L1 was mixed with L1L1, and applied to Superdex 30 chromatography before (A, C) or after (B, D) β-galactosidase digestion. The eluate was monitored by absorption at 210 nm (C, D), and radioactivity of each 0.5 ml fraction was determined (A, B).](image-url)
was observed at the position of \([^{3}H]\text{Gal}\beta_{1-4}\text{ManR}(6\text{SO}_{4})\).
These results indicate that the structure of the nonreducing terminal region of major parts of \(^{35}\text{S-}\)oligosaccharides released after Actinase E digestion was sialyl \(\text{Gal}\beta_{1-4}\text{GlcNAc}(6\text{SO}_{4})\).
Structures of minor components, however, remained to be determined because small peaks eluted at 12 min, 22 min and 27.5 min could not be assigned in this experiment. We used \(\text{Gal}(6\text{SO}_{4})\beta_{1-4}\text{ManR}\) and \(\text{Gal}\beta_{1-4}\text{ManR}(6\text{SO}_{4})\) prepared from keratan sulfate as standard materials in HPLC. Chromatographic behaviors of other possible disaccharide alditols such as \(\text{Gal}(2\text{SO}_{4})\beta_{1-4}\text{ManR}\) and \(\text{Gal}(4\text{SO}_{4})\beta_{1-4}\text{ManR}\) were not examined in this paper; therefore, the possibility that the reaction products might contain \(\text{Gal}(2\text{SO}_{4})\beta_{1-4}\text{ManR}\) or \(\text{Gal}(4\text{SO}_{4})\beta_{1-4}\text{ManR}\) could not be excluded.

Sulfation of oligosaccharides with \text{FLAGKSGal6ST} fusion protein

To confirm that the sulfotransferase activity toward sialyl N-acetyllactosamine oligosaccharides was due to the protein expressed from \text{KSGal6ST} cDNA, we expressed a recombinant \text{KSGal6ST} containing FLAG peptide at the N-terminal. The sulfotransferase activities toward keratan sulfate and SL2L4 were overexpressed about 5-fold and 19-fold, respectively, over the control. After the extracts of COS-7 cells were purified with anti-FLAG mAb-conjugated column, the sulfotransferase activity toward SL2L4 and keratan sulfate was detected only when COS-7 cells were transfected with \text{FLAGKSGal6ST} cDNA (Table II). The sulfotransferase activity toward chondroitin was not observed at all in the affinity-purified fractions. These results indicate that sulfation of SL2L4 and keratan sulfate was catalyzed by the same protein expressed from \text{KSGal6ST} cDNA. However, since the affinity-purified fraction was not homogeneous even after purification with FLAG-affinity column, the possibility that...
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either or both of the sulfotransferase activities might be due to proteins of the host cells, whose synthesis might have been enhanced by the transfection of the cDNA, could not be completely excluded.

Expression of KSGal6ST in immunologically relevant tissues

Since KSGal6ST was shown to be involved in the formation of L-selectin ligand (Bistrup et al., 1999), we investigated whether KSGal6ST is expressed in various immunologically relevant tissues. Among various human tissues, KSGal6ST mRNA with 2.9 kb was expressed in the spleen, lymph node, thymus and appendix (Figure 7). The size of the message was almost the same as that observed in the human brain (Fukuta et al., 1997). The expression of KSGal6ST in the various immunologically-relevant tissues suggests the important function in the immunological system.

Table II. Incorporation (pmol/min/mg protein) of 35SO4 into keratan sulfate, SL2L4 and chondroitin catalyzed by the extracts from COS-7 cells transfected with FLAGKSGal6ST

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Acceptor</th>
<th>Keratan sulfate</th>
<th>SL2L4</th>
<th>Chondroitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfected COS-7 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>4.4 ± 0.5</td>
<td>5.7 ± 0.6</td>
<td>1.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Affinity-purified fraction</td>
<td>1.6</td>
<td>2.9</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Control COS-7 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>0.8 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Affinity-purified fraction</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

*COS-7 cells transfected with FLAGKSGal6ST.

Transfection with FLAGKSGal6ST and preparation of the affinity purified fraction were described under Materials and methods. Data for the crude extract fractions were average ± SD of triplicate dishes. The affinity-purified fractions were prepared from one of the triplicate extracts. Data for the affinity-purified fractions were represented as pmol/min/mg protein of the original crude extracts.

Fig. 6. Separation by paper chromatography and HPLC of the products formed from 35S-labeled fetuin oligosaccharides after sequential reactions. (A) The fraction obtained after neuraminidase digestion (indicated by a horizontal bar in Figure 5B) was subjected to sequential reaction with hydrazine/hydrazine sulfate, nitrite at pH 4 and NaBH4 as described under Materials and methods. The final products were mixed with a mixture containing [3H] Gal(6SO4)β1-4AManR(6SO4), [3H] Gal(6SO4)β1-4AManR, [3H] Galβ1-4AManR(6SO4) and AManR(6SO4), and separated with paper chromatography. Peak 1, peak 2, and peak 3 indicate the migration positions of [3H] Gal(6SO4)β1-4AManR and [3H] Galβ1-4AManR(6SO4), a mixture of [3H] Gal(6SO4)β1-4AManR and [3H] Galβ1-4AManR(6SO4), and AManR(6SO4), respectively. (B) The radioactive fraction which comigrated with [3H] Gal(6SO4)β1-4AManR and [3H] Galβ1-4AManR(6SO4) (indicated by a horizontal bar in (A) was eluted from the paper, purified with paper electrophoresis and subjected to SAX-HPLC. Radioactivity of 3H (open circles) and 35S (solid circles) of each fraction was determined. Peak 4 and peak 5 in (B) were assigned as [3H] Gal(6SO4)β1-4AManR and [3H] Galβ1-4AManR(6SO4), respectively.

Fig. 7. Northern blot analysis of KSGal6ST messages in various immunologically relevant tissues. Northern blots with poly(A)+ RNA from the spleen (lane 1), lymph node (lane 2), thymus (lane 3), appendix (lane 4), peripheral blood leukocytes (lane 5), bone marrow (lane 6), and fetal liver (lane 7) were hybridized with 32P-labeled DNA probe for human KSGal6ST cDNA. Each lane contained 2 µg of poly(A)+ RNA. The positions of the molecular size standards (kb) are indicated at the right.
Discussion

In this article, we showed that KSGal6ST transferred sulfate not only to keratan sulfate but also to sialyl N-acetylglactosamine oligosaccharides. We have previously reported that C6ST also transferred sulfate to sialyl N-acetylactosamine oligosaccharides (Habuchi et al., 1997). KSGal6ST was found to share the substrate specificity with C6ST. Both KSGal6ST and C6ST catalyzed transfer sulfate to position 6 of Gal residue of 3′SLN. Sulfate moiety attached to GlcNAc residue adjacent to the targeted Gal residue caused marked stimulation of the both enzymes. Fucose attached to GlcNAc may inhibit both the activities, since both KSGal6ST and C6ST could transfer sulfate to 3′SLN, but not to SLε4. However, clear differences in the specificity were also observed between KSGal6ST and C6ST. The most prominent feature of KSGal6ST was that the rate of sulfation of SL2L4 was much higher than the rate of sulfation of keratan sulfate. In contrast, the rate of sulfation of SL2L4 by C6ST was 1.5% of the rate of sulfation of keratan sulfate (Habuchi et al., 1997). From these substrate specificities, KSGal6ST was supposed to be more suitable for the sulfation of oligosaccharides than C6ST. This assumption may be supported by the observation that oligosaccharides bound to intact fucin were sulfated by KSGal6ST as efficiently as nonsulfated free oligosaccharides. β-Galactosidase digestion of the sulfated oligosaccharides revealed that only reducing end side Gal residue of L1L1 was sulfated by KSGal6ST, while about one-third of 35SO4 incorporated to L1L1 by C6ST resided on the nonreducing terminal Gal residue. The affinity of KSGal6ST for the sulfated oligosaccharide bearing sialic acid on the nonreducing terminal (SL2L4) was much larger than that for the desialylated oligosaccharide (L2L4), suggesting that the nonreducing terminal sialic acid may cause the increase in the affinity for the acceptors when sulfate group is present on GlcNAc residue adjacent to the targeted Gal residue. We used partially purified KSGal6ST preparation expressed in COS-7 cells for the study of the substrate specificity. As described in our previous paper (Fukuta et al., 1997), the partially purified preparation was almost devoid of C6ST activity. However, the possibility that the transfection might have affected other endogenous sulfotransferase activity in COS-7 cells could not be excluded.

A microsomal sulfotransferase preparation obtained from the rat spleen was reported to have the ability to sulfate position 6 of Gal residues of glycoprotein oligosaccharides (Spiro and Bhoyroo, 1998). The substrate specificities of the rat spleen sulfotransferase was essentially the same as those of KSGal6ST; the rat spleen sulfotransferase catalyzed the transfer of sulfate to 3′SLN and fucin oligosaccharides but not to SLε4 tetrasaccharide. In the human tissues, KSGal6ST mRNA was expressed in the brain (Fukuta et al., 1997) and in the various immunologically relevant tissues including spleen (Figure 7). On the other hand, the activity of 6-O-sulfation of the Gal residue was also found in various tissues including the brain (Spiro and Bhoyroo, 1998). The similarity in the specificity and distribution between KSGal6ST and the rat Gal-6-O-sulfotransferase suggests that the rat Gal-6-O-sulfotransferase activity may be carried by a rat counterpart of KSGal6ST or by a hypothetical isoform of KSGal6ST with the specificity similar to that of KSGal6ST. However, the possibility that the Gal-6-O-sulfotransferase activity in the spleen may be partly due to C6ST, since C6ST is also expressed in the spleen (Fukuta et al., 1998).

GlyCAM-1 is one of high endothelial venule-associated ligands (Imai et al., 1991; Lasky et al., 1992) and was reported to contain O-linked sugar chains containing sulfated sialyl Lewis x structure (Hemmerich and Rosen, 1994; Hemmerich et al., 1995). Structural analysis of GlyCAM-1 has identified Gal(6SO4) and GlcNAc(6SO4) as the major sulfated sugars (Hemmerich et al., 1994). Recently, KSGal6ST has been shown to be able to sulfate L-selectin ligand when COS cells with a cDNA encoding a GlyCAM-1/IgG chimera were transfected with a cDNA encoding KSGal6ST (Bistrup et al., 1999). When CHO/fucosyltransferase VII/core 2 β-6-N-acetylglucosaminyl transferase were transfected with KSGal6ST and HEC-GlcNac6ST cDNAs (singly or in combination), the resulting cells showed positive binding to L-selectin/IgM. The combination of the two sulfotransferase cDNAs synergistically enhanced the binding of L-selectin/IgM. These interesting observations may be correlated with our findings that sulfation of Gal residues with KSGal6ST was strongly stimulated by the presence of sulfate group on the adjacent GlcNAc residue. Since KSGal6ST showed no activity toward SLε4 in vitro, introduction of sulfate to Gal residue catalyzed by KSGal6ST should precede the introduction of fucose.

Chiba et al. reported the structure of major oligosaccharides bound to α-dystroglycan, Neu2-3Galβ1-4GlcNAcβ1-2Man (Chiba et al., 1997). They showed that, even after the neuraminidase digestion, a significant portion of the oligosaccharide fractions released by β-elimination from α-dystroglycan was still absorbed to the anion exchange column. The structure of the neuraminidase-resistant negatively charged oligosaccharide is not known. It remains to be investigated whether sulfate group is present on the dystroglycan oligosaccharides. A mAb 3B3, which recognized agrin-binding proteins, was raised using the synaptic membrane proteins as the antigen (Bowe et al., 1994). One of the epitope-bearing proteins was found to be dystroglycan. The reactivity of the mAb was decreased by the digestion with neuraminidase but not affected by the digestion with chondroitinase or keratanase, suggesting that the epitope for the mAb may not be glycosaminoglycans but oligosaccharides with sialic acid. NSIST cDNA was cloned from Torpedo electric organ by detecting the expression of the epitope recognized by mAb 3B3 on the surface of COS cells (Nastuk et al., 1998). NSIST shows significant sequence homology to both C6ST and KSGal6ST; identity of amino acid sequence between NSIST and chick C6ST is 56% and identity of amino acid sequence between NSIST and human KSGal6ST is 38%. Such similarities in the amino acid sequence suggest that NSIST may be a novel sulfotransferase and that, in addition to sialic acid, sulfate group may be involved in the formation of the structure of the epitope for mAb 3B3. Although the substrate specificity of NSIST has not been revealed yet, it may possibly be similar to those of KSGal6ST.

Materials and methods

The following commercial materials were used: H235SO4 (NEN Life Science Products, Inc.) was from Daiichi Chemicals Co. Ltd. (Tokyo, Japan); [3H]NaBH4 (16.3 GBq/mmol) was from Amersham Japan (Tokyo, Japan). Unlabeled PAPS,
fetal calf serum fetuin (F3004), galactosamine 6-sulfate and Flag peptide were from Sigma (St. Louis, MO); Fast Desalting Column HR 10/10; Hiloal Superdex 30 16/60; DEAE-Sephadex A-50 and DEAE-Sephacel were from Pharmacia, Biotech (Tokyo, Japan); chondroitinase ABC, Streptococcus neuraminidase, Streptococcus β-galactosidase, keratanase I, keratanase II, NeuAc2-3Galβ1-4(Fucα1-3)GalNAc(SLex), and NeuAc2-3Galβ1-4GlcNAc(3'SLN) were from Seikagaku Corp. (Tokyo, Japan); Actinase E (proteinase from Streptomyces gricus) was from Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan); Partisol 10-SAX was from Whatman (Clifton, NJ); and Galβ1-4GlcNAc (LN) was from Funakoshi (Tokyo, Japan).

Keratan sulfate from bovine cornea and NeuAc2-3Galβ1-4GlcNAc(6SO4)β1-3Gal(6SO4)β1-4GlcNAc(6SO4)β1-4GlcNAc(6SO4) (SL2L4), which was prepared from keratan sulfate by keratanase II digestion (Nakazawa et al., 1989; Hashimoto et al., 1990), were products of Seikagaku Corporation and generously gifted from the company. NeuAc2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc(3'SLN) (L1L1) and Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc(L1L1), which were prepared by desulfation of the corresponding oligosaccharides, were generously gifted from Dr. Yutaka Kariya, Tokyo Research Institute of Seikagaku Corporation. [35S]PAPS was prepared as described previously (Delfert, 1997). The reaction mixture contained 2.5 μmol of imidazole-HCl, pH 6.4, 0.5 μmol of CaCl2, 0.1 μmol dithiothreitol, 0.025 μmol of oligosaccharides or 0.025 μmol (as glucosamine) of keratan sulfate or 0.025 μmol (as sialic acid) of fetuin, 50 pmol [35S]PAPS (about 5 × 105 cpm), and the partially purified KSGal6ST or FLAG-KSGal6ST in a final volume of 50 μl. The reaction mixtures were incubated at 37°C for 60 min for the partially purified KSGal6ST or 20 min for FLAG-KSGal6ST and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, 35S-labeled products were separated from 35SO4 and [35S]PAPS by Superdex 30 gel chromatography, and the radioactivity was determined. As a control, reaction mixture without acceptors was applied to the Superdex 30 column, and the radioactivity observed in the control was subtracted. When fetuin was used as an acceptor, the reaction was stopped by placing on ice and the reaction mixture was immediately injected into a Superdex 30 column. When keratan sulfate was used as an acceptor, sulfotransferase reaction proceeded linearly up to 1.5 μg of the partially purified KSGal6ST and up to 60 min under the conditions described above.

Preparation of a FLAG-KSGal6ST fusion protein

Recombinant KSGal6ST was also expressed as a fusion protein with Flag peptide. A DNA fragment which codes for full open reading frame was amplified by PCR using human KSGal6ST cDNA as a template. The 5' and 3' primers were CGCAAGCT-TATGCAATGTTCCTGGAAGGCC and CAGGAATTCTCA-CGAGAAGGGGCCGAACTC, respectively. At the 5'end of the oligonucleotide primers, restriction enzyme recognition sites were introduced; HindIII site for the sense primer and EcoRI site for the antisense primer. The PCR product was digested with EcoRI and HindIII, and subcloned into these sites of pFLAG-CMV-2 plasmid (Kodak, New Haven, CT). The resulting plasmid was transfected in COS-7 cells as described previously (Fukuta et al., 1997) and the fusion protein produced was extracted from the cells with buffer B containing 10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 10 mM MgCl2, 2 mM CaCl2, 0.5% Triton X-100, 20% glycerol by gentle shaking on a rotatory shaker for 30 min at 4°C. The extracts were centrifuged at 10,000 × g for 10 min. The supernatant fraction (crude extract) was applied to an anti-FLAG mAb-conjugated affinity column (Kodak) equilibrated with the buffer B. The absorbed materials were eluted with Flag peptide under the conditions recommended by the manufacturer.

Incorporation of [35S]SO4 into oligosaccharides, keratan sulfate, and fetuin

The reaction mixture contained 2.5 μmol of imidazole-HCl, pH 6.4, 0.5 μmol of CaCl2, 0.1 μmol dithiothreitol, 0.025 μmol of oligosaccharides or 0.025 μmol (as glucosamine) of keratan sulfate or 0.025 μmol (as sialic acid) of fetuin, 50 pmol [35S]PAPS (about 5 × 105 cpm), and the partially purified KSGal6ST or FLAG-KSGal6ST in a final volume of 50 μl. The reaction mixtures were incubated at 37°C for 60 min for the partially purified KSGal6ST or 20 min for FLAG-KSGal6ST and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, 35S-labeled products were separated from 35SO4 and [35S]PAPS by Superdex 30 gel chromatography, and the radioactivity was determined. As a control, reaction mixture without acceptors was applied to the Superdex 30 column, and the radioactivity observed in the control was subtracted. When fetuin was used as an acceptor, the reaction was stopped by placing on ice and the reaction mixture was immediately injected into a Superdex 30 column. When keratan sulfate was used as an acceptor, sulfotransferase reaction proceeded linearly up to 1.5 μg of the partially purified KSGal6ST and up to 60 min under the conditions described above.

Neuraminidase digestion and NaBH4 reduction of 35S-labeled 3'SLN

35S-labeled 3'SLN was prepared using the partially purified KSGal6ST (2 μg as protein) as described above except that concentration of [35S]PAPS was increased to 6.8-fold and incubation was carried out for 20 h. The 35S-labeled 3'SLN eluted from the Superdex 30 column was lyophilized, purified by paper electrophoresis, and digested with neuraminidase as described below. Aliquot of the sample after neuraminidase digestion was dried, dissolved in 10 μl of 0.5 M NaBH4/0.2 M Na2CO3, pH 10.2. After 2 h at 0°C, 10 μl of the same solution was added and the reduction was continued for further 2 h at

Construction of pCXNKSGal6ST and preparation of keratan sulfate Gal 6-sulfotransferase from COS-7 cells transfected with pCXNKSGal6ST

The human KSGal6ST cDNA was inserted in an expression vector pCXN2 (pCXN2 was generously donated from Dr. Jun-ichi Miyazaki, Department of Disease-related Gene Regulation, Faculty of Medicine, University of Tokyo) and pCXNKSGal6ST was constructed as described previously (Fukuta et al., 1997). Transient expression of KSGal6ST cDNA in COS-7 cells and the preparation of the partially purified KSGal6ST with DEAE-Sephadex A-50 and heparin-Sepharose CL 6B were described previously (Fukuta et al., 1997).
0°C. After the reduction, excess NaBH₄ was destroyed by addition of 10 µl of 3 M acetic acid. The reaction mixtures were dried under N₂ stream, dissolved in a small volume of water, and applied to a Dowex 50 H⁺ column (bed volume 0.2 ml). The flow through fraction was dried and suspended in methanol. Boric acid was removed as methyl ester by drying in vacuo.

N-Deacetylation, deamination, and NaBH₄ reduction of 35S-labeled oligosaccharides released from the sulfated fetuin

35S-Labeled oligosaccharides were released from the sulfated fetuin by the digestion with Actinase E and neuraminidase, and separated with Superdex 30 chromatography. The oligosaccharides were then subjected to N-deacetylation, deaminative cleavage and NaBH₄ reduction as described (Shaklee and Conrad, 1986; Habuchi et al., 1996) using nonradioactive NaBH₄. The final sample was dissolved in 60 µl of water and spotted on a strip of Whatman 3 paper and developed with the solvent described below.

Digestion with neuraminidase, β-galactosidase, keratanase I, and Actinase E

Digestion with neuraminidase was carried out for 60 min at 37°C in the reaction mixture containing, in a final volume of 50 µl, 35S-labeled oligosaccharide, 5 µmol of potassium acetate buffer, pH 6.5, 0.5 µmol of CaCl₂ and 20 mU of neuraminidase (Kiyohara et al., 1974). Reaction mixture for β-galactosidase digestion contained 35S-labeled L1L1, 25 nmol of L1L1, 2.5 µmol of sodium acetate buffer, pH 5.5, and 5 µU of the enzyme in a final volume of 50 µl (Kiyohara et al., 1976). The reaction mixtures were incubated at 37°C for 20 h. Keratanase I digestion was carried out for 15 h at 37°C in the reaction mixture containing, in a final volume of 25 µl, 35S-labeled keratan sulfate or 35S-labeled fetuin, 1.25 µmol of Tris-HCl, pH 7.4, 100 mU of keratanase I, and protease inhibitors (50 µM Na₂-p-tosyl-L-lysine chloromethyl ketone, 30 µM N-tosyl-L-phenylalanine chloromethyl ketone, 300 µM phenylmethyl sulfonfluoride). Reaction mixture for Actinase E contained 35S-labeled fetuin, 2 µmol of Tris-HCl, pH 8.0 and 250 µg of Actinase E in a final volume of 40 ml. The reaction mixtures were incubated at 37°C for 20 h.

Superdex 30 chromatography, paper electrophoresis, paper chromatography, and HPLC

Hiload Superdex 30 16/60 column was equilibrated with 0.2 M NH₄HCO₃. The flow rate was 1 ml/min; 1 ml or 0.5 ml fractions were collected, mixed with 4 ml Clearsol (Nakarai Tesque, Kyoto), and the radioactivity was determined. Oligosaccharides were monitored by absorption at 210 nm. Paper electrophoresis was carried out on Whatman No. 3 paper (2.5 cm x 57 cm) in pyridine/acetic acid/water (1:10:400, by volume, pH 4) at 30 V/cm for 40 min. Samples for paper chromatography was spotted on a Whatman No. 3 paper (2.5 cm x 57 cm) and developed with 1-butanol/acetic acid/1 M NH₄ (3:2:1, by volume). The dried paper strips after paper electrophoresis or paper chromatography were cut into 1.25 cm segments and radioactivity was determined by liquid scintillation counting. HPLC separation of 35S-labeled disaccharide alditols was carried out on a Whatman Partisil 10-SAX column (4.5 x 25 cm) equilibrated with 5 mM KH₂PO₄. The column was developed with 5 mM KH₂PO₄. The flow rate was 1 ml/min and the column temperature was 40°C; 0.5 ml fractions were collected, mixed with 4 ml Clearsol, and the radioactivity was determined.

Determination of glucosamine and sialic acid

The glucosamine contents of oligosaccharides were determined by the Elson-Morgan method as modified by Strominger et al. (Strominger et al., 1959) after hydrolysis of the glycosaminoglycans with 6 M HCl at 100°C for 4 h. Sialic acid was determined by thiobarbituric acid method (Aminoff, 1961) after hydrolysis with 0.1 M H₂SO₄ at 80°C for 60 min.

Northern blot hybridization

Human Multiple Tissue Northern Blot Filters (Clontech), on which 2 µg poly(A) RNAs from various adult human tissues were blotted, were prehybridized in a solution containing 50% formamidine, 5× SSPE, 5× Denhardt’s solution, 0.5% SDS, and 0.1 mg/ml of denatured salmon sperm DNA for 3 h at 42°C. Hybridization was carried out in the same buffer containing 35P-labeled probe for 16 h at 42°C. The radioactive probe was prepared from the EcoRI fragment containing 2415 bp cDNA (Fukuta et al., 1997) by the random oligonucleotide-primed labeling method using [α-32P]dCTP (Amersham) and a DNA random labeling kit (Takara Shuzo). The filters were washed at 65°C in 2× SSPE, 0.1% SDS, and subsequently in 1× SSPE, 0.1% SDS. The membrane was exposed to x-ray film for 26 h with an intensifying screen at –80°C.

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Abbreviations

KSGal6ST, keratan sulfate Gal-6-sulfotransferase; C6GST, chondroitin 6-sulfotransferase; PAPS, 3’-phosphoadenosine 5’-phosphosulfate; HPLC, high performance liquid chromatography; Gal(6SO₄), 6-O-sulfo-D-galactose; GlcNAc(6SO₄), 6-O-sulfo-N-acetyl-D-glucosamine; GlcNAc(6SO₄)R, 6-O-sulfo-N-acetyl-D-glucosaminitol; AManR, 2-3-anhydro-D-mannitol; AMan(6SO₄)R, 6-O-sulfo-2,3-anhydro-D-mannitol; LN, Galβ1-4GlcNAc; LNS, Galβ1-4GlcNAc(6SO₄); 3’S LN, NeuAcα2-3Galβ1-4GlcNAc; SLeα, NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc; L1L1, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc; SL1L1, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc; L2L2, Galβ1-4GlcNAc(6SO₄)β1-3Gal(6SO₄)β1-4GlcNAc(6SO₄); and 3’S LN, NeuAcα2-3Galβ1-4GlcNAc(6SO₄)β1-3Gal(6SO₄)β1-4GlcNAc(6SO₄).

References


