

Localization of human heparan glucosaminyl N-deacetylase/N-sulphotransferase to the trans-Golgi network

Donald E. HUMPHRIES*§, Brandon M. SULLIVAN†¶, M. Deize ALEIXO† and Jennifer L. STOW†‡||

*Department of Veterans Affairs Medical Center, Department of Medicine, Harvard Medical School, Boston, MA 02130, U.S.A., †Renal Unit, Massachusetts General Hospital, Boston, MA 02130, U.S.A., ‡Department of Pathology, Harvard Medical School, Boston, MA 02130, U.S.A., and ¶Centre for Molecular and Cellular Biology, University of Queensland, Brisbane 4072, Queensland, Australia

In order to determine the intracellular location of heparan N-deacetylase/N-sulphotransferase, cDNAs encoding human heparan glucosaminyl N-deacetylase/N-sulphotransferase were cloned from human umbilical vein endothelial cells. The deduced amino acid sequence was identical to that of the human heparan N-sulphotransferase cloned previously [Dixon, Loftus, Gladwin, Scambler, Wasmuth and Dixon (1995) *Genomics* **26**, 239–244]. RNA blot analysis indicated that two heparan N-sulphotransferase transcripts of approx. 8.5 and 4 kb were produced in all tissues. Expression was most abundant in heart, liver and

pancreas. A cDNA encoding a Flag-tagged human heparan N-sulphotransferase (where Flag is an epitope with the sequence DYKDDDDK) was transfected into mouse LTA cells. Immunofluorescence detection using anti-Flag monoclonal antibodies demonstrated that the enzyme was localized to the trans-Golgi network. A truncated Flag-tagged heparan N-sulphotransferase was also retained in the Golgi, indicating that, as for many other Golgi enzymes, the N-terminal region of heparan N-sulphotransferase is sufficient for retention in the Golgi apparatus.

INTRODUCTION

Heparin and heparan sulphate glycosaminoglycans are initially synthesized in the Golgi apparatus as polymers of alternating D-glucuronic acid and N-acetylglucosamine residues [1,2]. N-Deacetylation/N-sulphation of the heparan chains then initiates a series of epimerization and O-sulphation reactions that are necessary for the production of heparin and heparan sulphate. While the modification reactions involved in the synthesis of heparin and heparan sulphate are essentially the same and are well defined, the mechanisms that control the degree of sulphation, leading to differential production of heparin and heparan sulphate, are not. The initial N-deacetylation/N-sulphation reaction is catalysed by enzymes that contain both N-deacetylase and N-sulphotransferase activities [3–5].

The heparan N-deacetylase/N-sulphotransferases are resident Golgi enzymes. Two heparan N-deacetylase/N-sulphotransferase enzymes have been purified and cloned. The first enzyme (referred to as heparan N-sulphotransferase-1) was cloned from rat liver [6]. The second heparan N-sulphotransferase (referred to as heparan N-sulphotransferase-2) was cloned from a heparin-producing mouse mastocytoma cell line and is thought to be important for the synthesis of heparin [7,8]. The amino acid sequences of the two enzymes are 70% identical, but differences have been reported in their ratio of N-deacetylase/N-sulphotransferase activities [7] and their co-factor requirements [8].

Newly synthesized proteoglycans passing through the Golgi stacks are modified sequentially by a series of highly specific glycosyltransferases, which synthesize the linkage region and heparan polymer, and modification enzymes such as the epimerases and sulphotransferases. The precise localization of these enzymes within the membranes of the Golgi subcompartments is critical for ensuring the systematic assembly of heparin/heparan sulphate chains. The subcompartment(s) in

which the two heparan N-deacetylase/N-sulphotransferases are localized are not known. Although biochemical and histochemical studies have suggested that sulphation occurs as a late Golgi event [9–12], more recent studies have suggested that heparan N-sulphotransferase may be localized in a proximal Golgi subcompartment [13–15].

The mechanisms for the targeting and retention of enzymes in different positions within the Golgi complex are not fully understood. Localization of mutagenized or chimaeric membrane proteins such as a 2,6-sialyltransferase [16], β -1,4-galactosyltransferase [17,18] and N-acetylglucosaminyltransferase I [19] have revealed that sequences in and around the transmembrane domain are important in directing and retaining the proteins in the Golgi complex. Targeting signals in heparan N-deacetylase/N-sulphotransferase have not yet been analysed.

In the present study, we have cloned and expressed a cDNA encoding an epitope-tagged human heparan N-sulphotransferase-1. Data are presented on the localization of heparan N-sulphotransferase-1 in the trans-Golgi network (TGN) and on the localization of a truncated epitope-tagged N-terminal peptide of heparan N-sulphotransferase-1 containing the putative Golgi targeting signal.

EXPERIMENTAL

Cell culture and transfection

Human umbilical vein endothelial cells (HUVEC), attachment factors and medium were obtained from Cell Systems (Kirkland, WA, U.S.A.). Mouse fibroblast LTA cells [20,21] were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 units/ml streptomycin. Pig kidney-tubule-derived LLC-PK₁ cells were grown in Dulbecco's modified Eagle's medium with high glucose

Abbreviations used: HUVEC, human umbilical vein endothelial cells; TGN, trans-Golgi network; Flag, epitope with the sequence DYKDDDDK; N-sulphotransferase-1₁₆₉, peptide comprising the first 169 amino acids of heparan N-sulphotransferase; β -COP, β -subunit of the coatamer complex.

§ To whom correspondence should be addressed: Department of Veterans Affairs Medical Center, Research 151, 150 S. Huntington Ave., Boston, MA 02130, U.S.A.

|| Present address: Centre for Molecular and Cellular Biology, University of Queensland, Brisbane 4072, Queensland, Australia

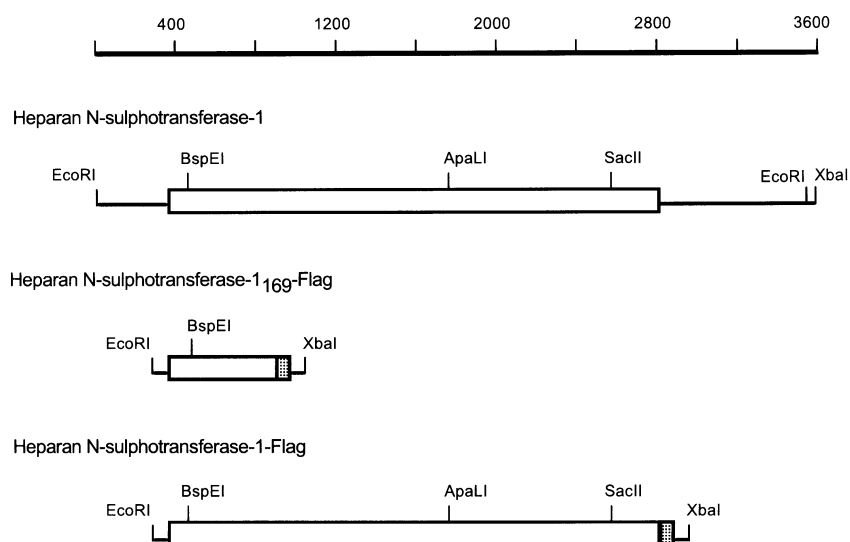


Figure 1 Schematic representation of human heparan N-sulphotransferase-1 cDNA and epitope-tagged constructs

The locations of restriction sites used to make the full-length cDNA and the Flag-tagged heparan N-sulphotransferases are shown. The full-coding-length human heparan N-sulphotransferase was made by ligating appropriate *EcoRI*–*ApaLI* DNA fragments of overlapping N-sulphotransferase-1 clones into pcDNA3. Nucleotides encoding the Flag epitope (shaded) were added by PCR as described in the Experimental section.

(4.5 g/l) and 10% fetal calf serum. Cells were incubated at 37 °C in 5% CO₂ at 95% relative humidity. LTA cells were transfected using the calcium phosphate technique [22,23]. LLC-PK₁ cells were transfected by lipofection (DOTMA). Stable transfectants were selected with neomycin (400 µg/ml G418; Gibco-BRL). Clonal cell lines were used for the immunolocalization studies.

Amplification of human heparan N-sulphotransferase cDNA

HUVEC RNA was isolated by the acid guanidinium isothiocyanate procedure of Chomczynski and Sacchi [24]. RNA (5 µg) was reverse-transcribed using a specific oligonucleotide [5'-GTGTT(G/C)(A/G)(A/T)ACCAI(C/G)A-3']. The 20 µl reaction mixture contained 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM of each dNTP, 20 pmol of primer and 200 units of Moloney murine leukaemia virus reverse transcriptase (Superscript II; Gibco-BRL) for 2 h at 42 °C. PCR reaction mixtures (GeneAmp Kit; Cetus) contained 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 4 mM dNTPs, 10% (2 µl) of the prepared HUVEC cDNA, 50 pmol of each oligonucleotide primer [5'-AA(A/G)GA(A/G)TT(C/T)TGGTGGTT(C/T)CC-3' and 5'-AA(A/G)AA(C/T)TCCAT(A/G)TACCA(A/G)TC-3'] and 2.5 units of *Taq* polymerase. Each cycle (35 in total) consisted of 30 s at 94 °C, 45 s at 50 °C and 2 min at 72 °C, followed by a final extension of 5 min at 72 °C. Oligonucleotides were based on the published cDNA sequence for rat liver heparan N-sulphotransferase [6]. The amplified DNA was subcloned into pCRII (Invitrogen) and sequenced.

Screening of a HUVEC cDNA library

Approx. 10⁶ HUVEC cDNA clones in λgt11 (Clontech) were transferred to nylon membranes (MSI) and screened by hybridization to the human heparan N-sulphotransferase PCR fragment [25]. Membranes were prehybridized for 2 h in 50% formamide, 0.75 M NaCl, 0.05 M sodium phosphate, 5 mM EDTA, 5× Denhardt's solution, 0.2% SDS and 100 µg/ml denatured

salmon sperm DNA at 43 °C prior to addition of [³²P]dCTP (DuPont–New England Nuclear)-labelled random primed probes. After 20 h, membranes were washed three times with 2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS for 10 min at 42 °C. This was followed by a single stringency wash with 0.2× SSC containing 0.1% SDS for 60 min at 60 °C. After purification of the clones, phage was isolated and cDNA inserts subcloned into pUC18.

DNA sequencing

Sequencing was carried out by the dideoxy chain-termination method [26,27] using Sequenase (United States Biochemical) and [³⁵S]dATP (DuPont–New England Nuclear). Compressions were resolved by repeating the reactions with deaza-7-dGTP (United States Biochemical) or the use of thermal cycling (New England Biolabs). Both strands of the cDNA were sequenced. Sequence alignments were performed with Geneworks software (Intelli-genetics).

RNA analysis

An RNA blot containing poly(A) mRNA from several tissues (each sample 2 µg) was obtained from Clontech. The membrane was prehybridized for 20 min in Quickhyb (Stratagene) at 68 °C prior to addition of [³²P]dCTP-labelled random-primed probes. After 2 h, membranes were washed three times with 2× SSC containing 0.1% SDS for 10 min at 42 °C. This was followed by a single stringency wash with 0.2× SSC containing 0.1% SDS for 60 min at 60 °C.

Construction of cDNAs encoding Flag-tagged heparan N-sulphotransferase-1 proteins

PCR was used to add nucleotides encoding the Flag epitope (DYKDDDDK) to the 3' end of a DNA fragment encoding the first 169 amino acids of human heparan N-sulphotransferase-1 (N-sulphotransferase-1₁₆₉-Flag; Figure 1). The forward oligo-

nucleotide primer (GGAATTCGGAGGCCAGGATGCCTG) contained an *EcoRI* site followed by sequence (nt 410–426) preceding the start codon. The reverse oligonucleotide (GCTCTAGACTACTTGTTCATCGTCGTCCTTGTAGTCGAAGCC-AATGATGCCAC) was an antisense primer containing sequence of heparan N-sulphotransferase (nt 909–926), sequence encoding the Flag epitope, a stop codon and a *XbaI* restriction site. PCR was performed for 35 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, with a final extension of 5 min at 72 °C. The amplified DNA was digested with *EcoRI* and *XbaI* and cloned into the eukaryotic expression vector pcDNA3 (Invitrogen).

Since none of the isolated heparan N-sulphotransferase clones encoded the entire enzyme, a construct containing the full coding sequence was made by ligating two *EcoRI*–*ApaI* DNA fragments into the *EcoRI* site in pcDNA3 (Figure 1). PCR was again used to add nucleotides encoding the Flag epitope, a stop codon and a *XbaI* restriction site. The forward and reverse oligonucleotide primers were 5'-TATCACAAAGGCATCGACTGG-3' and 5'-GCTCTAGACTACTTGTTCATCGTCGTCCTTGTAGTCCCTGGTGTCTGGAGGTC-3' respectively. PCR was performed as described above. After digestion with *SacII* and *XbaI*, the amplified DNA fragment was used to replace the *SacII*–*XbaI* fragment in the full-length heparan N-sulphotransferase construct. The *BspEI*–*XbaI* DNA fragment was then ligated into the *BspEI*–*XbaI*-digested heparan N-sulphotransferase₁₆₉-Flag construct to remove most of the 5' untranslated region.

Assay of N-sulphotransferase activity

N-sulphotransferase activity was measured by determining [³⁵S]sulphate incorporation into N-desulphated heparin (Sigma). Reaction mixtures (20 µl) contained 10–20 µg of protein from cell homogenates in 50 mM Hepes, pH 7.4, 10 mM MgCl₂, 5 mM CaCl₂, 0.4% Triton X-100, 15% glycerol and ³⁵S-labelled adenosine 3'-phosphate 5'-phosphosulphate (1 mM; 6 × 10⁶ c.p.m./µmol). After 1 h at 37 °C, the reaction mixtures were applied to Whatman No. 1 chromatography paper and developed overnight in 95% ethanol/1 M ammonium acetate, pH 7.8 (5:2, v/v), to separate unincorporated ³⁵S-labelled adenosine 3'-phosphate 5'-phosphosulphate from heparan [³⁵S]sulphate.

Immunoblotting

Cell proteins were solubilized in Laemmli sample buffer and electrophoresed in 4–15% (w/v) polyacrylamide gels. Proteins were transferred on to Immobilon membranes (Millipore) prior to incubation with the anti-Flag M2 monoclonal antibody. Flag-tagged proteins were detected with alkaline-phosphatase-conjugated antibodies.

Immunofluorescence staining

Transfected cells grown on glass coverslips were fixed for 10 min in cold (4 °C) methanol/acetic acid (3:1, v/v) or in 4% (v/v) paraformaldehyde for 1 h, and then rinsed with PBS. After several washes with PBS, the cells were rinsed once with PBS/0.5% BSA and once with PBS/0.1% Triton X-100 to permeabilize the cells. Cells were rinsed again with PBS/BSA and incubated with monoclonal anti-Flag antibody (Kodak/IBI) (diluted 1:100) or other specific antibodies followed by Cy3-conjugated secondary antibodies.

Some coverslips were double-labelled by sequential incubations

in anti-Flag monoclonal antibody, a polyclonal antibody raised against the TGN marker protein TGN38 and appropriate FITC- and Cy3-conjugated secondary antibodies. Coverslips were mounted in glycerol/*N*-propylgallate and viewed by epifluorescence.

RESULTS

Cloning of human heparan N-sulphotransferase-1

PCR amplification of HUVEC cDNA using primers based on the sequence of rat liver heparan N-sulphotransferase resulted in an 842 bp cDNA encoding a human heparan N-sulphotransferase. Screening of a HUVEC cDNA library with this DNA fragment resulted in the isolation of six overlapping clones which together contained a 5' untranslated region of 423 bp, an open reading frame of 2646 bp and a 3' untranslated region of 558 bp (GenBank accession no. U26600).

Differential expression of heparan N-sulphotransferase-1 mRNA in human tissues

Human mRNA from several tissues was analysed to determine the amount of heparan N-sulphotransferase-1 mRNA. A 5' untranslated region DNA fragment specific for heparan N-sulphotransferase-1 (nt 1–320) hybridized most strongly to an approx. 8.5 kb transcript in all tissues, and less strongly to an ~4 kb transcript (Figure 2). The two heparan N-sulphotransferase-1 mRNAs are the result of differential polyadenylation [28]. The GenBank EST database contains several cDNAs of both the short (R59982, HO9932) and the long (R43794, H43902) heparan N-sulphotransferase-1 mRNAs, confirming that two differently polyadenylated heparan N-sulphotransferase-1 mRNAs exist. Heparan N-sulphotransferase-1 transcripts were most abundant in heart, liver and pancreas, while lung contained the lowest levels.

Construction and expression of Flag-tagged heparan N-sulphotransferases

Since antibodies to heparan N-sulphotransferase were not available, cDNA constructs encoding Flag-tagged human heparan N-sulphotransferase-1 proteins were made (Figure 1). The full-

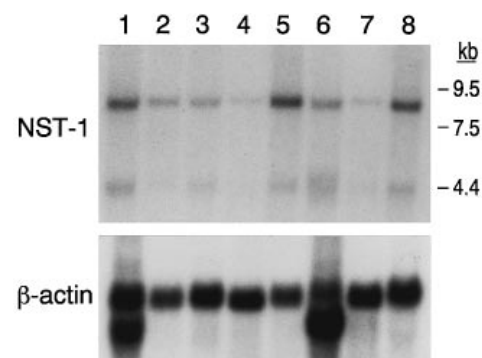


Figure 2 Expression of heparan N-sulphotransferase-1 mRNA in human tissues

A human multiple-tissue blot containing mRNA samples (2 µg) from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (lanes 1–8 respectively) was hybridized with a ³²P-labelled human heparan N-sulphotransferase-1 (NST-1) DNA fragment obtained by PCR (nt 1–320) and with β -actin. Migration positions of RNA standards are indicated on the right.

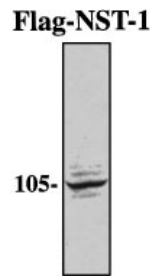


Figure 3 Immunoblotting of Flag-tagged heparan N-sulphotransferase-1

LTA cells stably expressing heparan N-sulphotransferase-1-Flag (Flag-NST-1) were extracted in SDS/PAGE sample buffer, and proteins in the extract were electrophoresed and transferred to Immobilon membranes for immunoblotting with the anti-Flag M2 antibody. The antibody detected a predominant band which migrated at approx. 105 kDa. The minor bands are probably due to Flagged-heparan N-sulphotransferases containing different amounts of carbohydrate.

Table 1 Overexpression of human heparan N-sulphotransferase-1-Flag in mouse LTA cells

Cell homogenates of a clonal LTA cell line stably transfected with human heparan N-sulphotransferase-1-Flag and of control cells transfected with vector alone were incubated at 37 °C for 1 h with N-desulphated heparin and ³⁵S-labelled adenosine 3'-phosphate 5'-phosphosulphate to measure N-sulphotransferase activity, as described in the Experimental section. Results are the means of four determinations.

Cells transfected with:	³⁵ S incorporated (c.p.m.)
Vector only	2080
Heparan N-sulphotransferase-1-Flag	22900

length heparan N-sulphotransferase-1-Flag construct was stably expressed in LTA cells and clonal lines were selected. The expressed protein was detected on immunoblots of cell extracts using the monoclonal antibody to the Flag epitope. The predominant band was detected at ~105 kDa (Figure 3). The minor bands are probably due to heparan N-sulphotransferase-Flag proteins with different amounts of glycosylation.

Flag-tagged heparan N-sulphotransferase is catalytically active

Cells stably expressing the full-length Flag-tagged heparan N-sulphotransferase-1 were tested for N-sulphotransferase activity. Assays of cell extracts indicated that stably transfected LTA cells expressing heparan N-sulphotransferase-Flag contained more than 10-fold higher N-sulphotransferase activity than cells transfected with vector alone (Table 1). Thus heparan N-sulphotransferase-Flag was an active enzyme, indicating that the Flag epitope did not interfere with the function of the protein.

Localization of heparan N-sulphotransferase-Flag to the TGN

LTA cells stably expressing Flag-tagged heparan N-sulphotransferase-1 were used for immunofluorescence labelling with the anti-Flag antibody to determine the intracellular location of the enzyme. Specific staining was seen in most cells in a defined perinuclear patch in the position of the Golgi complex. The reticular pattern of this Golgi staining was characteristic of the light-microscopic appearance of the TGN. This location was confirmed by demonstration of the precise co-localization of

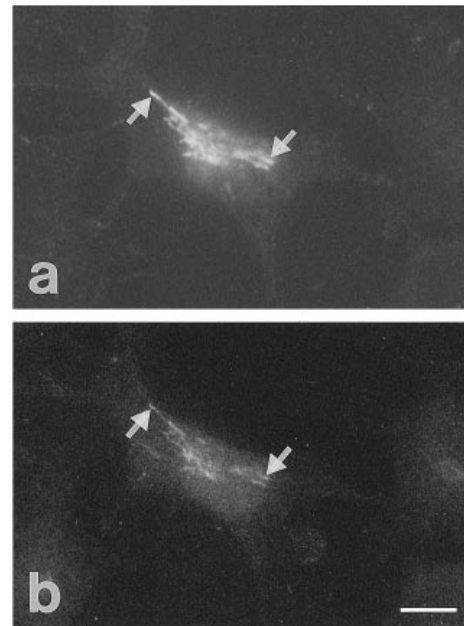


Figure 4 Co-localization of Flag-tagged heparan N-sulphotransferase-1 with TGN38 by immunofluorescence

Cells expressing heparan N-sulphotransferase-1-Flag were double-labelled with the anti-Flag monoclonal antibody and with polyclonal anti-TGN38 antibody, which were detected with (a) Cy3 and (b) FITC-labelled conjugates respectively. A highly magnified view of the perinuclear Golgi area of a stained cell shows that both antibodies labelled reticulated Golgi membranes and that the two proteins were co-localized on exactly the same portions of the membrane (arrows). These membranes can be identified as TGN by the presence of the TGN marker TGN38.

TGN38, a known TGN marker [29], and heparan N-sulphotransferase-1-Flag over the same perinuclear patch and extensions of the TGN (Figure 4). To further confirm the TGN localization of heparan N-sulphotransferase-1, cells were treated with the drug brefeldin A prior to fixation and staining. Brefeldin A induces the fusion of membranes, creating both a proximal endoplasmic reticulum/Golgi compartment and a distal TGN/endosome/plasma membrane compartment [30]. In cells treated for 30 min with brefeldin A, staining of TGN38 and of heparan N-sulphotransferase-1-Flag was found to be similarly redistributed to the same perinuclear patch at the microtubule-organizing centre (Figures 5b and 5d). In contrast, the vesicle coat protein β -COP (β -subunit of the coatomer complex), which associates with membranes of the cis-Golgi [31], was found in a different location following brefeldin A treatment (Figures 5e and 5f). The single perinuclear spot at the TGN (Figures 5b and 5d) and the scattered patches of cis-Golgi network staining (Figure 5f) serve to differentiate membranes of the TGN and the early Golgi stack [32]. The similar redistribution of heparan N-sulphotransferase-1-Flag and TGN38 in the presence of brefeldin A confirms that heparan N-sulphotransferase-1 is found predominantly in the TGN.

In the majority of cells expressing heparan N-sulphotransferase-1-Flag, the enzyme was confined to the TGN, as seen by immunofluorescence staining (Figure 4). However, in individual cells expressing very high levels of heparan N-sulphotransferase-1-Flag, the heparan N-sulphotransferase-1 enzyme was also localized on the plasma membrane (results not shown). This possibly represents a 'spill-over' of expressed enzyme out of the TGN, and further supports the presence of the enzyme in a distal Golgi compartment.

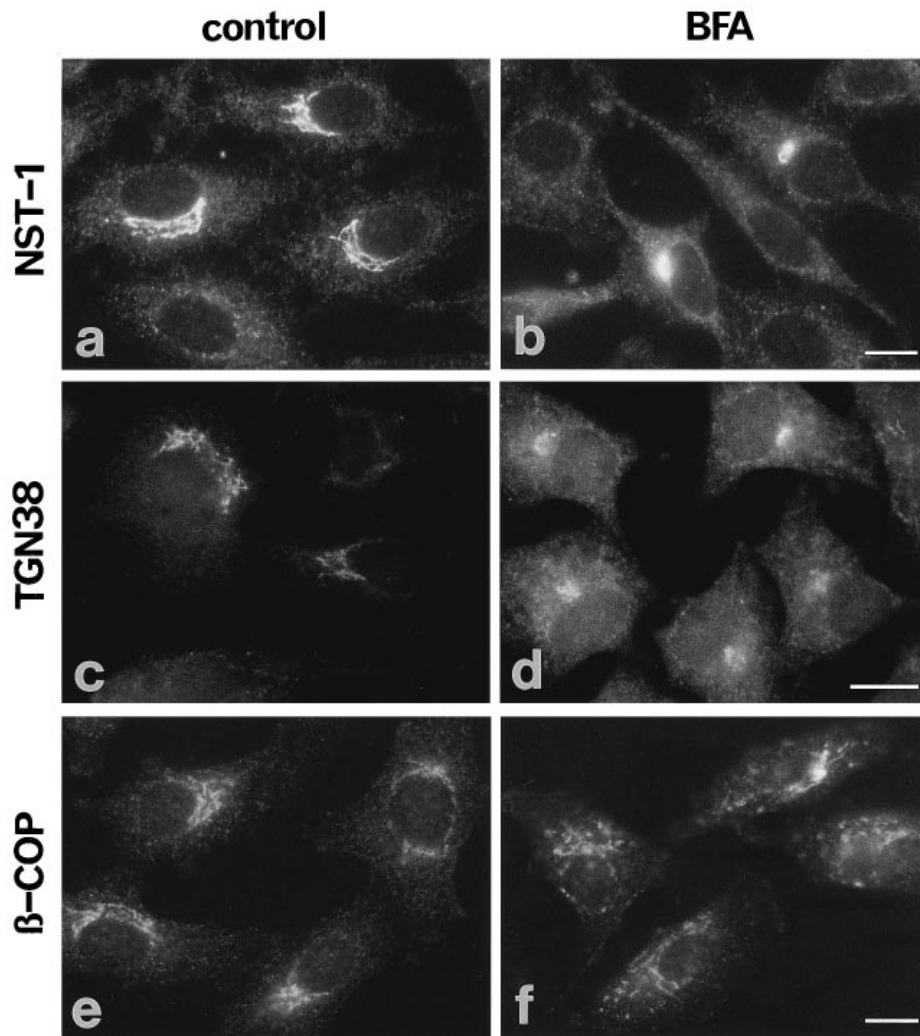


Figure 5 Immunofluorescence localization of Golgi-associated proteins in cells treated with brefeldin A

Control cells and cells treated with brefeldin A (BFA) were fixed and stained with anti-Flag antibody to detect heparan N-sulphotransferase-1-Flag (NST-1); with specific antibodies to detect TGN38, a marker of TGN membranes; or with antibodies against β -COP, a coat protein associated with vesicles in the cis-Golgi. All three antibodies give similar patterns of staining of reticulated membranes within the perinuclear Golgi complex. Disruption and reorganization of Golgi membranes following brefeldin A treatment resulted in the relocation of TGN38 staining to a single spot at the microtubule-organizing centre. In contrast, after brefeldin A treatment β -COP staining was found in scattered patches representing cis-Golgi membranes. The Flag staining pattern in brefeldin A-treated cells resembled that of TGN38, showing that heparan N-sulphotransferase-1-Flag is associated with TGN membranes.

Truncated heparan N-sulphotransferase-1₁₆₉-Flag is also localized to the Golgi complex

Amino acids in and around the transmembrane domain of Golgi enzymes have been shown to be important signals for Golgi localization and retention [33]. Therefore we analysed the localization of heparan N-sulphotransferase-1₁₆₉-Flag in stably transfected LLC-PK₁ cells. Cells expressing the Flag-tagged heparan N-sulphotransferase peptide were stained with the anti-Flag antibody. Specific immunofluorescence labelling was seen over the perinuclear Golgi complex, indicating that the truncated form of the enzyme was correctly targeted to the Golgi membranes (Figure 6). The bright staining of the LLC-PK₁ cells with anti-Flag antibodies indicated that very high levels of heparan N-sulphotransferase-1₁₆₉-Flag were expressed in these transfected cell lines. Even with such high levels of expression, however, all of the peptide was retained within the perinuclear

Golgi, without spill-over to other parts of the cell. The overly bright staining prevented further definition of the sub-compartmental localization of heparan N-sulphotransferase-1₁₆₉-Flag within the Golgi complex. Staining with an antibody to the Golgi marker p200, a vesicle-associated protein, confirmed that the staining of the perinuclear patches did represent labelling of the Golgi complex (Figure 6).

DISCUSSION

We have cloned a human heparan N-sulphotransferase from HUVEC which is very similar to the enzyme cloned from rat liver. We refer to this N-sulphotransferase as heparan N-sulphotransferase-1, since it was the first of two distinct heparan N-sulphotransferases to be cloned [6–8]. This same enzyme was recently cloned by investigators searching for the gene responsible

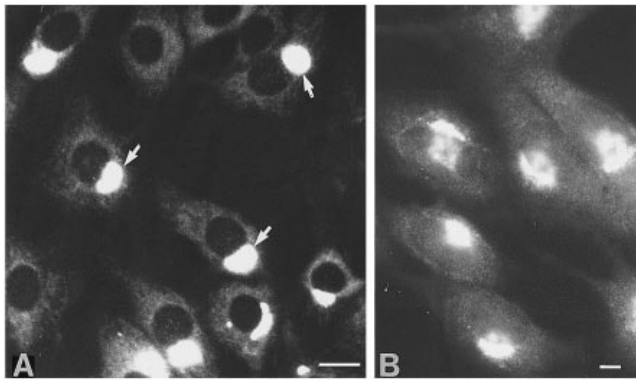


Figure 6 Immunofluorescence localization of truncated heparan N-sulphotransferase-1

LLC-PK₁ cells stably expressing heparan N-sulphotransferase-1₁₆₉-Flag peptide were stained with antibodies to Flag (A) to label heparan N-sulphotransferase-1₁₆₉-Flag, or with an antibody to the Golgi-associated protein p200 (B). The Flag-tagged N-sulphotransferase-1 peptide was localized in a single large patch in a perinuclear position (arrows) representing staining of the Golgi complex. Cells from the same clone also show bright staining of p200 over the perinuclear Golgi and diffuse staining of p200 through the cytoplasm.

for Treacher Collins syndrome on chromosome 5 [28]. Overall, the nucleotide sequences are 99.8% identical. Three nucleotide differences were noted within the coding region of the human heparan N-sulphotransferase-1 cDNA, but all of these substitutions are silent, so that the deduced amino acid sequences are identical. The human and rat heparan N-sulphotransferases differ in only 18 of the 883 amino acids that comprise the enzyme. The least conserved region of the human and rat heparan N-sulphotransferases is the amino acids in and around the transmembrane domain, an area shown to be important for retention in the Golgi complex of enzymes involved in glycoprotein biosynthesis [16–19,33,34].

The heparan N-deacetylase/N-sulphotransferases appear to have a domain structure similar to that of the glycosyltransferases cloned previously [8,35]. Like the glycosyltransferases involved in glycoprotein and glycolipid biosynthesis, the heparan N-sulphotransferases are thought to be type II membrane proteins with a short N-terminal cytoplasmic tail, a single transmembrane domain, a stem region and a catalytic domain.

While many of the glycosyltransferases involved in glycoprotein synthesis have been cloned and localized to discrete subcompartments in the Golgi complex, none of the enzymes involved in glycosaminoglycan biosynthesis have been definitively localized to a Golgi subcompartment. Studies with brefeldin A, a fungal metabolite which causes the redistribution of Golgi enzymes, have suggested that chondroitin sulphate and heparan sulphate synthesis may occur in different subcompartments of the Golgi complex [13–15,36]. Most chondroitin sulphate biosynthesis appears to occur in the TGN, whereas heparan sulphate biosynthesis appears to occur in a more proximal compartment. However, at least in cartilage, some chondroitin sulphate is also synthesized in a more proximal subcompartment [15], and treatment of cells with brefeldin A reduces, but does not eliminate, synthesis of heparan sulphate [13,14].

Immunocytochemical localization of specific enzymes offers the most direct means of determining the subcompartmental distribution and sequence of the processing enzymes in the Golgi. Since antibodies to heparan N-sulphotransferase were not

available, cDNA constructs encoding a Flag-tagged full-length human heparan N-sulphotransferase-1 and a short fragment of the enzyme were expressed in cells to allow localization of the enzyme by immunocytochemical techniques. Heparan N-sulphotransferase-Flag was an active enzyme, suggesting that addition of the Flag epitope did not greatly alter the structure of the enzyme and is thus not likely to interfere with its targeting. This Flag-tagged heparan N-sulphotransferase was co-localized with TGN38, a known TGN marker protein [29], in both untreated cells and cells treated with brefeldin A. Thus heparan N-sulphotransferase-1 is localized to the TGN in mouse LTA cells. At the light-microscopy level our data provide the first definitive evidence for the localization of a sulphotransferase to the TGN. We cannot, however, rule out the presence of small amounts of N-sulphotransferase-1 in earlier Golgi compartments, since immunofluorescence labelling reveals only compartments with heavy concentrations of protein. It is also possible that the localization of heparan N-sulphotransferase-1 within the Golgi stack could vary between cell types, as has been found with other Golgi enzymes such as α -mannosidase [37]. A complete survey of the localization of heparan N-sulphotransferase can only be carried out at the electron-microscopy level, and this will be pursued in future studies. In some cells which greatly overexpressed heparan N-sulphotransferase-1-Flag, the Flag-tagged enzyme was detected at the cell surface. Thus the production of large amounts of N-sulphotransferase-1 results in some of the enzyme escaping the TGN by entering a secretory or recycling pathway to the cell surface. Similar results were obtained on overexpression of the TGN membrane protein TGN38 [38], which is normally resident in the TGN but cycles transiently to the cell surface.

Amino acids in and around the transmembrane domain have been shown to be responsible for the localization and retention of proteins in the Golgi complex. To determine if the N-terminus of heparan N-sulphotransferase-1 contains sufficient information to retain the protein in the Golgi, nucleotides encoding the Flag epitope were added to a sequence encoding the first 169 amino acids of human heparan N-sulphotransferase-1. Staining of stably transfected LLC-PK₁ cells showed labelling over the perinuclear Golgi complex, indicating that this truncated form of the enzyme was also targeted correctly to the Golgi membranes. Although localization of full-length heparan N-sulphotransferase-1-Flag and N-sulphotransferase-1₁₆₉-Flag was not directly compared in the same cells, these data indicate that the N-terminal and transmembrane domains of heparan N-sulphotransferase-1 are sufficient for a general Golgi localization, consistent with targeting information in other Golgi enzymes. For instance, the N-terminus of galactosyltransferase was found to be sufficient to convey Golgi retention of a chimaeric construct of ovalbumin [17]. This same region has been shown to be important for the targeting of a number of other glycosyltransferases to different cisternae within the Golgi.

No consensus sequence has been found in the targeting domains of various glycosyltransferases. Several hypotheses have been forwarded, including aggregation, homodimer formation and lipid sorting (reviewed in [33,34]), to explain how these N-terminal sequences operate to retain enzymes in specific Golgi subcompartments. As yet, no consensus has emerged that predicts a mechanism for the retention of heparan N-sulphotransferase-1 in the TGN. A cytoplasmic sequence, Tyr-Gln-Arg-Leu, has been shown to be necessary for localization of TGN38 to the TGN [34]; the heparan N-sulphotransferase amino acid sequence predicted to extend into the cytoplasm does not contain a similar motif. Lack of the Tyr-Gln-Arg-Leu sequence suggests that heparan N-sulphotransferase-1 may have another targeting sig-

nal, and that resident and recycling Golgi proteins may have different signals for retention in the TGN. The TGN houses a disparate array of functions, including the modification of newly synthesized glycoproteins, remodelling of internalized or recycled proteins, protein sorting and selective loading of transport vesicles [12]. The TGN itself may be subdivided into distinct subcompartments for resident Golgi enzymes, such as heparan N-sulphotransferase-1, and for recycling proteins. Studies utilizing mutated heparan N-sulphotransferase-1 will be required to identify the signal(s) that direct the enzyme to the TGN.

The present studies have demonstrated for the first time that human heparan N-sulphotransferase-1 is retained in the TGN. Since N-sulphation is a prerequisite for the further processing of heparan glycosaminoglycans, it is likely that the heparan glucuronosyl epimerase and O-sulphotransferase enzymes will also be localized in the TGN. Likewise, since studies have suggested that sulphation and elongation are linked [39], the glucuronosyl/*N*-acetylglucosaminyltransferase may also be found in this compartment. Similar studies with the second heparan N-sulphotransferase will determine whether it is also localized in the TGN or in a more proximal Golgi sub-compartment.

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