Characterization of a rat liver Golgi sulphotransferase responsible for the 6-O-sulphation of *N*-acetylglucosamine residues in β -linkage to mannose: role in assembly of sialyl-galactosyl-*N*-acetylglucosamine 6-sulphate sequence of N-linked oligosaccharides

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Rat liver Golgi membranes were found to contain an enzyme that can transfer sulphate from 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to C-6 of the terminal GlcNAc in β -linkage to mannose and has properties indicating that it is involved in the synthesis of the NeuAc α 2-3(6)Gal β 1-4GlcNAc(6-SO₄) sequences observed in the N-linked carbohydrate units of various glycoproteins. Assays performed with [³⁵S]PAPS (*K*_m 0.67 μ M) and GlcNAc β 1-6Man α 1-O-Me (GnMaMe) acceptor (*K*_m 0.71 mM) indicated that the sulphotransferase had a pH optimum of approx. 7.0 and is markedly stimulated by Mn²⁺ ions (maximum approx. 15 mM) and Triton X-100 (0.05–0.1 %). Hydrazine/nitrous acid/NaBH₄ treatment of the ³⁵S-labelled product yielded radiolabelled 2,5-anhydromannitol(6-SO₄). The sulphated GnMaMe product of the GlcNAc-6-O-sulphotrans-

ferase could be galactosylated by a rat liver Golgi enzyme that was shown to have the same properties as the UDP-Gal:GlcNAc β -1,4-galactosyltransferase from bovine milk. Competition studies performed with GlcNAc and GlcNAc-6-SO₄ furthermore indicated that the same liver enzyme acted on both acceptors to produce Gal β 1-4GlcNAc and Gal β 1-4GlcNAc(6-SO₄) with *K*_m values of 1.04 and 1.68 mM respectively. Because the sulphated *N*-acetyl-lactosamine could in turn serve as an acceptor for rat liver sialyltransferase, it seems that this enzyme, together with the Golgi galactosyltransferase and the GlcNAc-6-O-sulphotransferase, could act in concert in assembling the NeuAc α 2-3(6)Gal β 1-4GlcNAc(6-SO₄) branches of complex N-linked oligosaccharides.

INTRODUCTION

It is now generally recognized that the attachment of sulphate groups constitutes a modification that N-linked oligosaccharides in a variety of secreted and cell surface glycoproteins can undergo during their biosynthesis. Although such anionic substituents have been observed in defined positions on the Gal [1–3], GalNAc [3,4] and GlcNAc [1,2,5–8] residues in complex carbohydrate units as well as on Man constituents of some hybrid [9] and polymannose [10] oligosaccharides, only a very limited number of the sulphotransferases involved in bringing about these additions have been identified. These previous investigations have characterized the enzymes responsible for the 3-O-sulphation of Gal [11] and the 4-O-sulphation of GalNAc [12] residues such as occur in terminal locations on the complex oligosaccharides of thyroglobulin [1] and pituitary glycoprotein hormones [4] respectively. Information, however, is not yet available with regard to the enzyme involved in the formation of GlcNAc(6-SO₄) groups that have also been observed in the branches of N-linked carbohydrate units of a number of glycoproteins including thyroglobulin [1,2] and components of HIV envelope [5], pig zona pellucida [6] and various cell surfaces [7,8]. Furthermore the manner of assembly of the sialylated and galactosylated chains in which these sulphated GlcNAc residues are usually found [NeuAc α 2-3(6)Gal β 1-4GlcNAc(6-SO₄)] remains unknown.

The present study, which was performed with rat liver Golgi membranes and exogenous acceptors, has provided insights into the sulphation of these GlcNAc residues as well as subsequent events that could bring them into internal positions in the

oligosaccharide chains. We have identified and characterized an enzyme that transfers sulphate from PAPS to the C-6 position of GlcNAc in β -linkage to mannose. Furthermore we find that Golgi galactosyltransferase can then effectively act on these terminal GlcNAc(6-SO₄) residues to establish a sulphated *N*-acetyl-lactosamine sequence, which in turn can serve as a substrate for rat liver sialyltransferase.

EXPERIMENTAL

Preparation of rat liver membranes

Golgi membranes and also rough and smooth endoplasmic reticulum fractions were prepared from the livers of fasted male rats (220–260 g; CD strain; Taconic, Inc.) as previously described [13] employing the procedures of Tulsiani et al. [14] and Depierre and Dallner [15] respectively; total microsomes were obtained by differential centrifugation [13]. All of the fractions were suspended in 50 mM Tris/acetate buffer, pH 7.0, containing 25 mM potassium acetate; they were stored at –80 °C until used.

Sulphotransferase assays

In the standard assay, rat liver Golgi membranes (40–60 μ g of protein) were incubated with 0.3–0.4 μ Ci [³⁵S]3'-phosphoadenosine 5'-phosphosulphate (PAPS; 1.6–2.4 Ci/mmol; Dupont–New England Nuclear) and 80 nmol of GlcNAc β 1-6Man α 1-O-Me (GnMaMe) for 2 h at 30 °C in 80 μ l of 50 mM Tris/acetate buffer, pH 7.0, containing 100 mM NaF, 2 mM

Abbreviations used: AnManH₂, 2,5-anhydromannitol; GnMaMe, GlcNAc β 1-6Man α 1-O-Me; GnMa, GlcNAc β 1-2Man; PAPS, 3'-phosphoadenosine 5'-phosphosulphate; *R*_{LacH₂}, chromatographic mobility relative to lactitol. All sugars are presumed to be in the D-configuration.

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ATP, 20 mM manganese acetate and 0.1 % (v/v) Triton X-100. The reaction was terminated by the addition of 5 μ l of 100 μ g/ μ l BSA followed by 3 ml of ice-cold 75 % (v/v) ethanol. After vigorous mixing, the samples were centrifuged at 600 *g* for 15 min at 2 °C and the protein pellet was subsequently washed twice with 2 ml of ice-cold 75 % ethanol. The combined supernatants were dried under vacuum at 35 °C, dissolved in 3 ml of 0.2 M NaCl and applied to a column (10 mm $\times$ 3 mm) of charcoal/Celite [Darco G-60/Celite 535, 1:1 (w/w)] [16] from which, after the passage of 10 ml of 0.1 M NaCl and 15 ml of water, the saccharides were eluted with 12 ml of 30 % (v/v) ethanol. Chromatography of the eluted material was performed on cellulose-coated plates in solvent system A (see below) and after detection by fluorography the sulphated saccharide products were quantified by liquid-scintillation counting after being scraped into vials to which 0.5 ml of water followed by 4 ml of scintillation fluid (Monofluor, National Diagnostics) were added.

In instances when the sulphated saccharide product migrated close to the region of PAPS on TLC, the charcoal/Celite-eluted fraction was applied to a column of Dowex 1-X2 (acetate form, 200–400 mesh) and selectively eluted with 0.1 M pyridine acetate, pH 5.0, as previously described [11] before being chromatographed in solvent system B (see below).

For acceptor specificity studies, saccharides were obtained from the following sources: GnMaMe, GlcNAc α 1-O-Me and GlcNAc β 1-O-Me from Sigma; GlcNAc β 1-2Man and (GlcNAc β 1-2Man) $_2\alpha$ 1-3,6Man from Dextra Laboratories; GlcNAc β 1-3Gal β 1-O-Me and GalNAc β 1-3Gal- β 1-O-Me from Pierce Chemical Co.; Gal β 1-4GlcNAc β 1-O-Et from Carbohydrates International; NeuAc2-3Gal β 1-4GlcNAc and NeuAc α 2-6Gal β 1-4GlcNAc from Oxford Glycosystems. GlcNAc β 1-3Gal β 1-4Glc and GalNAc β 1-4Gal β 1-4Glc were prepared by endoglycoceramidase treatment of the lactotriose (Lc $_3$) and gangliotriose (Gg $_3$) glycosphingolipids [17] respectively (gifts from Dr. S. Hakomori, Biomembrane Institute, University of Washington, Seattle, WA, U.S.A.). The source for the non-radiolabelled PAPS for the K_m studies was Sigma.

Galactosyltransferase assays

For specificity studies, rat liver Golgi membranes (30 μ g of protein) were incubated with 1.0 μ Ci of UDP-[1- 3 H]Gal (36.7 Ci/mmol; DuPont–New England Nuclear) and 100 nmol of GlcNAc or monosaccharide acceptors, which were sulphated or phosphorylated for 60 min at 37 °C in 80 μ l of 50 mM Tris/acetate buffer, pH 7.0, containing 2 mM ATP, 20 mM manganese acetate and 0.1 % (v/v) Triton X-100. The reaction was terminated in the same manner as described for the sulphotransferase, and the samples, after being dissolved in 3 ml of water, were applied to charcoal/Celite columns. After an 18 ml water wash followed by 12 ml of 2 % (v/v) ethanol, the galactosylated saccharides were eluted with 12 ml of 20 % (v/v) ethanol and subjected to TLC in solvent system A. After detection by fluorography, the radiolabelled saccharide components were scraped from the plates and quantified by scintillation counting. The saccharides tested as acceptors for the galactosyltransferase included GlcNAc, GlcNAc-6-SO $_4$, GlcNAc-3-SO $_4$, Gal-6-SO $_4$, Glc-6-SO $_4$ and GlcNAc-6-PO $_4$ from Sigma, and Gal-4-SO $_4$, Man-6-SO $_4$ and GalNAc-6-SO $_4$ from Dextra Laboratories.

The assay of the galactosyltransferase for the determination of K_m values and for acceptor competition studies involved a shorter incubation time (20 min), smaller volume (60 μ l) and UDP-[1- 3 H]Gal of lower specific radioactivity (0.85 mCi/mmol). Purified bovine milk β -1,4-galactosyltransferase was purchased from Sigma for comparative experiments.

Preparation of nonradiolabelled sulphated GnMaMe

To obtain GlcNAc(6-SO $_4$) β 1-6Man1-O-Me to serve as a galactosyltransferase acceptor, GnMaMe (80 nmol) was incubated in 90 μ l of the sulphotransferase buffer solution with rat liver Golgi membranes (360 μ g of protein) and a total of 120 nmol of unlabelled PAPS (added in four portions of 30 nmol each) for 8 h at 30 °C. After deproteinization and charcoal/Celite fractionation, the sulphated GnMaMe was applied to a column of Dowex 1-X2 (acetate form, 200–400 mesh) from which it was eluted under conditions (0.1 M pyridine acetate, pH 5.0) that retained the PAPS on the anion exchanger [11].

Sialylation of radiolabelled galactosyltransferase products

Chromatographically purified 3 H-labelled products of Golgi galactosyltransferase action (approx. 3×10^5 d.p.m.) were incubated for 6 h at 37 °C with 6 m-units of rat liver sialyltransferase from Sigma (added in two portions of 3 m-units each) and 180 nmol of non-radiolabelled CMP-NeuAc (added in three portions of 60 nmol each) in 70 μ l of 30 mM Tris/acetate buffer, pH 7.0, containing 0.15 % (v/v) Triton X-100. After deproteinization and charcoal/Celite column fractionation, as described for the sulphotransferase assays, the oligosaccharide products were subjected to TLC in solvent system A.

Chemical procedures

Chromatographically purified [35 S]sulphate-labelled oligosaccharides and glycosides were submitted to hydrazine/nitrous acid/NaBH $_4$ treatment as previously described [1,11] and the radiolabelled fragments were examined by TLC in solvent system C (see below); 3 H-labelled sulphated 2,5-anhydromannitol (AnManH $_2$) standards were prepared in a manner already reported [18]. Radiolabelling of various oligosaccharide standards for chromatography was achieved by reduction with NaB 3 H $_4$ [1]. Desulphation was accomplished at pH 4.0 with a DMSO reagent as previously described [1]. Protein determinations were performed by the procedure of Peterson [19] with BSA as a standard.

TLC

Chromatography was performed on plastic sheets precoated with cellulose 0.1 mm thick (Merck) in pyridine/ethyl acetate/water/acetic acid (5:5:3:1, by vol.) (solvent system A), pyridine/ethyl acetate/water/acetic acid (6:3:3:1, by vol.) (solvent system B), or ethyl acetate/pyridine/tetrahydrofuran/water/acetic acid (50:22:15:15:4, by vol.) (solvent system C). A wick of Whatman no. 3 paper was clamped to the top of the plate during chromatography; components were revealed by fluorography and quantified by scintillation counting after elution with water. For preparative purposes oligosaccharides were solubilized with water and the eluates were extracted with peroxide-free ether to remove scintillants. Radioactive lactitol, which was employed as a migration marker, was prepared by reduction of the parent disaccharide with NaB 3 H $_4$ [1].

Radioactivity measurements

Liquid-scintillation counting was performed in Monofluor scintillant with a Beckman LS 7500 instrument. Components on thin-layer plates were located by fluorography at –80 °C on X-Omatic AR film (Eastman Kodak) after being sprayed with a scintillation mixture containing α -methyl-naphthalene [20].

RESULTS

Assay of sulphotransferase activity towards β -Man-linked GlcNAc acceptors

On incubation of rat liver Golgi membranes with GnMaMe or GlcNAc β 1-2Man (GnMa) and [35 S]PAPS, a radioactive com-

Figure 1 TLC examination of the products from the action of rat liver Golgi sulphotransferase on GnMaMe and GnMa

Incubations with Golgi enzyme (40 μ g of protein) were performed with [35 S]PAPS (0.45 μ Ci) in the presence of acceptor (80 nmol of GnMaMe or GnMa) or its absence (—) at 30 °C as described in the Experimental section. After deproteinization and fractionation on charcoal/Celite columns, equal aliquots of the samples were applied to a cellulose-coated plate for chromatography in solvent system A for 32 h, after which the components were revealed by fluorography. The positions to which standard lactitol (arrow) and PAPS migrated are indicated.

Figure 2 TLC of the Dowex 1-purified products of the rat liver Golgi sulphotransferase

After incubations of Golgi enzyme in the presence of acceptor [80 nmol of GnMaMe or 40 nmol of (GlcNAc β 1-2Man) $_2$ α 1-3,6Man (Penta)] or its absence (—) under the conditions described in Figure 1, the deproteinized charcoal/Celite eluates were further fractionated on Dowex 1-X2 (acetate) columns to remove the PAPS as described in the Experimental section. The 100 mM pyridine acetate, pH 5.0, eluates from these columns were subjected to TLC on a cellulose-coated plate for 26 h in solvent system B and the components were detected by fluorography. The migration of standard lactitol is indicated by the arrow.

Figure 3 Effect of time on the transfer of sulphate from [35 S]PAPS to GnMaMe by rat liver Golgi enzyme

Incubations with Golgi enzyme (40 μ g of protein) were performed with 0.3 μ Ci of [35 S]PAPS and 80 nmol of acceptor as described in the Experimental section and the product was quantified by liquid-scintillation counting after charcoal/Celite fractionation and TLC (see Figure 1). Values obtained in the absence of exogenous acceptor are plotted as open symbols.

ponent was evident on TLC that migrated with a chromatographic mobility relative to lactitol (R_{LacH_2}) of 1.22 and 0.95 respectively and was not observed in an incubation without acceptor (Figure 1). Although in the standard assay the sulphated saccharide product was clearly resolved from [35 S]PAPS (Figure 1), an additional Dowex 1 chromatographic step was employed to remove the latter component when larger oligosaccharide acceptors were tested (Figure 2).

Properties of GlcNAc-sulphotransferase

Enzymic activity towards GnMaMe increased with time for more than 3 h at 30 °C and no detectable activity was evident in the absence of the exogenous acceptor (Figure 3). Moreover,

Figure 4 Effect of pH on the activity of rat liver Golgi sulphotransferase towards GnMaMe

Incubations were performed at the indicated pH values under the standard assay conditions in sodium acetate (■), sodium Mes (▲), and Tris/acetate (●) buffers.

Figure 5 Effect of divalent cations on the transfer of sulphate from [^{35}S]PAPS to GnMaMe by rat liver Golgi enzyme

Incubations were performed under standard assay conditions at various concentrations of Mn^{2+} and Mg^{2+} as well as at 20 mM Ca^{2+} . The effect of EDTA (10 mM) was assessed after a 30 min incubation at 2 °C before the addition of [^{35}S]PAPS.

under the conditions of the standard assay the sulphotransferase activity increased in a linear manner in the range 10–80 μg of protein (results not shown).

The enzyme showed activity between pH 6.2 and 8.0 with a quite pronounced maximum close to pH 7.0 (Figure 4). Sulphation of GnMaMe was stimulated by manganese in the range 1–50 mM, reaching an optimum at approx. 15 mM (Figure 5). Magnesium was substantially less effective than manganese in promoting sulphotransferase activity, and calcium was essentially ineffective; moreover the presence of EDTA abolished measurable activity (Figure 5). The inclusion of Triton X-100 in the assays substantially stimulated the sulphate transfer with a maximum in the range 0.05–0.1 % (v/v) (Figure 6).

Kinetic measurements of sulphotransferase activity with increasing concentrations of GnMaMe acceptor or PAPS permitted the calculation from Lineweaver–Burk plots of K_m values of 0.71 mM and 6.7 μM respectively (Figure 7).

Figure 6 Effect of Triton X-100 on rat liver Golgi sulphate transfer to GnMaMe

Incubations at various concentrations of the detergent were performed by the standard assay.

Figure 7 Effect of acceptor and PAPS concentrations on rat liver Golgi GlcNAc-6-O-sulphotransferase activity

Golgi enzyme was incubated with various concentrations of GnMaMe (upper panel) or [^{35}S]PAPS (lower panel) under standard assay conditions. The insets show the Lineweaver–Burk plots of the data.

As previously observed for the Gal-3-sulphotransferase [11], both ATP and NaF had to be included in the incubations to prevent the degradation of PAPS by sulphohydrolases and phosphohydrolases. In the absence of either ATP (2 mM) or NaF (100 mM), sulphotransferase activity decreased to 16% and 53% respectively of that observed in the complete system; when both reagents were excluded from the assay essentially no transfer of sulphate to GnMaMe was observed. On storage of the rat liver Golgi membranes (2 mg/ml protein) at -80 °C in the Tris/acetate buffer, pH 7.0, containing 25 mM potassium acetate, over 90% of the sulphotransferase activity was preserved for at least 18 months.

Substrate specificity of sulphotransferase

From an examination of a number of acceptors it became apparent that the rat liver Golgi sulphotransferase acted optimally on saccharides terminating in GlcNAc in β -glycosidic linkage to a Man residue (Table 1). It is possible that the presence of a hydrophobic methyl group in GnMaMe promoted its acceptor activity over that of GnMa, although we could not determine with the available saccharide substrates whether the enzyme acts preferentially on positional isomers of the GlcNAc-Man sequence. Clearly the β -anomeric configuration of the GlcNAc plays a role in the enzyme's specificity as the α -methyl glycoside, in contrast with the β -form, had no acceptor activity. Pertinently, GlcNAc β 1-3Gal sequences that are known to occur in sulphated poly-*N*-acetyl-lactosamine oligosaccharides

Table 1 Specificity of rat liver Golgi GlcNAc-6-O-sulphotransferase toward various saccharide acceptors

Incubations were performed with rat liver Golgi membrane (60 µg of protein) and [³⁵S]PAPS (0.3 µCi) for 2 h at 30 °C with the indicated acceptor (80 nmol; 1 mM) under the conditions described in the Experimental section. The products were determined by liquid-scintillation counting after column and thin-layer chromatographic purifications. The values are expressed relative to GlcNAcβ1-6Manα1-O-Me, into which 25850 d.p.m. was incorporated during the period of the assay. Evaluation of (GlcNAcβ1-2Man)₂α1-3,6Man was performed at 0.5 mM (40 nmol) and the relative activity was normalized to a 1.0 mM concentration. The relative activity of Galβ1-4GlcNAcβ1-O-Et was 7 but hydrazine/nitrous acid/NaBH₄ treatment of the product [1] indicated the sulphate was present exclusively as Gal-3-SO₄.

Acceptor	Relative activity
GlcNAcβ1-6Manα1-O-Me	100
GlcNAcβ1-2Man	46
GlcNAcβ1-3Galβ1-O-Me	4
GlcNAcα1-O-Me	0
GlcNAcβ1-O-Me	26
(GlcNAcβ1-2Man) ₂ α1-3,6Man	44
GlcNAcβ1-3Galβ1-4Glc	0
NeuAcα2-3Galβ1-4GlcNAc	2
NeuAcα2-6Galβ1-4GlcNAc	0
NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc	0
GalNAcβ1-3Galα1-O-Me	0
GalNAcβ1-4Galβ1-4Glc	0
Galβ1-4GlcNAcβ1-O-Et	0

Figure 8 Identification of the radiolabelled fragment formed by hydrazine/nitrous acid/NaBH₄ treatment of [³⁵S]sulphated GnMaMe

The chromatographically purified product of rat liver Golgi sulphate transfer to GnMaMe was treated by the hydrazine/nitrous acid/NaBH₄ procedure as described in the Experimental section and subsequently chromatographed on a cellulose-coated plate in solvent system C together with a standard (STD) of ³H-labelled AnManH₂(6-SO₄). The components were revealed by fluorography; migration of standard lactitol is indicated by the arrow.

[6,21,22] did not serve as acceptors; neither did oligosaccharides terminating in GalNAc (Table 1). The Galβ1-4GlcNAcβ1-O-Et acceptor did incorporate a modest amount of sulphate radioactivity but upon hydrazine/nitrous acid/NaBH₄ fragmentation of this product it became evident that this was a function of Gal-3-sulphotransferase, which has previously been shown to have substantial affinity for this disaccharide [11]. Although GlcNAc-

Table 2 Subcellular distribution of rat liver GlcNAc-6-O-sulphotransferase

Fractions were prepared as described in the Experimental section. For specific activities, assays were performed with GnMaMe as described in the Experimental section; the results are the radioactivity incorporated into this acceptor per mg of protein during the period of the assay. Relative activities are the specific activities relative to a microsome value of 1.0.

Fraction	10 ³ × Specific activity (d.p.m./mg)	Relative activity
Golgi	519	47.2
Smooth endoplasmic reticulum	16	1.5
Rough endoplasmic reticulum	4	0.4
Microsomes	11	1.0

6-SO₄ is generally found in sialyl-N-acetyl-lactosamine sequences in N-linked carbohydrate units [1,2,5,8], oligosaccharides that contained this constellation of sugars were found to be inactive as acceptors (Table 1).

Characterization of sulphotransferase product

Treatment of the chromatographically purified ³⁵S-sulphated GnMaMe product by the hydrazine/nitrous acid/NaBH₄ procedure yielded a radiolabelled component that migrated to the same position (*R*_{LacH₂} 3.50) on TLC as an AnManH₂(6-SO₄) standard (Figure 8). This indicates that the sulphate group is located on C-6 of the GlcNAc in the GnMaMe; a C-3 or C-4 position of the sulphate would have yielded a product, AnManH₂(3-SO₄) or AnManH₂(4-SO₄) (indistinguishable owing to the symmetry of the anhydromannitol) that migrated in solvent system C with an *R*_{LacH₂} of 5.06. Treatment of the ³⁵S-sulphated GlcNAcβ-O-Me product of the sulphotransferase also yielded AnManH₂(6-SO₄) on examination by TLC (results not shown).

Subcellular localization of GlcNAc-6-O-sulphotransferase

Although our studies were performed with Golgi membranes on the basis that enzymes involved in the later stages of N-linked oligosaccharide processing are situated in this cellular compartment [23,24], we nevertheless assayed the sulphotransferase activity of several other subcellular fractions. These determinations did indeed demonstrate that the Golgi membranes are substantially enriched with the GlcNAc-6-O-sulphotransferase (Table 2), as we had previously noted for the Gal-3-O-sulphotransferase [11].

Galactosylation of GlcNAc-6-SO₄ by rat liver Golgi transferase

Because GlcNAc-6-SO₄ generally occurs as a component of an N-acetyl-lactosamine sequence, we explored the possibility that this sulphated sugar could serve as an acceptor for a Golgi galactosyltransferase. Indeed, we observed that on incubation of Golgi membranes with GlcNAc-6-SO₄ and UDP-[³H]Gal, a radiolabelled component was formed that migrated on TLC (*R*_{LacH₂} 1.12) in solvent system A more slowly than Galβ1-4GlcNAc (Figure 9), which was the expected product from the well-known β-1,4-galactosyltransferase [25–27]. The radiolabelled product formed by the action of the Golgi galactosyltransferase on the sulphated GlcNAc acceptor is believed to be Galβ1-4GlcNAc(6-SO₄) as it would bind to Dowex 1 (acetate), from which it could be eluted with 0.2 M pyridine acetate, pH

Figure 9 Demonstration by TLC that rat liver Golgi enzyme can galactosylate GlcNAc-6-SO₄ in a manner comparable to GlcNAc

Rat liver Golgi membranes (30 μ g of protein) were incubated with UDP-[³H]Gal (1.0 μ Ci) in the presence of 100 nmol of GlcNAc (Gn) or GlcNAc-6-SO₄ [Gn(6S)] or in the absence of acceptor (—) for 60 min at 37 °C as described in the Experimental section. Subsequently, equal aliquots of the deproteinized and charcoal/Celite-fractionated samples were chromatographed on a cellulose-coated plate in solvent system A for 32 h and the components were revealed by fluorography. The positions to which standard Gal β 1-4GlcNAc (GaGn) and lactitol (arrow) migrated are indicated.

5.0, and was converted to the neutral Gal β 1-4GlcNAc after treatment with a DMSO desulphation procedure (results not shown).

Characterization of rat liver Golgi UDP-Gal:GlcNAc-6-SO₄ transferase

To test the specificity of the transfer of galactose to GlcNAc-6-SO₄ a number of other acidic monosaccharides including GlcNAc-3-SO₄, GlcNAc-6-PO₄, Glc-6-SO₄, Man-6-SO₄, Gal-6-SO₄ and GalNAc-6-SO₄ were incubated with the Golgi membranes but none of these sugars served as acceptors. In view of these findings, the possibility that the galactosylation of GlcNAc and GlcNAc-6-SO₄ was catalysed by the same enzyme was evaluated. Comparative kinetic studies performed with GlcNAc-6-SO₄ and GlcNAc acceptors revealed similar K_m values (1.68 and 1.04 mM respectively), although the V_{max} values were quite different (1.5×10^5 d.p.m./20 min and 17.1×10^5 d.p.m./20 min respectively).

The likelihood that the galactosylation of GlcNAc-6-SO₄ was brought about by UDP-Gal:GlcNAc β -1,4-galactosyltransferase was supported by the finding that the purified enzyme from milk effectively acted on GlcNAc in its sulphated as well as its unsubstituted form in a manner comparable to the rat liver Golgi membranes (Figure 10); wide separation of the two products was achieved by TLC in solvent system B (Figure 10) and moreover the NaBH₄-reduced transfer product from GlcNAc-6-SO₄ was found to migrate to the same position as a chemically synthesized Gal β 1-4GlcNAc(6-SO₄) standard (made available by Dr. K. L. Matta, Roswell Park Cancer Institute, Buffalo, NY, U.S.A.) radiolabelled by NaB³H₄ reduction.

Further evidence favouring the contention that the UDP-Gal:GlcNAc β -1,4-galactosyltransferase was involved in the galactosylation of both substrates was obtained by competition

Figure 10 Comparison of the actions of rat liver Golgi and bovine milk galactosyltransferases on GlcNAc and GlcNAc-6-SO₄ acceptors

The purified enzyme from bovine milk (Milk, 0.05 unit) or rat liver Golgi membranes (Liver, 30 μ g of protein) were incubated with UDP-[³H]Gal (1.2 μ Ci) in the presence of GlcNAc (Gn) or GlcNAc-6-SO₄ [Gn(6S)] under the conditions described in Figure 9. After NaBH₄ reduction of the products, TLC was performed on equal aliquots in solvent system B for 32 h. Detection of the components was achieved by fluorography; the migration of NaB³H₄-reduced standard Gal β 1-4GlcNAc (GaGn) and Glc β 1-4GlcNAc(6-SO₄) [GaGn(6S)] as well as lactitol (arrow) are indicated.

experiments, which clearly indicated that GlcNAc and GlcNAc-6-SO₄ were mutually inhibitory (Figure 11).

Formation of Gal β 1-4GlcNAc(6-SO₄)/ β 1-6Man-O-Me by rat liver Golgi enzymes

To determine whether the rat liver galactosyltransferase could participate in the biosynthesis of sulphated *N*-acetyl-lactosamine linked to a mannose residue, a GlcNAc(6-SO₄)/ β 1-6Man-O-Me

Figure 11 Competition between GlcNAc and GlcNAc-6-SO₄ for rat liver Golgi galactosyltransferase

Rat liver Golgi enzyme (60 μ g of protein) was incubated with either 5 mM GlcNAc(6S) (left panel) or 0.05 mM GlcNAc (right panel) together with increasing concentrations of GlcNAc or GlcNAc(6S) respectively, as well as 1.9 nmol of UDP-[³H]Gal (1.68 μ Ci) for 20 min at 37 °C. After deproteinization and fractionation on a charcoal/Celite column, the samples were subjected to TLC in solvent system A (see Figure 9) and the radioactivity incorporated into Gal β 1-4GlcNAc(6-SO₄) [Gal-GlcNAc(6S)] and Gal β 1-4GlcNAc (Gal-GlcNAc) was determined by liquid-scintillation counting after elution of the components from the plates. The results are expressed relative to the activity in the product in the absence of competitor [1.0 represents 176 600 d.p.m. and 154 030 d.p.m. for Gal-GlcNAc(6S) and Gal-GlcNAc respectively] as well as the activity in the competitor at its lowest concentration, which was 174 040 d.p.m. for Gal-GlcNAc (0.1 mM GlcNAc, left panel) and 16 420 d.p.m. for Gal-GlcNAc(6S) [0.06 mM GlcNAc(6S), right panel].

Figure 12 Demonstration that rat liver Golgi GlcNAc-6-O-sulphotransferase and galactosyltransferase can function in concert to synthesize an oligosaccharide with a Gal β 1-4GlcNAc(6-SO₄) β 1-6Man1-O-Me sequence

Rat liver Golgi membranes (60 μ g of protein) were incubated with UDP-[³H]Gal (8.0 μ Ci) for 3 h at 37 °C (Galtrans) with a GnMaMe acceptor (120 nmoles) that either had (+) or had not (–) undergone prior sulphonylation (Sulfotrans) as described in the Experimental section. After deproteinization and fractionation by charcoal/Celite, the galactosyltransferase incubations were applied to a Dowex 1-X2 (acetate form) column to yield the neutral galactosylated non-presulphated oligosaccharide (Gal β 1-4GlcNAc β 1-6Man1-O-Me) and the acidic galactosylated presulphated oligosaccharide (Gal β 1-4GlcNAc(6-SO₄) β 1-6Man1-O-Me) in the 5 mM pyridine acetate, pH 5.0, wash and the 0.2 M pyridine acetate, pH 5.0, eluate respectively. TLC of an aliquot of these fractions was performed in solvent system A for 40 h and the radioactive components were revealed by fluorography; the migration of standard lactitol is indicated by the arrow.

acceptor was prepared with the Golgi sulphotransferase and non-radiolabelled PAPS. Subsequent incubation of this sulphated component with the Golgi membranes and UDP-[³H]Gal resulted in the formation of an anionic radiolabelled product that migrated on TLC in solvent system A to a position (R_{LacH2} 0.77) consistent with the methyl glycoside of the sulphated trisaccharide, Gal β 1-4GlcNAc(6-SO₄) β 1-6Man-O-Me (Figure 12). This finding contrasted with a neutral product resulting from the action of the galactosyltransferase on the unsulphated GnMaMe acceptor, which demonstrated a faster chromatographic mobility (R_{LacH2} 1.13) (Figure 12).

Sialylation of the Gal β 1-4GlcNAc(6-SO₄) sequence

To determine whether the sulphated *N*-acetyl-lactosamine sequence could be modified by sialyltransferase, we incubated [³H]Gal β 1-4GlcNAc(6-SO₄) with the enzyme from rat liver and non-radiolabelled CMP-NeuAc. A product was formed by this interaction that migrated on TLC in solvent system A at a substantially slower rate (R_{LacH2} 0.18) than the acceptor (Figure 13). Incubation of radiolabelled non-sulphated *N*-acetyl-lactosamine with the sialyltransferase, in contrast, yielded a component that migrated to the position of a NeuAc α 2-6Gal β 1-4GlcNAc standard (R_{LacH2} 0.33) as expected from the enzyme preparation used (Figure 13), which is reported to have predominantly (approx. 99 %) 2,6-sialyltransferase activity. These observations indicate that a NeuAc α 2-6Gal β 1-4GlcNAc(6-SO₄) sequence was established by the sialyltransferase action.

Figure 13 Sialylation of rat liver Golgi neutral and sulphated disaccharide products lead to the formation of NeuAc α 2-6Gal β 1-4GlcNAc and NeuAc α 2-6Gal β 1-4GlcNAc(6SO₄) sequences respectively

Approx. 3×10^5 d.p.m. of chromatographically purified [³H]Gal β 1-4GlcNAc (GaGn) and [³H]Gal β 1-4GlcNAc(6-SO₄) [GaGn(6S)] were incubated with (+) or without (–) rat liver sialyltransferase (Sialtrans) (6 m-units; Sigma) and 180 nmol of non-radiolabelled CMP-NeuAc in a 70 μ l total volume of 30 mM Tris/acetate buffer, pH 7.0, containing 0.15% (v/v) Triton X-100 for 6 h at 37 °C. After deproteinization and fractionation on a charcoal/Celite column as described in the Experimental section, equal aliquots of the samples were applied to a cellulose-coated plate and chromatographed in solvent system A for 39 h; the components were then detected by fluorography. Comparison with standards indicated that the sialylated product of GaGn (Sialtrans+) migrated to the same position as NeuAc α 2-6Gal β 1-4GlcNAc; in this solvent system NeuAc α 2-3Gal β 1-4GlcNAc migrates 1.6-fold faster than the α 2-6 isomer.

DISCUSSION

The investigations reported here indicate that rat liver Golgi membranes contain an enzyme that has the capacity to transfer sulphate from PAPS to C-6 of terminal GlcNAc residues in β -linkage to mannose. The substrate specificity of this sulphotransferase suggests that it participates in the assembly of the sialyl-*N*-acetyl-lactosamine branches of N-linked carbohydrate units in which GlcNAc-6-SO₄ residues have been observed in a number of glycoproteins [1,2,5,8].

Although GlcNAc-6-SO₄ is usually situated internally in N-linked oligosaccharides, in contrast with the previously described Gal-3-SO₄ [1] and GalNAc-4-SO₄ [4] capping groups, it nevertheless seems from our studies that the sulphonylation occurs on terminal GlcNAc residues with the subsequent addition of Gal and NeuAc constituents to produce the NeuAc α 2-3(6) β 1-4GlcNAc(6-SO₄) sequence. This contention is supported by the observation that GlcNAc in either *N*-acetyl-lactosamine or sialyl-*N*-acetyl-lactosamine groupings did not serve as a sulphate acceptor and furthermore that rat liver Golgi galactosyltransferase and sialyltransferase could effectively act on GlcNAc-6-SO₄-containing substrates.

Our acceptor competition studies indicated that the galactosyltransferase which functioned on free or Man-linked GlcNAc-6-SO₄ was the well-characterized UDP-Gal:GlcNAc β -1,4-galactosyltransferase [25–27]. Indeed, the purified enzyme from bovine milk was found to galactosylate both GlcNAc and GlcNAc-6-SO₄ in a manner similar to that from the rat liver Golgi membranes. Although a number of studies have shown that the acceptor specificity of the β -1,4-galactosyltransferase is quite broad [28,29], and indeed neutral synthetic 6-O-substituted

derivatives of GlcNAc have been shown to be acted on by this enzyme [30], the galactosylation of the anionic GlcNAc-6-SO₄ was quite surprising. Our assays did not find acceptor activity in any other acidic monosaccharide esters tested, including GlcNAc-6-PO₄.

The liver sialyltransferase and galactosyltransferase, together with the GlcNAc-6-O-sulphotransferase, would have the potential for a concerted action in assembling the NeuAc α 2-3(6)Gal β 1-4GlcNAc(6-SO₄) branches of complex N-linked oligosaccharides. Although it is believed that the sialylation and galactosylation steps take place in a *trans*-Golgi compartment [23,24], the localization within this network of the sulphotransferase, the action of which must precede that of the other two enzymes, needs to be determined. In addition, future studies will be directed towards evaluating the physiological function of the GlcNAc-6-O-sulphotransferase *in vivo*.

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REFERENCES

- 1 Spiro, R. G. and Bhoyroo, V. D. (1988) *J. Biol. Chem.* **263**, 14351–14358
- 2 DeWaard, P., Koorevaar, A., Kamerling, J. P. and Vliegthart, J. F. G. (1991) *J. Biol. Chem.* **266**, 4237–4243
- 3 Hård, K., Van Zadelhoff, G., Moonen, P., Kamerling, J. P. and Vliegthart, J. F. G. (1992) *Eur. J. Biochem.* **209**, 895–915
- 4 Green, E. D., Boime, I. and Baenziger, J. U. (1986) *Mol. Cell. Biochem.* **72**, 81–100
- 5 Shilatfard, A., Merkle, R. K., Helland, D. E., Welles, J. L., Haseltine, W. A. and Cummings, R. D. (1993) *J. Virol.* **67**, 943–952
- 6 Noguchi, S. and Nakano, M. (1992) *Eur. J. Biochem.* **209**, 883–894
- 7 Edge, A. S. B. and Spiro, R. G. (1984) *J. Biol. Chem.* **259**, 4710–4713
- 8 Roux, L., Holojda, S., Sundblad, G., Freeze, H. H. and Varki, A. (1988) *J. Biol. Chem.* **263**, 8879–8889
- 9 Yamashita, K., Ueda, I. and Kobata, A. (1983) *J. Biol. Chem.* **258**, 14144–14147
- 10 Freeze, H. H. and Wolgast, D. (1986) *J. Biol. Chem.* **261**, 127–134
- 11 Kato, Y. and Spiro, R. G. (1989) *J. Biol. Chem.* **264**, 3364–3371
- 12 Skeleton, T. P., Hooper, L. V., Srivastava, V., Hindsgaul, O. and Baenziger, J. U. (1991) *J. Biol. Chem.* **266**, 17142–17150
- 13 Lubas, W. A. and Spiro, R. G. (1987) *J. Biol. Chem.* **262**, 3775–3781
- 14 Tulsiani, D. R. P., Opheim, D. J. and Touster, O. (1977) *J. Biol. Chem.* **252**, 3227–3233
- 15 Depierre, J. and Dallner, G. (1976) in *Biochemical Analysis of Membranes* (Maddy, A. H., ed.), pp. 79–131, John Wiley and Sons, New York
- 16 Spiro, R. G. (1966) *Methods Enzymol.* **8**, 26–52
- 17 Lavery, S. B., Holmes, E. H., Harris, D. D. and Hakomori, S. (1992) *Biochemistry* **31**, 1069–1080
- 18 Edge, A. S. B. and Spiro, R. G. (1985) *Arch. Biochem. Biophys.* **240**, 560–572
- 19 Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- 20 Spiro, M. J. and Spiro, R. G. (1985) *J. Biol. Chem.* **260**, 5808–5818
- 21 Hokke, C. H., Damm, J. B. L., Kamerling, J. B. and Vliegthart, J. F. G. (1993) *FEBS Lett.* **329**, 29–34
- 22 Fukushima, K., Okhura, T., Kanai, M., Kuroki, M., Matsuoka, Y., Kobata, A. and Yamashita, K. (1995) *Glycobiology* **5**, 105–115
- 23 Melman, I. and Simons, K. (1992) *Cell* **68**, 829–840
- 24 Sampath, D., Varki, A. and Freeze, H. H. (1992) *J. Biol. Chem.* **267**, 4440–4455
- 25 Brew, K., Vanaman, T. C. and Hill, R. L. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **59**, 491–497
- 26 Spiro, M. J. and Spiro, R. G. (1968) *J. Biol. Chem.* **243**, 6529–6537
- 27 Schanbacher, F. L. and Ebner, K. E. (1970) *J. Biol. Chem.* **245**, 5057–5061
- 28 Ichikawa, Y., Look, G. C. and Wong, C.-H. (1992) *Anal. Biochem.* **202**, 215–238
- 29 Palcic, M. M. and Hindsgaul, O., (1996) *Trends Glycosci. Glycotech.* **8**, 37–49
- 30 Palcic, M. M., Srivastava, O. P. and Hindsgaul, O. (1987) *Carbohydr. Res.* **159**, 315–324