N-glycosylation is required for full enzymic activity of the murine galactosylceramide sulphotransferase

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3-*O*-Sulphogalactosylceramide (sulphatide) is a major lipid component of myelin membranes, and is required for proper myelin formation. Sulphatide is synthesized in the Golgi apparatus by galactosylceramide sulphotransferase (CST; EC 2.8.2.11). Murine and human CSTs contain two putative N-glycosylation sites (Asn-66 and Asn-312). The second site is conserved among all galactose 3-O-sulphotransferases cloned to date. In order to study the functional relevance of N-glycosylation, we generated epitope-tagged CST and soluble Protein A–CST fusion proteins lacking both N-glycosylation sites, separately or in combination. Our results show that both sites are glycosylated when CST is expressed in Chinese hamster ovary (CHO) or COS cells. Moreover, transfecting CST mutants lacking both N-glycosylation sites, or only Asn-312, reduced significantly the amount of sulphatide synthesized, whereas substituting Asn-66 with a glutamine residue did not. In contrast, activity *in vitro* was reduced by approx. 50 % in the Asn-66 → Gln (N66Q) mutant, and was almost undetectable in N312Q and N66/312Q transfectants. Furthermore, soluble Protein A–CST expressed in the presence of tunicamycin was almost inactive, and accumulated in transfected cells. Expression of fully active CST in a CHO-glycosylation mutant lacking N-acetylglucosaminyltransferase I demonstrated that condensation of the N-linked pentamannosyl-core structure is sufficient to form a fully active enzyme.

Key words: cerebroside, glycolipid, sulphatide.

INTRODUCTION

Sulphated glycolipids are found in high concentrations in the nervous system, kidney and other vertebrate tissues. The most abundant sulphated glycolipids are the glycosphingolipid 3-*O*-sulphogalactosyl ceramide (sulphatide) and the glycerolipid 3-*O*-sulphogalactosyl-1-alkyl-2-acyl-glycerol (seminolipid). In the brain and the peripheral nervous system, sulphatide is a major component of the myelin membrane of oligodendrocytes and Schwann cells. Seminolipid [1] is present in small amounts in the brain, but is the major glycolipid synthesized by spermatocytes and is essential for normal spermatogenesis [2, 3]. Sulphatide and seminolipid are degraded in the lysosomes by arylsulphatase A [4,5]. Storage of sulphatide as a result of arylsulphatase A deficiency in metachromatic leukodystrophy causes degeneration of oligodendrocytes, leading to a progressive demyelination and a spectrum of neurological symptoms [6].

The formation of sulphatide and seminolipid by the transfer of sulphate from adenosine 3′-phosphate 5′-phosphosulphate (PAPS) to galactocerebroside and galactosylalkylacylglycerol respectively is catalysed by the same enzyme, galactosylceramide sulphotransferase (CST), a Golgi type II membrane protein [7,8]. CST cDNAs have been cloned from a human renal cancer cell line [8] and from the mouse kidney [9]. The gene is highly expressed in kidney, brain and testis, in accordance with the high level of CST activity in these tissues, suggesting that sulphatide production is mainly regulated at the transcriptional level [9]. Targeted disruption of the CST gene in mice causes deficiency in sulphatide and seminolipid, resulting in disorganized myelin at the paranodal junction of the node of Ranvier, and in abnormalities in spermatogenesis [3].

In addition to the galactolipid-specific CST, three distinct sulphotransferases transferring sulphate to the 3-hydroxy group of galactose in glycoproteins have been cloned recently [10–13]. They all show significant sequence similarity throughout their catalytic domains. In contrast, sequence similarity among the galactose 3-O-sulphotransferases (Gal-3STs) and other Golgi-apparatus sulphotransferases, e.g. HNK-1 sulphotransferase [14], is restricted to two small, N-terminal motifs that represent the PAPS-binding site [15].

All sulphotransferases of the Golgi apparatus contain putative N-glycosylation sites, although in most cases it is unknown which of these sites are glycosylated. Although the functional relevance of the N-glycosylation of sulphotransferases has not been addressed so far, several reports have shown clearly that glycosylation is important for the activity of Golgi-resident glycosyltransferases [16–23]. It is well established that glycosylation is important for quality control and protein folding in the endoplasmic reticulum (ER) [24]. In addition, glycosylation has been found to be required for the correct subcellular targeting of some proteins, for the stabilization of enzymes, or to be directly involved in forming an active enzyme (see [25] for further references).

Human and murine CSTs contain two putative N-glycosylation sites (at amino acid positions 66 and 312) [8,9]. We show in the present study that both potential N-glycosylation sites of CST are glycosylated when expressed in Chinese hamster ovary (CHO) or COS cells. Furthermore, using site-directed

Abbreviations used: BCIP, 5-bromo-4-chloroindol-3-yl phosphate; CHO, Chinese hamster ovary; CST, galactosylceramide sulphotransferase; ER, endoplasmic reticulum; GalC, galactocerebroside; GalT, UDP-galactose:ceramide galactosyltransferase; Gal-3ST, galactose 3-O-sulphotransferase; HA, haemagglutinin; HPTLC, high-performance TLC; NBT, Nitro Blue Tetrazolium; N66Q, etc., site-directed mutant bearing an Asn-66 → Gln substitution, etc.; PAPS, adenosine 3′-phosphate 5′-phosphosulphate; TRITC, tetramethylrhodamine β-isothiocyanate.

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mutagenesis and inhibition of glycosylation by tunicamycin, we found that N-glycosylation of Asn-312, and to a lesser extent Asn-66, is required for the full expression of catalytic activity.

EXPERIMENTAL

Materials

All cell-culture media and reagents were obtained from Invitrogen. Fetal calf serum was obtained from Biochrom (Berlin, Germany). Galactocerebroside (GalC) and sulphatide were purchased from Sigma. ^{35}S-labelled PAPS (52.9 GBq/mmol) was obtained from PerkinElmer or NEN Life Science Products. Alternatively, [^{35}S]PAPS was synthesized from [^{35}S]sulphate and ATP, as described previously [26]. L-[U-^{14}C]serine (5.5 GBq/mmol) that was labelled with ^{14}C at all positions was obtained from Amersham Biosciences. High-performance TLC (HPTLC) silica-gel 60 plates were purchased from Merck. PNGase F was purchased from New England Biolabs. Pre-stained molecular-size markers were purchased from Biorad. Antibody SulphI [27] against sulphatide was kindly given by Dr P. Fredman (University of Göteborg, Sweden). Antiserum against α-mannosidase II [28] was provided by Dr K. Moremen (University of Georgia, GA, U.S.A.). Anti-haemagglutinin (HA) antibody 12CA5 was purchased from Roche. Secondary antibodies were obtained from the following companies: anti-(mouse Ig)–horseradish peroxidase was from Amersham Biosciences; anti-(mouse Ig)–FITC was from Dianova; anti-(rabbit Ig)–horseradish peroxidase and anti-(rabbit Ig)–tetramethylrhodamine β-isothiocyanate (TRITC) were from Jackson Immunoresearch; and anti-(mouse Ig)–alkaline phosphatase was from Dako. The plasmid pPROTA [29] was kindly given by Dr R. Breathnach (INSERM U463, Institute of Biology, Nantes, France). CHO cells stably transfected with rat UDP-galactose:ceramide galactosyltransferase (GalT; EC 2.4.1.45) were kindly provided by Dr B. Popko (University of North Carolina, NC, U.S.A.). A subclone of this line, CHO-GalT-A6, showing a more stable expression of galactocerebroside, was subsequently isolated by limiting dilution and used for all experiments. CHO-15B cells were provided by Dr F. Wieland (University of Heidelberg, Heidelberg, Germany).

DNA constructs and site-directed mutagenesis

Murine CST was cloned from oligo(dT)-primed murine cDNA (from postnatal day 18) by PCR, using the oligonucleotides CST1 (5′-ATGACTCTGCTGCCAAAGAAGC-3′) and CST2 (5′-CCACCTTAGAAAGTCCCTAAGG-3′) deduced from the published cDNA sequence [9]. The PCR product was ligated into pCR4-TOPO (Invitrogen), followed by subcloning into the *Eco*RI site of pcDNA3.1-zeo (Invitrogen), resulting in the plasmid pcDNA3.1-zeo-CST. In addition, CST was subcloned into expression vector pTRE-HA, a derivative of the pTRE plasmid (Clontech). pTRE-HA was generated by ligation of the HA adapter (composed of the following phosphorylated oligonucleotides: 5′-GATCTGCCACCATGGATATCTACCCTTATGACGTCCCCGATTACGCCAGCCG-3′ and 5′-GATCCGGCTGGCGTAATCGGGGACGTCATAAGGGTAGATATCCATGGTGGCA-3′) between the two *Bam*HI sites of the pTREplasmid. To generate pTRE-CSTHA, CST cDNA was amplified byPCR using oligonucleotides CST5 (5′-CGAATTCACCATGACTCTGCTGCCAAAG-3′) and CST6 (5′-CTGCCCCATGGCCCACCTTAGAAAGTCCC-3′) introducing *Eco*RI and *Nco*I sites (shown underlined). The PCR product was digested with *Eco*RI and *Nco*I and ligated into the *Eco*RI and *Nco*I sites of pTRE-HA, resulting in plasmid pTRE-CSTHA. This plasmid encodes CST,with a C-terminal sequence of AMDIYPYDVPDYASRIQT (the HA epitope is underlined). To generate plasmid pPROTA-CST, driving the expression of soluble Protein A–CST fusion protein, CST cDNA encoding amino acids 38 to 423 was amplified by PCR using the oligonucleotides CST3 (5′-GCGAATTCTCCCAACATGGCCTTCACG-3′) and CST4 (5′-GCGAATTCACCACCTTAGAAAGTCCC-3′), which introduced *Eco*RI sites (shown underlined). The PCR product was digested with *Eco*RI, and ligated into the *Eco*RI site of pPROTA [29].

Site-directed mutagenesis of putative N-glycosylation sites was performed by PCR, as described recently [30]. The following oligonucleotides were used to substitute asparagine residues at positions 66 and 312 with glutamines [giving rise to the site-directed mutants Asn-66 → Gln and Asn-312 → Gln (N66Q and N312Q respectively]: N66Qa (5′-TCCCGCCCAGGGCTCAGCAG-3′), N66Qb (5′-CTGAGCCCTGGGCGGGAGTG-3′), N312Qa (5′-GTCACTTCCAGGCCAGCTTC-3′) and N312Qb (5′-GAAGCTGGCCTGGAAGTGAC-3′). All sequences were confirmed by DNA sequencing using the BigDye terminator cycle sequencing kit and an ABI PRISM 310 analyser (PerkinElmer Applied Biosystems).

Cell culture and transfections

CHO and COS-7 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1) and Dulbecco's modified Eagle's medium alone, respectively, supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

Cells (3×10^5/well) were seeded in six-well plates 24 h before transfection. Plasmid DNA (2 μg) was mixed with 6.6 μl of ExGen 500 transfection reagent (MBI Fermentas, Munich, Germany) and added to the cells, following the manufacturer's instructions. pTRE-CSTHA plasmid and the corresponding N-glycosylation mutants or the empty vector pTRE-HA were co-transfected with equal amounts of plasmid pTet-off (Clontech). To generate stably transfected CHO-GalT cells expressing CST (CHO-Sulph), cells were transfected with pcDNA3.1-zeo-CST, and stable transfected cells were selected with 400 μg/ml zeocin. Transiently transfected cells or cell-culture supernatants were analysed 40 to 48 h after transfection. Cells from a 35 mm well were lysed in 50 μl of 20 mM Tris/HCl, pH 8.0 containing 1% (v/v) Triton X-100, 50 mM NaCl, 2 mM EDTA, 1 mM PMSF and 50 μg/ml aprotinin. Lysates were cleared by centrifugation (15000 ***g*** for 10 min), and were either used immediately for sulphotransferase assay or stored at −20 °C. Protein concentrations were determined using the BioRad D_C protein assay.

CST assay

CST assay was performed as described by Jungalwala et al. [31], with minor modifications. Briefly, 5 μl of GalC (4 mg/ml) and 10 μl of 1% (v/v) Triton X-100 in chloroform/methanol (2:1, v/v) were dried under a vacuum. A 40 μl aliquot of 100 mM Tris/HCl, pH 7.0, containing 2.5 mM ATP, 20 mM $MgCl_2$, 2 μM PAPS (approx. 450 MBq/mmol [^{35}S]PAPS) and 10 μl of cell lysate (50–100 μg of protein) or culture supernatant was added, and the reaction mixture was incubated for 2 h at 37 °C. Reactions were stopped by adding 1 ml of chloroform/methanol (2:1, v/v), followed by 200 μl of 150 mM NaCl. The mixture was centrifuged, and the organic phase was washed once with methanol/150 mM NaCl/chloroform (48:47:3, by vol.) and then dried under a vacuum. Dried lipids were dissolved in 20 μl of chloroform/methanol (2:1, v/v) and applied on to HPTLC

silica-gel 60 plates, followed by separation in chloroform/methanol/water (65:25:4, by vol.). After drying the plates, radioactive products were visualized using a phosphorimager or by autoradiography.

SDS/PAGE and Western blot analysis

SDS/PAGE ([10 % (w/v) polyacrylamide gels] and Western blotting on to nitrocellulose membranes were performed as described previously [30]. Proteins were detected with monoclonal antibody 12CA5 directed against HA-tag or normal rabbit Igs (to stain for Protein A-fusion proteins), and the appropriate horseradish-peroxidase-labelled secondary antibodies. Bound antibodies were visualized using West Pico chemiluminescence reagent (Pierce).

TLC

Lipids were extracted from cells with chloroform/methanol/water (8:4:3, by vol.) and separated on HPTLC plates in chloroform/methanol/water (65:25:4, by vol.). Glycolipids were visualized using orcinol staining [32]. In order to detect sulphatide by immunostaining, HPTLC plates were treated with Plexigum P28 and dried overnight at 37 °C. Non-specific binding sites were blocked by incubation with 2 % (w/v) BSA/0.05 % (v/v) Tween 20 in PBS. Immunostaining was accomplished by sequential incubations with anti-sulphatide antibody SulphI (1.7 μg/ml) and alkaline phosphatase-conjugated anti-mouse Ig in blocking solution, as described by Bethke et al. [33]. Bound alkaline phosphatase was visualized using 100 μg/ml 5-bromo-4-chloroindol-3-yl phosphate (BCIP) in 200 mM Tris/HCl, pH 8.5.

Immunofluorescence and immunocytochemistry

CHO-GalT cells were seeded on to glass coverslips and transfected as described above. Cells were washed twice with PBS and fixed in 2 % (w/v) paraformaldehyde in PBS for 10 min. After incubating with 50 mM NH_4Cl in PBS for 20 min, non-specific binding sites were blocked with 1 % (w/v) BSA in PBS. To detect intracellular antigens, cells were permeabilized using 0.1 % (v/v) Triton X-100. Cells were stained with anti-sulphatide monoclonal antibody SulphI [27], anti-HA monoclonal antibody 12CA5 and α-mannosidase II antiserum [28], followed by anti-mouse Ig–TRITC and anti-mouse Ig–FITC/anti-rabbit Ig–TRITC respectively. All antibodies were diluted in 1 % BSA in PBS. For the immunocytochemical detection of sulphatide, cells were treated in the same way, except that anti-(mouse Ig)–alkaline phosphatase was used as a secondary antibody. Bound alkaline phosphatase was detected using 165 μg/ml BCIP and 330 μg/ml Nitro Blue Tetrazolium (NBT) in 100 mM Tris/HCl, pH 9.5, containing 100 mM NaCl, 5 mM $MgCl_2$ and 1 mM levamisol.

Deglycosylation of protein

Cell lysates from transiently transfected COS-7 cells were boiled for 10 min in the presence of 0.5 % (w/v) SDS and 1 % 2-mercaptoethanol to denature proteins. After adding 1 % Nonidet P40, 400 μg of protein was incubated with 500 units of PNGase F at 37 °C for 2 h. Treated and untreated samples were analysed by Western blotting with antibody 12CA5, as described above.

Tunicamycin treatment

CHO-GalT cells were transfected with pPROTA-CST as described above. At 4 h after transfection, the medium was replaced with normal medium containing tunicamycin (10 μg/ml). Cells were cultivated for a further 44 h, and culture supernatants were then harvested and cells were lysed as described above. Supernatants and lysates were stored at −20 °C and subsequently used for Western blotting and sulphotransferase assay.

Metabolic labelling of cells

At 1 day after transfection, transiently transfected CHO cells in six-well plates were incubated with 55 kBq [^{14}C]serine/well for 16 h. Lipids were isolated by chloroform/methanol (2:1, v/v) extraction and resolved by HPTLC, as described above. Radioactivity was visualized using a PhosphorImager.

RESULTS

N-glycosylation of murine CST

Murine CST was cloned by PCR using primers designed according to the sequence published previously [9] and expressed either in its native form or as a fusion protein with a C-terminal HA-tag (CST-HA) to allow immunodetection of the enzyme. After stable transfection of CHO-GalT cells, which stably express GalT, sulphatide could be identified by indirect immunofluorescence with the sulphatide-specific antibody SulphI (Figure 1A). Mock-transfected CHO-GalT cells were sulphatide-negative (Figure 1B). Synthesis of sulphatide was confirmed by TLC, orcinol staining and immunodetection (Figures 1C and 1D).

Two N-glycosylation sites are present in human and murine CST at amino acid positions 66 and 312 [8, 9]. In order to prove N-glycosylation of CST, COS-7 cells were transiently transfected with the plasmid encoding CST-HA. Cell lysates from transfected cells were treated with PNGase F to remove all asparagine-linked glycans. Treated and untreated samples were analysed by Western blotting with anti HA-tag antibody 12CA5 (Figure 2A). Untreated CST-HA migrated with an apparent molecular mass of 54 kDa. After PNGase F treatment, the molecular mass was decreased to 49 kDa, which is in good agreement with the molecular mass predicted from the amino acid sequence. The molecular-mass shift of 5 kDa suggests the presence of two N-glycans of 23 kDa each. To confirm this result and to investigate further the functional consequences of N-glycan removal, we changed Asn-66 and Asn-312 to glutamine residues, both separately and together in combination. The three resulting mutants (N66Q, N312Q, N66/312Q) and wild-type CST were transiently expressed in CHO-GalT cells and analysed by Western blotting with an anti HA-tag antibody (Figure 2B). The apparent molecular masses of N66Q and N312Q mutants were reduced by approx. 3 kDa, whereas the double mutant N66/312Q migrated at 49 kDa. These results confirmed that both putative N-glycosylation sites were actually glycosylated.

In vivo activity and subcellular localization of wild-type and mutant CSTs

In order to investigate whether the removal of N-glycans influences the activity of CST, CHO-GalT cells were transiently transfected with wild-type and mutant (N66Q, N312Q, N66/312Q) CST as described above and immunostained for sulphatide using SulphI antibody (Figure 3). All constructs were found to be active in this assay (Figures 3A–3D), whereas untransfected CHO-GalT cells were devoid of sulphatide (Figure 3E). The number of sulphatide-positive cells transfected with the N312Q and N66/312Q constructs (Figures 3C and 3D), however, was reduced by approx. 50 % compared with wild-type CST and mutant N66Q (Figures 3A and 3B). The reduction of sulphatide synthesis in transfectants expressing mutant CST was even more

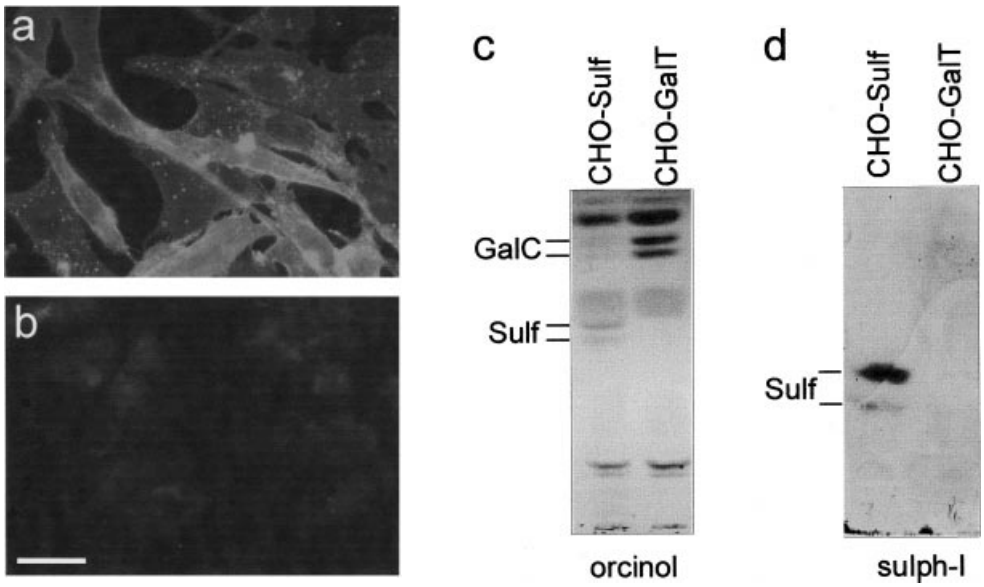

Figure 1 CHO-GalT cells expressing CST synthesize sulphatide

CHO-GalT cells stably transfected with CST (CHO-Sulf) (**a**) or untransfected CHO-GalT (**b**) cells were stained with anti-sulphatide antibody SulphI. The bar in (**b**) represents 20 μm. (**c**) Lipids isolated from CHO-GalT or CHO-Sulf cells were separated on HPTLC plates. Glycolipids were visualized by orcinol staining. (**d**) Sulphatide was detected by immunostaining with antibody SulphI. The positions of sulphatide (Sulf) and GalC standards are indicated on the left of the gels in (**c**) and (**d**).

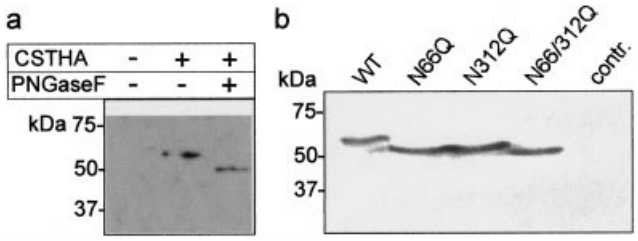

Figure 2 N-Glycosylation of CST at Asp-66 and Asp-312

(**a**) COS-7 cells were transiently transfected with CST-HA. Cell lysates from transfected cells or untransfected controls were incubated with (+) or without (−) PNGase F and analysed for HA-tagged CST with 10% (w/v) SDS/PAGE (40 μg of protein per lane) and Western blotting. (**b**) Lysates from CHO-GalT cells transiently transfected with wild-type CST-HA (wt), mutants N66Q, N312Q, N66/312Q or empty vector (contr.) were analysed for HA-tagged CST by Western blotting using antibody 12CA5.

pronounced when the rate of sulphatide synthesis was determined by metabolic labelling of sphingolipids using [^{14}C]serine (Figure 3F). Compared with wild-type CST transfectants, sulphatide synthesis was 90% (N66Q), 30% (N312Q) and 10% (N66/312Q) of the control value respectively.

Since it has been reported that removal of N-glycosylation sites from glycosyltransferases can interfere with subcellular targeting [17,18], we determined the subcellular localization of wild-type CST and the mutants by indirect immunofluorescence. Following transient transfection, CHO-GalT cells were fixed with paraformaldehyde, permeabilized with Triton X-100 and stained for the HA-tag. Simultaneously, cells were stained for the Golgi marker α-mannosidase II. Wild-type CST-HA co-localized with α-mannosidase II (Figure 4). There was also, however, immunoreactivity in ER-like structures. The staining pattern was similar when the mutants N312Q and N66/312Q were analysed. Thus it seems that removal of the N-glycans did not influence localization of the enzyme. Because of the considerable immunoreactivity in ER-like structures, however, we cannot rule out the possibility that a higher proportion of the N-glycosylation mutants than of the wild-type CST is retained in the ER.

Expression of functional soluble CST as a fusion protein with Protein A

In order to facilitate CST activity assays *in vitro*, we tested whether CST can be expressed as a soluble protein that can be secreted by CHO cells. With this aim in mind, we subcloned CST lacking the N-terminal 37 amino acids (encoding the transmembrane domain) into the pPROTA expression vector [29]. As shown in Figure 5(A), CHO-GalT cells transfected with this construct secreted a Protein A-fusion protein with an apparent molecular mass of approx. 100 kDa. TLC of lipids isolated from the transfected cells revealed that sulphatide was being synthesized (Figure 5B). Furthermore, culture supernatants from pPROTA-CST-transfected cells exhibited CST activity, whereas it was undetectable in supernatants from cells transfected with the empty vector pPROTA (Figure 5C). Thus CST can be expressed in a soluble form that is active both *in vivo* and *in vitro*.

In vitro activity of CST N-glycosylation mutants

The three N-glycosylation mutants described above were subcloned into the pPROTA vector and transiently expressed in CHO-GalT cells. Expression of the fusion proteins was monitored by Western blotting of cell lysates and culture supernatants (Figure 6A). No significant difference between wild-type and mutant CST was observed in terms of the amount of fusion protein synthesized. Furthermore, all mutants were secreted efficiently. We estimated that, 2 days after transfection, approximately half of the proteins had been secreted while the other half remained inside the cells.

CST activity was determined in culture supernatants and in cell lysates from transfected cells (Figure 6B and Table 1). Unexpectedly, activity was almost undetectable in culture super-

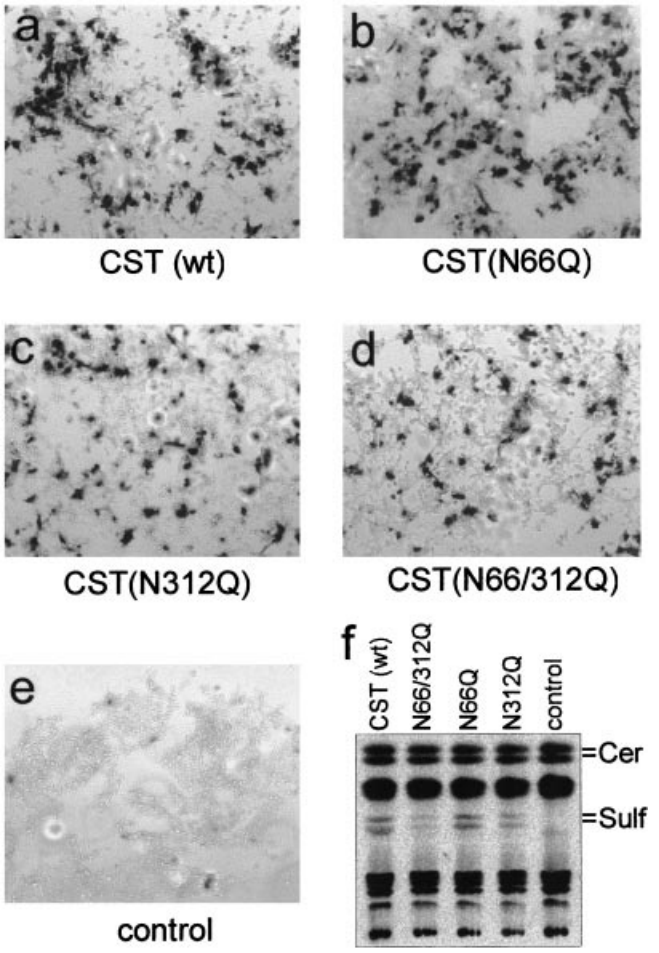

Figure 3 Sulphatide synthesis in CHO cells expressing CST N-glycosylation mutants

Plasmids coding for wild-type CST (wt) (**a**) or CST N-glycosylation mutants [N66Q (**b**), N312Q (**c**) or N66/312Q (**d**)] were transiently expressed in CHO-GalT cells. Sulphatide synthesis of the CST constructs was determined by their ability to induce sulphatide synthesis. Cells transfected with the empty vector served as negative control (**e**). Cells were fixed in 2% (w/v) paraformaldehyde 2 days after transfection and sulphatide was visualized immunocytochemically with antibody SulphI. Bound primary antibody was revealed using anti-(mouse-Ig)–alkaline-phosphatase and NBT/BCIP as substrates. (**f**) CHO-GalT cells transiently expressing wild-type CST (wt), mutants N66Q, N312Q, N66/312Q, or mock-transfected controls were metabolically labelled with [^{14}C]serine, and one-half of the lipids isolated from one 35-mm culture dish was separated by TLC and visualized using a PhosphorImager. Cer, cerebrosides; Sulf, sulphatide.

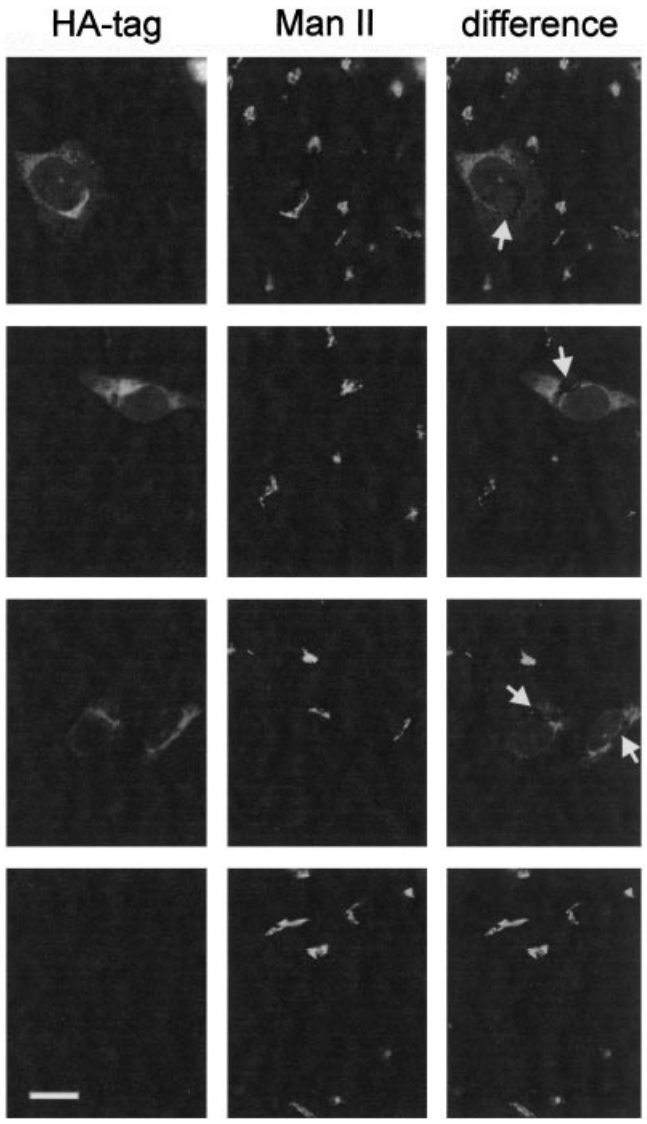

Figure 4 Subcellular localization of HA-tagged wild-type CST and N312Q mutant in transiently transfected CHO cells

CHO-GalT cells were transiently transfected with wild-type CST-HA (top three panels), mutants N312Q or N66/312Q (upper middle and lower middle three panels respectively), or the empty vector pTRE-HA (as a control; bottom three panels). Permeabilized cells were stained for HA-tagged CST with 12CA5/anti-(mouse Ig)–FITC (HA-tag) and rabbit anti-(α-mannosidase II)/TRITC-conjugated anti-(rabbit Ig) (Man II). The difference between FITC and TRITC signals is shown to indicate co-localization (white arrows). The bar represents 20 μm.

natants from N312Q and N66/312Q transfectants (1% for N312Q and < 1% for N66/312Q). When cell lysates were analysed, activity was found to be reduced by 94% (N312Q) and 98% (N66/312Q) respectively compared with the wild-type enzyme. Activity of the N66Q mutant was less perturbed, reaching 40% of the activity of the wild-type enzyme in the culture supernatant, and approx. 80% when analysed in cell lysates.

To ensure that the low level of activity observed with the N312Q and N66/312Q mutants was not due to the absence of the membrane domain or the presence of Protein A, we measured CST activity in lysates from CHO cells transfected with the membrane-bound HA-tagged wild-type and mutant CSTs. Again, activity was significantly decreased in N66Q and was almost undetectable in N312Q and N66/312Q transfectants (Table 1).

Expression of CST in the presence of tunicamycin

In order to confirm using an independent approach that the removal of the N-glycosylation sites was responsible for the impaired activity of the N312Q and N66/312Q mutants, we tested the activity of wild-type Protein A–CST expressed in the presence or absence of tunicamycin, which is known to inhibit the transfer of *N*-acetylglucosamine 1-phosphate (GlcNAc1P) from UDP-GlcNAc to dolichol phosphate to form dolichyl-diphosphate-GlcNAc. When cells expressing Protein A–CST were treated with 10 μg/ml tunicamycin, the apparent molecular mass was decreased by approx. 10 kDa, suggesting that the Protein A moiety was also N-glycosylated (there are four

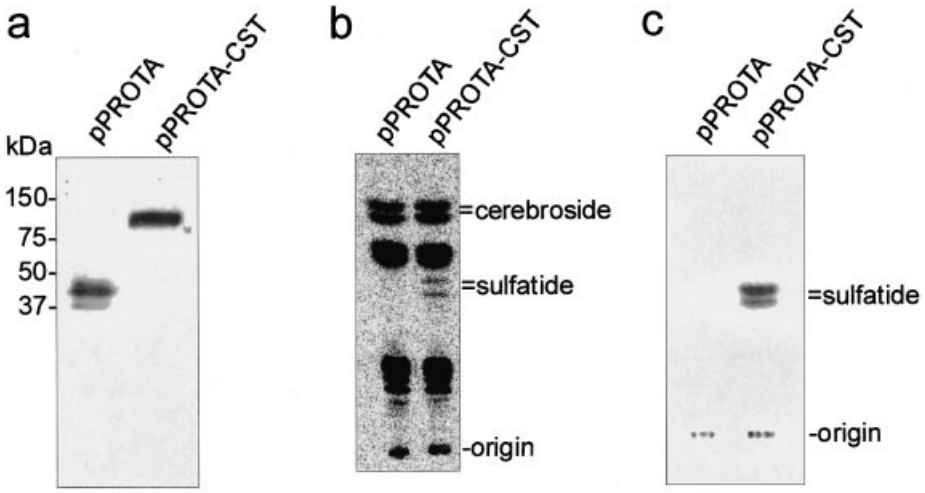

Figure 5 Expression of a catalytically active soluble Protein A–CST fusion protein

(**a**) CHO-GalT cells were transiently transfected with pPROTA or pPROTA-CST, and culture supernatants were analysed by Western blotting 48 h later using rabbit Ig and anti-(rabbit-Ig)–horseradish peroxidase. (**b**) Lipids isolated from [^{14}C]serine-labelled CHO-GalT cells transiently transfected with pPROTA or pPROTA-CST were separated on HPTLC plates. Radioactivity was detected using a PhosphorImager. (**c**) Aliquots of culture supernatants (10 μl) were incubated with [^{35}S]PAPS and galactocerebroside in the standard CST assay, as described in the Materials and methods section. Reaction products were analysed by HPTLC, followed by autoradiography on X-ray film.

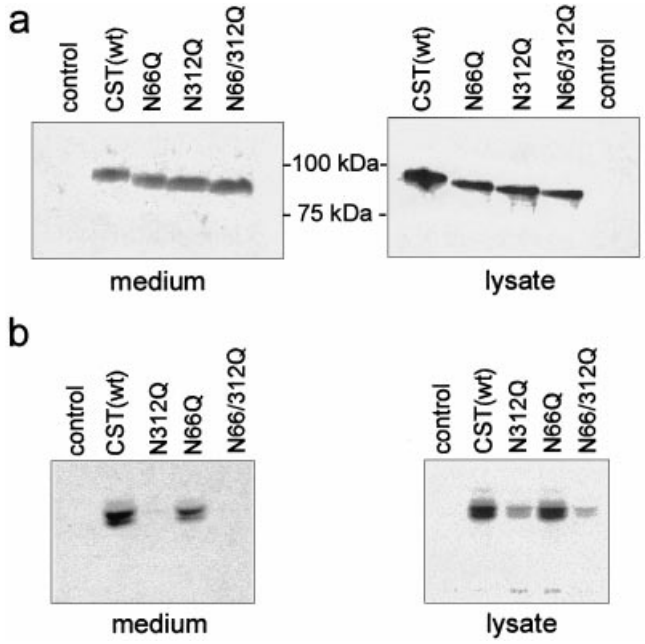

Figure 6 *In vitro* activity of Protein A–CST fusion protein glycosylation mutants

CHO-GalT cells in 35 mm wells were transiently transfected with wild-type pPROTA-CST (wt) or the corresponding glycosylation mutants (N66Q, N312Q or N66/312Q). Mock-transfected cells served as a control. At 48 h after transfection, culture supernatants (total volume of 1 ml) were collected, and cells were lysed with 1% Triton X-100 in a final volume of 50 μl. (**a**) Aliquots (10 μl) of culture supernatants (medium) or 0.5 μl of cell lysates (lysates) were subjected to Western blot analysis with rabbit IgG and anti-(rabbit Ig)–horseradish peroxidase antibodies, followed by chemiluminescence detection. (**b**) Aliquots (10 μl) of culture supernatants (medium) or 10 μl of cell lysates were incubated with [^{35}S]PAPS and galactocerebroside in the standard CST assay, as described in the Materials and methods section. Reaction products were resolved by HPTLC and detected using a PhosphorImager. Quantification was performed using phosphorimager software, and is shown in Table 1.

potential N-glycosylation motifs in the Protein A sequence). Treatment with tunicamycin lowered significantly the amount of Protein A–CST in the medium, whereas there was a marked increase in the intracellular concentration of the fusion protein (Figure 7A). Thus tunicamycin inhibits secretion of the Protein A–CST fusion protein. Furthermore, activity could be detected neither in the medium nor in cell lysates from tunicamycin-treated cells (Figure 7B). Because tunicamycin blocks efficient synthesis of the CST acceptor substrate galactocerebroside by inhibiting the UDP-galactose transporter [34], it was not possible to detect *in vivo* activity of CST in tunicamycin-treated cells.

Table 1 Catalytic activity of CST glycosylation mutants

Assays were performed with culture supernatant (10 μl) or cell lysates (50–100 μg, matched to give equal amounts of the CST proteins, as determined by Western blotting) as described in the Materials and methods section. Reaction products were separated by TLC and visualized by phosphorimaging, as shown in Figure 6. Data shown with * are the means for at least three independent experiments; those shown with † are the means for two independent experiments. –, not performed; wt, wild-type.

		Relative activity (%)	
Cell line	Construct transfected	Medium	Cell lysate
CHO-GalT	Protein A–CST(wt)	100*	100*
CHO-GalT	Protein A–N66Q	40*	78*
CHO-GalT	Protein A–N312Q	1*	6*
CHO-GalT	Protein A–N66/312Q	< 1*	2*
CHO-15B	Protein A–CST(wt)	113†	–
CHO-GalT	CST-HA(wt)	–	100†
CHO-GalT	N66Q-HA	–	45†
CHO-GalT	N312Q-HA	–	2†
CHO-GalT	N66/312Q-HA	–	<1†

N-glycans of the complex type are not necessary to form a fully active CST

In order to obtain a further insight into the structural requirements of the N-glycans required to form a fully active enzyme, we expressed Protein A–CST in a CHO-glycosylation mutant (15B) lacking a functional *N*-acetylglucosaminyl transferase I gene [35]. Owing to this defect, CHO-15B cells can only synthesize

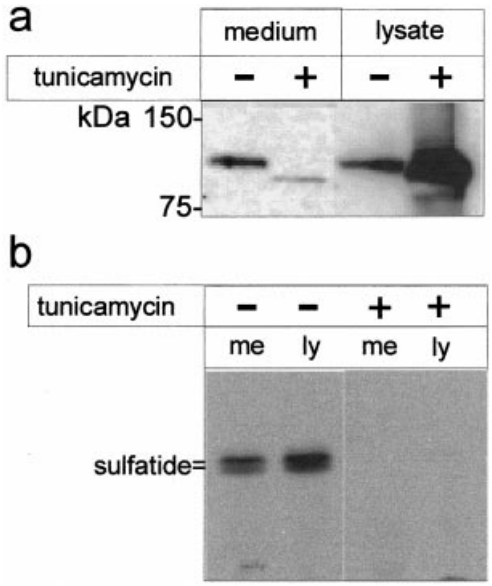

Figure 7 Expression of Protein-A–CST in the presence of tunicamycin

pPROTA-CST was transiently expressed in CHO-GalT. The transfection was stopped after 4 h, and medium with or without 10 μg/ml tunicamycin was added. After 48 h, Protein A–CST expression in the culture supernatant (medium) and in cell lysates was monitored by Western blotting (**a**). CST activity in the medium (me) and in cell lysates (ly) was determined as described above (**b**).

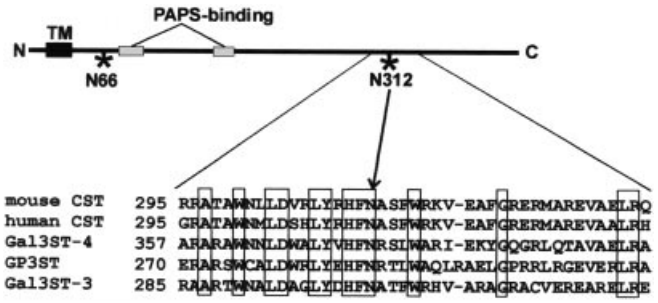

Figure 8 Schematic representation of CST and partial sequence alignment of sulphotransferases transferring sulphate to the 3-hydroxy group of galactose

Asterisks indicate the two N-glycosylation sites of CST (Asn-66 and Asn-312). The conserved motifs involved in PAPS binding are indicated by grey-shaded boxes. The sequences shown are for mouse CST [9], human CST [8], human GP3ST [10], human Gal3ST-3 [11,12] and human Gal3ST-4 [13]. Amino acids identical in all sequences are boxed.

N-glycans with terminal mannose residues. CST expressed in CHO-15B cells was fully active (Table 1). Thus condensation of the high mannose-core structure and the subsequent modification by mannosidases and glucosidases are sufficient to form a fully active CST.

DISCUSSION

Several Golgi-associated carbohydrate-specific sulphotransferases have been cloned in the last 10 years ([36], references therein). CST is a member of a group of at least four sulphotransferases that catalyse the transfer of sulphate to the 3-hydroxy group of galactose (Gal-3STs) in glycolipids or glycoproteins. All of them have potential N-glycosylation sites within their catalytic domains. Which of these sites are actually modified, however, and the nature of their functional role has not been addressed so far. We show in this study that the two potential N-glycosylation sites that are present in the murine CST become modified when expressed in mammalian cell lines. Moreover, our data show that N-glycosylation of Asn-312 and, to a lesser extent, Asn-66, is necessary to form a fully active CST. CST lacking both N-glycosylation sites was even less active than mutant N312Q, suggesting that the effects of the mutations were additive. N-glycosylation has been found to be important for the activity of several glycosyltransferases [16–21]. Some of them require extensive processing of the core structure, like addition of terminal GlcNAc [37] or galactose residues [38] to reach 100% activity. In order to generate a fully active CST, condensation of the core structure, and possibly also processing by glucosidases and mannosidases, is sufficient, because the CST expressed in N-acetylglucosaminyltransferase I-deficient CHO cells is as active as the enzyme expressed in wild-type CHO cells.

N-glycosylation of CST might be required directly for its activity. Accordingly, a partial sequence alignment (Figure 8) shows that the N-glycosylation site at position 312 of CST is conserved among all Gal-3STs. Moreover, the amino acid sequences surrounding this site exhibit a relatively high degree of similarity. Because hardly any of these amino acid residues are conserved in other sulphotransferases, it is reasonable to assume that this part of the enzyme is involved in the binding of the galactose residue of the acceptor substrate. The N312Q mutation might change the affinity for the acceptor substrate galactocerebroside.

Alternatively, instead of directly affecting the catalytic activity, the removal of the N-glycans could alter the activity of CST by increasing its susceptibility to degradation or aggregation. Glycosylation of Asn-66 and Asn-312, however, seems not to be important in preventing degradation, because the expression levels of wild-type and glycosylation mutants did not differ significantly. Furthermore, because membrane-bound mutant CSTs still localize to the Golgi apparatus and soluble forms of these mutants are efficiently secreted, it is unlikely that they are strongly misfolded immediately after synthesis.

Removing all N-glycans by tunicamycin treatment strongly reduced the amount of Protein A–CST fusion protein that was secreted, suggesting that N-glycans are important for efficient secretion. The observation that Protein A–CST(N66/312Q) was secreted efficiently could be explained by the presence of N-glycans attached to the Protein A moiety of the fusion protein. Inhibition of secretion by tunicamycin or removal of N-glycosylation sites has been described for other glycoproteins [39,40]. Treatment with tunicamycin might cause a very rapid destabilization/aggregation of the Protein A fusion protein, thus preventing secretion and leading to the observed intracellular accumulation of the enzyme. The presence of N-glycans in the Protein A moiety of the N66/312Q fusion protein could delay aggregation, and thus allow efficient secretion. This assumption would explain why the relative activity of Protein A–CST(N66/312Q) was significantly higher in cell lysates compared with wild-type Protein A–CST synthesized in the presence of tunicamycin, although the intracellular amount of the latter was strongly increased.

One important finding of the present study is that CST can be expressed in a soluble form. This will facilitate large-scale production of CST for detailed structural analysis. Moreover, this observation suggests that the enzyme does not recognize the lipid part of the cerebroside acceptor molecule, which is hidden

in the lipid bilayer. Thus it should be possible to identify water-soluble substrates for CST, which would facilitate kinetic studies and the development of specific inhibitors of CST. Such inhibitors are promising reagents for the treatment of metachromatic leukodystrophy by substrate-deprivation therapy, as has been shown for other lysosomal-storage disorders [41].

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