Biochemical and Biophysical Research Communications **255,** 182–187 (1999)
Article ID bbrc.1999.0151, available online at http://www.idealibrary.com on IDEAL®

Molecular Cloning and Expression of a Second Glucuronyltransferase Involved in the Biosynthesis of the HNK-1 Carbohydrate Epitope[1]

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Received December 23, 1998

A cDNA encoding a novel glucuronyltransferase was cloned from a rat brain cDNA library. The cDNA sequence contained an open reading frame encoding 324 amino acids, with type II transmembrane topology. The amino acid sequence reveled 49% homology to rat GlcAT-P, a glucuronyltransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope of glycoproteins, [Terayama *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94, 6093–6098] and the highest sequence homology was found in the catalytic region. Northern blot analysis indicated that this newly cloned glucuronyltransferase is expressed in the nervous system, consistent with the selective localization of the HNK-1 carbohydrate epitope in the nervous system. Transfection of this cDNA into COS-1 cells induced the expression of the HNK-1 carbohydrate epitope on cell surfaces, and induced the morphological changes in these cells. These results indicated that this newly cloned cDNA is a second glucuronyltransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope.

The HNK-1 carbohydrate epitope, which is recognized by the monoclonal antibody HNK-1 (1), is characteristically expressed on a large number of cell adhesion molecules, such as on the neural cell adhesion molecule (NCAM), L1, myelin-associated glycoprotein (MAG), transiently expressed axonal glycoprotein-1 (TAG-1) and PO (2–5), and on some proteoglycans (6) in the nervous system, all of which are involved in cell-cell and/or cell-substratum interactions. This epitope is also expressed on some glycolipids (SGGL-1 and -2) (7,8). The expression of the HNK-1 carbohydrate epitope on these glycoconjugates is spatially and temporally regulated during the development of the nervous system (9,10). Studies using either the HNK-1 antibody or HNK-1 reactive glycolipids demonstrated that the HNK-1 carbohydrate epitope plays crucial roles in cell-cell and/or cell-substrate interactions such as cell adhesion, migration and neurite extension (11–13).

The HNK-1 epitope has been demonstrated to be composed of the sulfated trisaccharides, sulfate-3GlcAβ1-3Galβ1-4GlcNAc, which is shared with glycolipids and glycoproteins (7,8,14). Since Gal β1-4 GlcNAc is found commonly in various glycoproteins and glycolipids, glucuronyltransferase is considered to be a key enzyme for the biosynthesis of the HNK-1 epitope. In our previous study, we demonstrated that there are at least two types of glucuronyltransferases associated with biosynthesis of the HNK-1 carbohydrate epitope in the rat brain (15). Recently we purified a glucuronyltransferase specific for glycoprotein acceptors (GlcAT-P) from the rat brain (16), and cloned its cDNA (17).

In this study, we screened a rat brain cDNA library using the GlcAT-P cDNA as a probe under low stringency conditions, to obtain other glucuronyltransferase(s) involved in the biosynthesis of the HNK-1 epitope. We succeeded in the cloning of a novel glucuronyltransferase cDNA, which encoded a type II membrane protein with four highly conserved motifs found in GlcAT-P. Transfection of this newly cloned cDNA into COS-1 cells induced the formation of HNK-1 carbohydrate epitopes on the cell surfaces. This novel glucuronyltransferase was tentatively called GlcAT-S.

MATERIALS AND METHODS

Materials. The mammalian expression vector pEF-BOS (18) was kindly provided by S. Nagata (Osaka Bioscience Institute). The monoclonal antibody M6749 (19) was a generous gift from H. Tanaka (Kumamoto University). The Chinese Hamster Ovary mutant cell

[1] The nucleotide sequence data reported in this paper has been submitted to the DDBJ/EMBL/GenBank databases with the Accession Number AB010441.

0006-291X/99 $30.00

line, Lec2, and a hybridoma cell line producing the HNK-1 antibody were purchased from the American Type Culture Collection.

Cloning of a novel glucuronyltransferase cDNA. A λgt11 18th day rat embryonic cDNA library (CLONTECH, 5 x 10^5 phages) was screened using the catalytic domain of GlcAT-P as a probe (a 832 bp fragment from nucleotide number 222 to 1053 of the GlcAT-P cDNA (17)). The membranes with transferred phage DNA were hybridized at 42°C in 5x SSC, 50mM NaH_2PO_4, pH7.0, 0.5% non-fat dried milk, 0.1% SDS, 100 μg/ml yeast RNA and 50% formamide overnight, and then washed under low stringency conditions at 42°C in 2x SSC and 0.1% SDS. Seventeen positive clones were detected under these conditions. The membranes were further washed under high stringency conditions at 65°C in 0.2x SSC and 0.1% SDS. Positive clones detected under high stringency conditions were found to be GlcAT-P cDNA. Two clones which were positive under low stringency conditions and negative under high stringency conditions were isolated. Upon sequence analysis one was identified to be GlcAT-P cDNA, and the other was found to be a novel cDNA. The 5′ end of the coding region, which was absent in the cloned cDNA, was directly amplified and determined by the cloning strategy of rapid amplification of cDNA ends using cerebral cortex mRNA from 2-week-old rats as a template.

DNA sequencing. DNA sequences were determined by the dideoxynucleotide chain termination method (20) with an Applied Biosystems model 373 DNA sequencer.

Northern blot hybridization. Total RNA was extracted by the acidic guanidium thiocyanate/phenol/chloroform method (21). Equal amounts of total RNA (10 μg in each lane) from various adult rat tissues were size-fractionated in a 1.2% agarose-formaldehyde gel and blotted onto nylon membranes (Hibond N^+, Amersham). The blots were hybridized with ^{32}P-labeled GlcAT-S cDNA overnight at 65°C in 0.5M NaH_2PO_4, pH7.2, containing 7% SDS, 1mM EDTA and 1% BSA (22), and then washed in 0.1x SSC, and 0.1% SDS at 65°C. Radioactivity was visualized using an image analyzer (Fuji Photo Film, BAS2000).

Construction of an expression vector containing the full length cDNA. A DNA fragment of 1024 bp of the newly isolated cDNA from nucleotide number -30 to 994 was amplified by the polymerase chain reaction from cDNAs transcribed from the total RNA of 19th day embryonic rat cerebellum, using primers (5′-CCTTCTAGA-GGGTGGTGTCCGAGACGCTGGGA-3′ and 5′-TTCTCTAGACAGC-GGCTGCTCCTGGTGGCTACACCT-3′) containing *Xba*I sites (underlined). Polymerase chain reactions were carried out using *Tth* polymerase, with 38 cycles at 94°C for 30 sec and at 68°C for 180 sec. The amplified DNA fragments were digested with *Xba*I, and cloned into the *Xba*I site of pEF-BOS. Several clones were isolated and sequenced, and a clone containing the correct sequence was used in the following experiments.

Cell culture and cDNA transfection. COS-1 and Lec2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium Alpha Medium (alpha MEM) containing 10% fetal bovine serum, respectively. Tissue culture dishes (60 mm) were seeded with Lec2 or COS-1 cells trypsinized 24 hr before transfection with 3 μg of GlcAT-S cDNA and GlcAT-P cDNA in pEF-BOS, or an equal amount of the empty vector using lipofectAMINE (Life Technologies) according to the instructions provided by the manufacturer. These cells were used for flow cytometry analysis, immunostaining and immunoblot analysis 48 hr after transfection.

Flow cytometry analysis. Transfected Lec2 cells were collected in PBS containing EDTA and incubated with the monoclonal antibody M6749 (10 μg/ml) for 1hr on ice. After washing with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM antibodies (Cappel) for 1hr on ice, and then analyzed by flow cytometry with a FACScan (Becton Dickenson, Mountain View, CA).

Immunofluorescence staining. For immunofluorescence staining, COS-1 cells were seeded on cover glasses immersed in culture dishes. The transfected COS-1 cells were fixed with methanol at −20°C for 20 min. The fixed cells were treated with 3% BSA in PBS and then incubated with the HNK-1 antibody (10 μg/ml) for 90 min at room temperature. After washing with PBS, the cells were visualized with FITC-conjugated goat anti-mouse IgM antibodies (Cappel), and observed with a confocal laser scan microscope, Fluoview (Olympus, Tokyo, Japan).

Western blot analysis. For immunoblot experiments, the membrane fraction of the transfected COS-1 cells were extracted with 10 mM/HCl buffer, pH7.5, containing 1% SDS and 5% β-mercaptoethanol. Proteins were separated by 5-20% gradient SDS-polyacrylamide gel electrophoresis with the buffer system of Laemmli (23) and transferred onto nitrocellulose membranes. After blocking with 5% non-fat dried milk in PBS, the membranes were incubated with the HNK-1 antibody (10 μg/ml) followed by HRP-conjugated goat anti-mouse IgM antibodies, and protein bands were then visualized with the 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB) substrate.

RESULTS

Cloning of the cDNA for a Novel Glucuronyltransferase (GlcAT-S)

Previous studies had indicated that there are at least two glucuronyltransferases in the rat brain, which are associated with the biosynthesis of the HNK-1 carbohydrate epitope (15). Recently, we purified a GlcAT-P which is specific for glycoprotein substrates (16). The GlcAT-P cDNA was cloned using its partial amino acid sequence information (17). In this study, we screened a rat brain cDNA library to look for other glucuronyltransferase(s) involved in the biosynthesis of the HNK-1 carbohydrate epitope, using the cDNA corresponding to the catalytic domain of GlcAT-P as a probe. We found one positive clone with a sequence similar to but distinct from that of GlcAT-P. Because of the lack of the 5′ end of the coding region in this cloned cDNA, it was directly amplified and determined by the rapid amplification of cDNA ends strategy using cerebral cortex mRNA from 2-week-old rats.

Primary Structure of the Novel GlcAT cDNA

The sequence of the cloned cDNA indicated an open reading frame of 972-bp, encoding a protein of 324 amino acids including two potential N-glycosylation sites (Fig. 1). The expected molecular weight of this protein is 37,219 Da. Two potential initiation codons are found within the first 66 nucleotides. However, only the first methionine at nucleotide position 1 is in a favorable context for translation initiation (24). Hydropathy analysis revealed a single potential transmembrane region consisting of 17 amino acid residues, and the protein appeared to have type II transmembrane orientation characteristics, like many other glycosyltransferases cloned to date (data not shown) (25). The newly cloned cDNA encoded a proline-rich domain next to the transmembrane region (from Asp-26 to

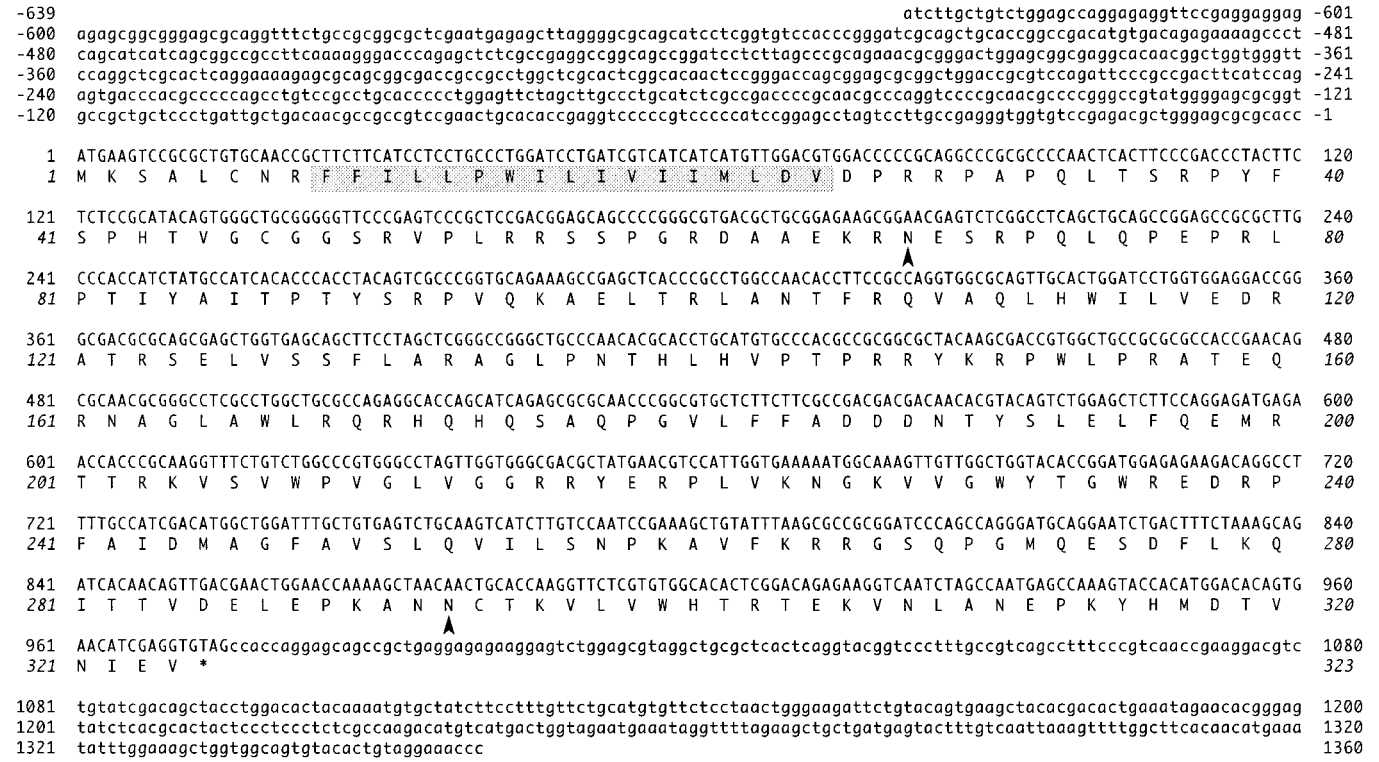

FIG. 1. The nucleotide and deduced amino acid sequence of the novel glucuronyltransferase cDNA. The putative transmembrane region is shadow boxed. Two potential N-glycosylation sites are indicated by arrowheads. The sequences are numbered at the right and left, starting from the first initiation codon.

Pro-81), as is seen in several other glycosyltransferases, including rat GlcAT-P (17,26). Amino acid sequence comparison of GlcAT-S and GlcAT-P is shown in Fig. 2. The overall identity was 49%, and the highest identity was found in the COOH-terminal catalytic domain containing the previously identified four highly conserved motifs (I-IV), following the proline-rich region (amino acids from Pro-81 to Val-324 overlap with 58.6% homology). This sequence conservation strongly suggests that the newly cloned cDNA encodes a novel glucuronyltransferase. We tentatively called this newly cloned cDNA GlcAT-S (the second HNK-1 associated GlcAT).

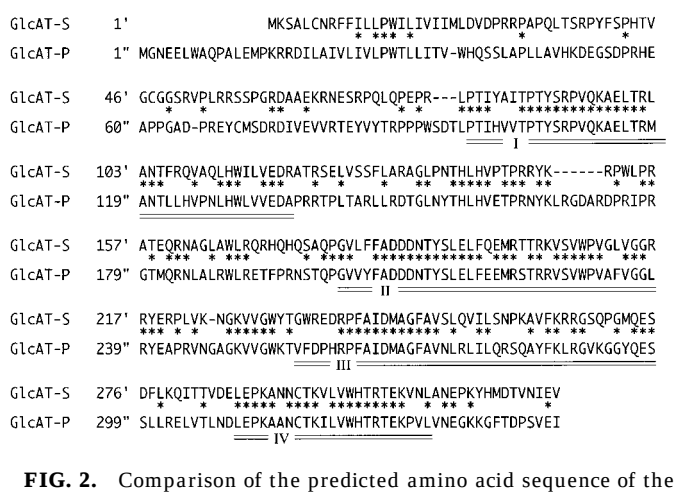

FIG. 2. Comparison of the predicted amino acid sequence of the novel glucuronyltransferase (GlcAT-S) and GlcAT-P. Conserved amino acids between the two sequences are indicated by asterisks, and gaps are introduced for maximal alignment by dashes. The amino acid sequences of four highly conserved regions, named motifs I-IV (17) are indicated by double underlines.

Tissue Distribution of the Novel Glucuronyltransferase mRNA

To study tissue-specific expression of the GlcAT-S mRNA in the adult rat, total RNAs from various tissues were subjected to Northern blot analysis using a cDNA fragment corresponding to the catalytic domain of the GlcAT-S cDNA (Asp-62 to Val-324) as a probe. As shown in Fig. 3, two transcripts of 2.3 kb and 1.1 kb were detected in the cerebral cortex (lane 1), cerebellum (lane 2) and whole brain (lane 3), in which the HNK-1 carbohydrate epitope was specifically expressed. On the other hand, no bands were detected in HNK-1 negative tissues, such as in the lung, liver, kidney, ileum, testis, lymphoid nodule, thymus, spleen, heart and macrophages (lanes 4–13). Tissue expression patterns of GlcAT-S mRNA were in good agreement with that of the HNK-1 carbohydrate epitope,

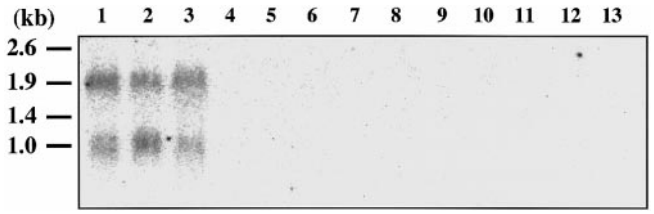

FIG. 3. Expression of GlcAT-S mRNA in adult rat tissues. Samples (10 μg) of total RNA were electrophoresed and hybridized using the catalytic domain of GlcAT-S as a probe. The source of total RNA from adult rat tissues were as follows: lane 1; cerebral cortex, lane 2; cerebellum, lane 3; whole brain, lane 4; lung, lane 5; liver, lane 6; kidney, lane 7; ileum, lane 8; testis, lane 9; lymphoid nodule, lane 10; thymus, lane 11; spleen, lane 12; heart and lane 13; macrophages. The positions of marker RNAs are indicated on the left.

suggesting that GlcAT-S encodes a novel glucuronyltransferase which is involved in the biosynthesis of the HNK-1 carbohydrate epitope.

Transfection of GlcAT-S cDNA into Lec2 Cells

To prove the enzymatic activity of the newly cloned GlcAT-S cDNA product *in situ*, full-length cDNAs were transfected into Lec2 cells that lack the CMP-sialic acid transporter (27). This mutation results in an increase of *N*-acetyllactosamine termini in glycoproteins and glycolipids, which are good substrates for HNK-1 associated glucuronyltransferase. The transfer of GlcA to *N*-acetyllactosamine termini on Lec 2 cell surfaces was studied using the M6749 antibody, which reacts with non-sulfated GlcA transferred to *N*-acetyllactosamine residues. As shown in Fig. 4, about half of the Lec2 cells transfected with GlcAT-S cDNA were stained with the M6749 antibody, whereas mock-transfected cells were negative, indicating that GlcAT-S cDNA produce active glucuronyltransferases *in situ*.

Transfection of the Newly Cloned GlcAT cDNA into COS-1 Cells

We previously reported that the expression of the HNK-1 epitope on cell surfaces was observed by transfection of GlcAT-P cDNA into COS-1 cells, indicating that COS-1 cells have a sulfotransferase(s) that transfers sulfate to glycoconjugates bearing GlcA, to complete the HNK-1 epitope (17). To demonstrate that the newly cloned GlcAT-S is an enzyme involved in the biosynthesis of the HNK-1 carbohydrate epitope, GlcAT-S cDNA was transfected into COS-1 cells. Two days after transfection, the cells were stained with the HNK-1 antibody. As shown in the Fig. 5A, GlcAT-S cDNA transfected COS-1 cells were intensely stained with the HNK-1 antibody, while mock transfected cells showed no staining with this antibody (data not shown). COS-1 cells expressing the HNK-1 epitope exhibited morphological changes as previously reported for the transfection of GlcAT-P cDNA (17). Western blot analyses using the HNK-1 antibody (Fig. 5B), of membrane fractions prepared from COS-1 cells transfected with either the GlcAT-S cDNA or GlcAT-P cDNA, both showed similar profiles of several protein bands ranging from 70 kDa to over 200 kDa, indicating that the acceptor specificity of GlcAT-S towards glycoproteins is similar with that of GlcAT-P. These results suggest that GlcAT-S is a second glucuronyltransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope on glycoproteins.

DISCUSSION

Our previous results indicated that two respective glucuronyltransferases are involved in the biosynthesis of the HNK-1 carbohydrate epitope on glycoproteins (GlcAT-P) and glycolipids (GlcAT-L) (15,28). On the basis of glucuronyltransferase activity toward glycoprotein acceptors, we had previously been successful in purifying GlcAT-P to apparent homogeneity from the rat brain (16). The purified GlcAT-P showed little activity toward glycolipid acceptors. Using the partial amino acid sequence of the purified GlcAT-P, we were able to clone GlcAT-P cDNA from a rat brain cDNA library (17). In order to clone other GlcAT cDNAs, including the GlcAT-L cDNA, we screened a rat brain cDNA library using GlcAT-P cDNA corresponding to the catalytic region of GlcAT-P as a probe, under low stringency conditions. We obtained a novel cDNA clone which had 49% identity with GlcAT-P cDNA at the amino acid sequence level. The highest sequence homology was observed in the catalytic region containing the previously identified four highly conserved motifs. This cDNA was shown to encode a glucuronyltransferase involved in the expression of the HNK-1 carbohydrate epitope on the surface of COS-1 cells. Thus, the newly cloned GlcAT proved to be a novel glucuronyltransferase responsible for the biosynthesis of the HNK-1 carbohydrate epitope. In order to study

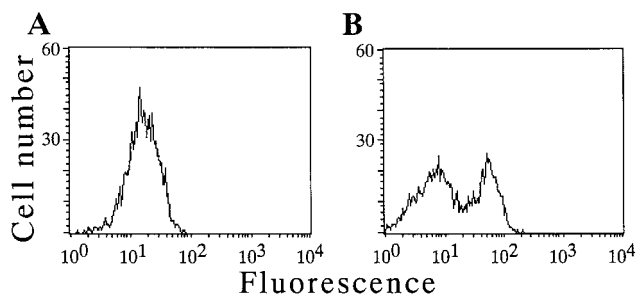

FIG. 4. Transient expression of GlcAT-S cDNA in Lec2 cells. Lec2 cells transfected with the vector alone (A) or with GlcAT-S cDNA in pEF-BOS (B) were stained with the M6749 antibody, followed by FITC-conjugated anti-mouse IgM, and then subjected to flow cytometric analysis.

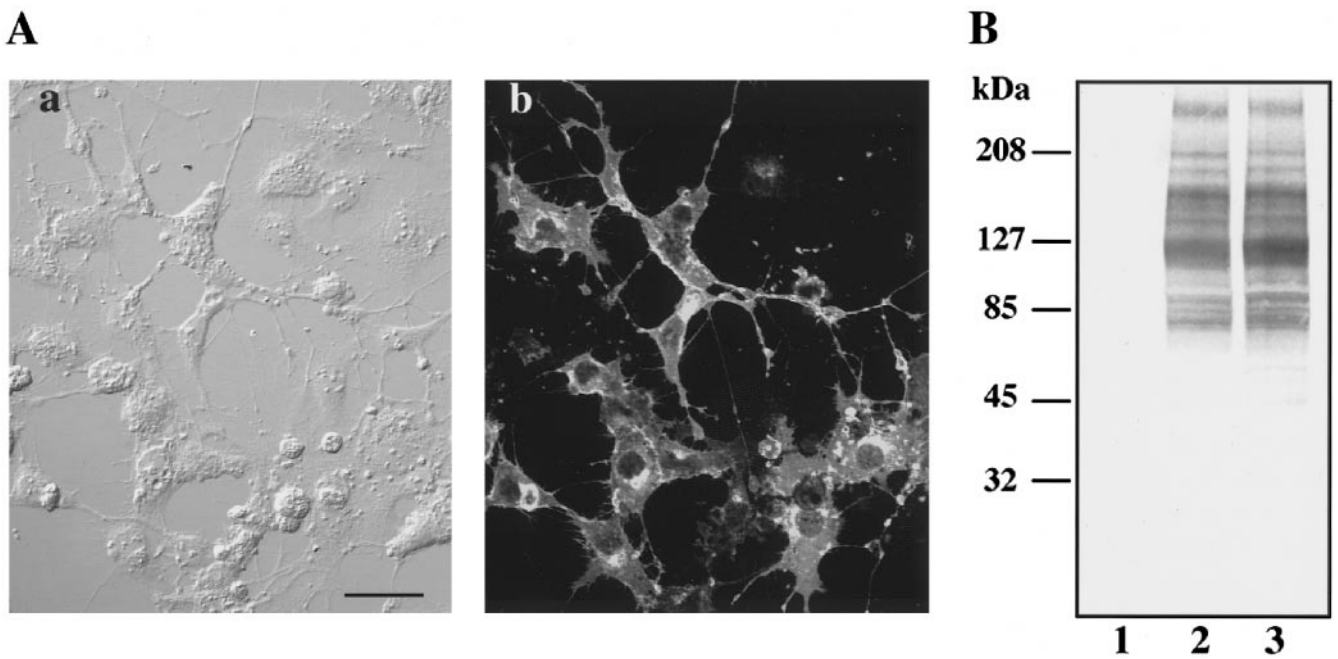

FIG. 5. (A) Transient expression of GlcAT-S cDNA in COS-1 cells. COS-1 cells were transfected with GlcAT-S cDNA in pEF-BOS, as described under Materials and Methods. Indirect immunofluorescence staining of the HNK-1 epitope (b) and the corresponding Nomarski DIC image (a). COS-1 cells transfected with GlcAT-S were intensely stained with the HNK-1 antibody and had altered morphologies (a and b). Scale bar, 50 μm. (B) Immunoblot analysis of COS-1 cell lysates using the HNK-1 antibody. membrane fractions prepared from COS-1 cells transfected with the vector alone (lane 1), GlcAT-S cDNA in pEF-BOS (lane 2), and GlcAT-P cDNA in pEF-BOS were used for immunoblot analysis with the HNK-1 antibody as described under Materials and Methods. The positions of marker proteins are indicated on the left.

whether this new GlcAT can transfer GlcA to glycolipid acceptors or glycoprotein acceptors or both, a soluble form of the enzyme was generated by replacing the N-terminal 23 amino acids of GlcAT-S with protein A. The fusion protein expressed in COS-1 cells was absorbed on IgG-Sepharose beads, and the enzyme-bound beads were used as the enzyme source under standard glucuronyltransferase assay conditions described previously (15,28). The soluble form of GlcAT-S exhibited glucuronyltransferase activity toward asialoorosomucoid, a glycoprotein acceptor (417 pmol/ml medium/h) but no detectable activity toward paragloboside, a glycolipid acceptor ($<$1 pmol/ml medium/h). In addition, Western blot analyses of the membrane fractions of COS-1 cells transfected with GlcAT-S cDNA indicated that the enzyme is able to induce the expression of the HNK-1 epitope on various glycoproteins (Fig. 5). Taken together, these results, indicate that the new GlcAT is a second enzyme involved in the biosynthesis of the HNK-1 carbohydrate epitope on glycoproteins.

Upon screening of a cDNA library by plaque hybridization under low stringency conditions using GlcAT-P cDNA as a probe, seventeen positive clones were detected. One of them was the GlcAT-S cDNA, and the rest were all GlcAT-P cDNA. These results indicated that the content of GlcAT-S in the rat brain at embryonic day 18 is much less than that of GlcAT-P. Furthermore, the Northern blot signals of GlcAT-S mRNA detected in the adult rat brain were weaker than those of GlcAT-P. These results suggest that GlcAT-P is the major glucuronyl-transferase involved in the biosynthesis of the HNK-1 carbohydrate epitope in the adult rat brain and/or that GlcAT-S might be expressed in restricted brain regions. Our preliminary *in situ* hybridization results using sections of the adult rat brain supports this idea.

A PCR-based cloning approach directed towards highly conserved regions in primary amino acid sequences has so far been successful for the cloning of many sialyltransferases (29,30) and galactosyltransferases (31). These glycosyltransferases have been demonstrated to form a large gene family (32,33). Regarding the glucuronyltransferases, this approach has been successful for the cloning of a novel glucuronyltransferase (GlcAT-I) involved in the biosynthesis of the glycosaminoglycan-protein linkage region of proteoglycans (34). The presence of GlcAT-S in the rat brain suggests that the glucuronyltransferases also form a gene family.

The reason as to why two different glucuronyltransferases (GlcAT-P and GlcAT-S) are involved in the biosynthesis of the HNK-1 epitope on glycoproteins in the rat brain is not clear at the moment. In order to

understand the function of the respective GlcATs and/or the biological roles of the HNK-1 epitope which are formed by respective GlcATs in the nervous system, it is essential to investigate the acceptor specificities and expression patterns of GlcAT-P and GlcA-S in more detail.

ACKNOWLEDGMENTS

We thank Ms. H. Nishigaki for secretarial assistance. This work was supported in part by the Grant-in-Aid for Scientific Research on Priority Area No. 10178104, from the Ministry of Education, Science, Sports, and Culture of Japan.

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