Expression Cloning of a cDNA Encoding a Sulfotransferase Involved in the Biosynthesis of the HNK-1 Carbohydrate Epitope*

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The HNK-1 carbohydrate epitope is expressed on several neural adhesion glycoproteins and as a glycolipid, and is involved in cell interactions. The structural element of the epitope common to glycoproteins and glycolipids has been determined to be sulfate-3-GlcAβ1→3Galβ1→4GlcNAc. The glucuronyltransferase and sulfotransferase are considered to be the key enzymes in the biosynthesis of this epitope because the rest of the structure occurs often in glycoconjugates. Here we describe the isolation of the rat sulfotransferase cDNA via an expression cloning strategy. The clone finally isolated predicts a protein of 356 amino acids, with characteristics of a type II transmembrane protein and with no sequence similarity to other known sulfotransferases. Both the enzyme expressed as a soluble fusion protein and homogenates of cells transfected with the full-length cDNA could transfer sulfate from a sulfate donor to acceptor substrates containing terminal glucuronic acid.

The carbohydrate antigen recognized by the monoclonal antibody HNK-1 was originally described as a marker for human natural killer cells (1). Later it was shown to be expressed predominantly on glycolipids and glycoproteins from nervous tissue (2–5). The expression pattern of the HNK-1 carbohydrate in both the central and peripheral nervous system is spatially and developmentally regulated (6–11). The HNK-1 carbohydrate epitope is carried by many, but not all, neural recognition glycoproteins and is involved in homophilic and heterophilic binding of these proteins (for a review, see Ref. 12). Of special interest is the association of the epitope with Schwann cells myelinating motor but not sensory axons (10), where it may be involved in the preferential reinnervation of muscle nerves by motor axons after lesion (13, 14).

Determination of the structure of the glycolipid (15) and glycoprotein (16) forms has shown that both carry sulfate-3-GlcAβ1→3Galβ1→4GlcNAc. The glucuronyltransferase and sulfotransferase are considered to be the key enzymes in the biosynthesis of this epitope because the rest of the structure occurs often in glycoconjugates. Here we describe the isolation of the rat sulfotransferase cDNA via an expression cloning strategy. The clone finally isolated predicts a protein of 356 amino acids, with characteristics of a type II transmembrane protein and with no sequence similarity to other known sulfotransferases. Both the enzyme expressed as a soluble fusion protein and homogenates of cells transfected with the full-length cDNA could transfer sulfate from a sulfate donor to acceptor substrates containing terminal glucuronic acid.

The antibody only binds to the sulfated form (17). Several other monoclonal antibodies have been isolated that recognize identical or similar structures (4, 18); of these, L2–412 is important for this study, because it also recognizes the non-sulfated form of the carbohydrate (19).

The key enzymes in the biosynthesis of HNK-1 carbohydrates are a glucuronyltransferase (20, 21), transferring GlcA in β1→3 linkage to a terminal galactose, and a sulfotransferase (22), responsible for coupling sulfate to the C-3 position of this GlcA residue. A cDNA encoding the glucuronyltransferase involved in the biosynthesis of at least the HNK-1 glycoprotein epitope has recently been cloned (23). We describe here the cloning of a cDNA coding for a sulfotransferase active on terminal glucuronic acid residues and whose expression can render cells immunoreactive with HNK-1 antibody when co-transfected with a glucuronyltransferase cDNA.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Plasmids—CHOP2 cells (24) were grown in minimal essential medium supplemented with 10% fetal calf serum, penicillin/streptomycin, and 200 μg/ml G418 (all from Life Technologies, Basel, Switzerland). For transfections, G418 was omitted. Hybridoma supernatants containing antibodies HNK-1 (mouse, Ref. 1) and L2–412 (rat, Ref. 4) were produced as described (18) and used without further purification. The glucuronyltransferase cDNA was in the mammalian expression vector pEF-BOS (23).

Poly(A)* RNA from cerebral cortex of newborn rats was converted to double-stranded cDNA using a kit from Stratagene, and a 1–3 kb fragment was prepared by gel electrophoresis. The sulfotransferase cDNA plasmid. After centrifugation, the cells from an 80-cm2 flask were taken up in 250 μl of 100 mM Bis-TRIS, pH 6.6, containing mixed protease inhibitors (chymostatin/pepstatin A/leupeptin/antipain/aprotinin, all at 10 μg/ml). Aliquots were stored at −20 °C and only

* This work was supported by the Jules Thorn Trust and by the Russian Foundation for Basic Research Grant N97–03-33037a. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession numbers AF022729.

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RECEIVED FOR PUBLICATION, SEPTEMBER 12, 1997

Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 47, Issue of November 21, pp. 29942–29946, 1997

Printed in U.S.A.

29942 This paper is available on line at http://www.jbc.org
A GlcA-dependent Sulfotransferase Activity Is Found in Transfected Cells—The HNK-1 reactivity after transfection with the sulfotransferase alone (Fig. 1B) but clearly higher than in mock transfected cells, is seen after transfection with the glucuronyltransferase cDNA clone, therefore, is likely to encode the HNK-1 sulfotransferase and then to several percent of all cells (Fig. 1C). Western blotting of proteins isolated from transfected cells (Fig. 2) confirms these results. Mock transfected cells gave no signal with L2–412 or HNK-1, cells transfected with glucuronyltransferase cDNA alone showed only L2–412 reactive proteins, and cells transfected with both glucuronyltransferase and sulfotransferase cDNAs were positive with both antibodies. Faint staining of probably a single protein is seen with both L2–412 and HNK-1 in blots of proteins from cells transfected with only the glucuronyltransferase cDNA; EST, expressed sequence tag.

RESULTS

Expression Cloning of the HNK-1 Sulfotransferase cDNA—For expression screening, we used the cell line CHOP2 (24), a derivative of the Lec2 cell line (29) that lacks the CMP-sialic acid transporter. This mutation results in an increase in glycoproteins and glycolipids terminating in β4-galactose, potentially increasing the amount of substrate available to the glucuronyl and subsequently acting sulfotransferase. Transfection of CHOP2 cells with the recently cloned glucuronyltransferase (25) indeed led to very clear surface staining of the cells with antibody L2–412, which recognizes the nonsulfated form of the carbohydrate (Fig. 1F). A single clone was isolated from the transfected cell homogenate (50 μl of human IgG-agarose beads (Sigma) after addition of 0.05% sodium azide and mixed protease inhibitors (see above). The beads were washed 3 times with 1 ml of 100 mM Bis-TRIS, pH 6.6, containing 0.05% sodium azide, 1 mM MnCl2, and 1 mg/ml bovine serum albumin and then stored in this buffer at 4 °C in the same volume as the cell pellets. Assays were carried out directly with the beads as well as with cell homogenates, except that Triton X-100 was omitted.

For the protein A fusion protein, the medium of transfected cells was replaced 1 day after transfection by medium with 5% low immunoglobulin calf serum (Life Technologies, Basel, Switzerland) and incubated for 2 more days. The medium (20 ml) was filtered (5 μl) and incubated overnight with 100 μl of human IgG-agarose beads (Sigma) after addition of 0.05% sodium azide and mixed protease inhibitors (see above). The beads were washed 3 times with 1 ml of 100 mM Bis-TRIS, pH 6.6, containing 0.05% sodium azide, 1 mM MnCl2, and 1 mg/ml bovine serum albumin and then stored in this buffer at 4 °C in the same volume as the cell pellets. Assays were carried out directly with the beads as well as with cell homogenates, except that Triton X-100 was omitted.

The sulfotransferase assays were done in 100 mM Bis-TRIS (pH 6.6), 10 mM MnCl2, 2.5 mM ATP, and 0.1% Triton X-100, in a final volume of 20 μl including 10 μl of the transfected cell homogenate (50 μg protein) or 5 μl of 25% brain homogenate (30 μg protein); 100 pmol of [35S]PAPS1 (900 Bq; from New England Nuclear, diluted with unlabeled PAPS from Sigma), and 10 nmol of acceptor substrate. The acceptors used were 4-nitrophenyl-β-D-galactose (Galβ-pNP), 4-nitrophosphoryl-β-D-glucuronic acid (GlcAβ-pNP) (both Fluka) or 2-heptanoylamidetheryl-(3-O-β-D-glucuronyl)-β-D-galactose (GlcAβ1→3Galβ-R). The mixture was incubated for 2 h at 37 °C with mild shaking. Then 100 μl of 4:6 water:methanol was added to the samples and centrifuged, and the residue was reextracted with 100 μl of 1:1 water:methanol. Combined supernatants were dried, redissolved in 10 μl of 1:1 water:methanol, and run on aluminum-supported HPTLC plates (Silica Gel 60, Merck) in 5:4:1, chloroform:methanol:0.25% KCl/water. Activity was assessed using a Phosphorimager (Molecular Dynamics) or by autoradiography.

1 The abbreviations used are: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; Galβ-pNP, 4-nitrophosphoryl-β-D-galactose; GlcAβ→3Galβ-R, 2-heptanoylamidetheryl-(3-O-β-D-glucuronyl)-β-D-galactose; GlcAβ-pNP, 4-nitrophosphoryl-β-D-glucuronic acid; HPTLC, high performance-thin layer chromatography; EST, expressed sequence tag.  

2 A. V. Kornilov, L. O. Kononov, A. A. Sherman, and N. E. Nifant’ev, unpublished data.
sulfotransferase cDNA (Fig. 2, A and B, lanes 3), suggesting that Chinese hamster ovary cells already expose a low level of acceptor that can be used by the transfected sulfotransferase. This protein is presumably responsible for the faint HNK-1 staining observed on whole cells transfected only with sulfotransferase cDNA.

The presence of sulfotransferase activity was confirmed by enzyme assays in vitro. Sulfotransferase activity was determined with Galβ1→3Galβ-R and GlcAβ1→3Galβ-R as acceptors (Fig. 3). Homogenates of cells transfected with the isolated sulfotransferase clone showed activity toward GlcAβ1→3Galβ-R as acceptor substrate. IgG beads were incubated with medium from cells expressing either the nonsecreted form of the sulfotransferase, without protein A (lane 1), the secreted fusion protein (lane 2), or the translation product from a gPROTA vector containing the sulfotransferase in antisense orientation (lane 3).

DISCUSSION

Several cDNAs encoding enzymes involved in glycosylation have been isolated by expression cloning (30). The most often used technique is panning and plasmid recovery from transfected mammalian cells. However, although the panning procedure will enrich the desired plasmid, after one or several rounds of panning, recovered plasmids are still divided into pools and tested for expression of the sugar epitope (sibling selection) (30–32). We found it much more efficient to directly start a sibling selection procedure.

The cloned sulfotransferase cDNA was shown to induce HNK-1 reactivity in CHOP2 cells only in combination with a glucuronyltransferase, indicating that these two enzymes, together with common enzymes already present in CHOP2 cells, are required and sufficient for the biosynthesis of the HNK-1 epitope on glycoproteins. It is, however, not known if these two enzymes are responsible for the synthesis of all HNK-1 carbohydrate epitopes observed in nervous tissue. While the HNK-1 carbohydrate epitope in nervous tissues is only observed on a limited number of proteins (11, 12), very many proteins seem to carry the epitope after expression of the enzymes in CHOP2 cells. The situation in CHOP2 cells may be abnormal, owing to lack of competition for acceptor by other enzymes, such as sialyltransferases and fucosyltransferases. In vitro sulfotransferase assays showed that the cloned cDNA encodes an enzyme capable of transferring sulfate from PAPS to acceptor substrates containing terminal GlcA. The disaccharide GlcAβ1→3Galβ-R is as good an acceptor as the complete glycolipid used previously to characterize the natural enzyme (22), and the acceptor preferences of the enzyme encoded by the cloned cDNA are very similar to those seen with brain homogenate. The enzyme therefore seems potentially capable of synthesizing the known HNK-1 structures on glycolipids and glycoproteins (15, 16).

Surprisingly, the cloned sulfotransferase showed no significant sequence similarity to other sulfotransferases, not even to the recently cloned sulfatide sulfotransferase (33). The latter enzyme transfers sulfate from PAPS to the C-3 of galactose residues, a reaction very similar to that of the HNK-1 sulfotransferase, expected to transfer the sulfate to C-3 of GlcA residues. As cytoplasmic sulfotransferases that use various substrates all have some sequence similarity (34), it might be expected that sulfotransferases acting on carbohydrate struc-
to also be structurally related. This is, however, not found among the enzymes whose cDNAs have been cloned so far. These comprise two different N-heparan sulfate sulfotransferases (35, 36) showing 70% sequence identity with each other, chondroitin 6-sulfotransferase (37), sulfatide sulfotransferase (33), and the HNK-1 sulfotransferase. However, all these enzymes show the same membrane topology and are predicted to be type II membrane proteins with a short N-terminal cytoplasmic domain and a larger luminal catalytic domain. This structure is typical of Golgi glycosyltransferases (38).

Several human ESTs are very similar to the cloned rat HNK-1 sulfotransferase cDNA, and probably encode the same enzyme in humans. Surprisingly, no such mouse ESTs are present in the data bases. Further ESTs, both human and mouse, are found that may encode more distant relatives of the enzyme. Although these transcripts can of course encode completely different enzymes, it is interesting that for both the HNK-1 glucuronyltransferase (23) and the HNK-1 sulfotransferase a family of related genes seems to exist. A similar situation may occur as for the fucosyltransferase gene family (39), wherein the enzymes differ only slightly in acceptor specificity. Such variations might be responsible for at least some of the marked differences observed in developmental and spatial patterns of HNK-1 immunostaining when different species are compared (7, 11).

The availability of clones for both a glucuronyltransferase and a sulfotransferase responsible for the biosynthesis of the HNK-1 epitope will greatly enhance studies on the regulation of the expression of these carbohydrate structures. The enzymes can be used to produce substantial amounts of HNK-1 carbohydrate, allowing more detailed testing than heretofore possible of its role in the nervous system.

Acknowledgments—We thank Sandra Ka¨lin and Barbara Wa¨fler for excellent technical assistance and Dr. James Dennis of the University of Toronto for the CHOP2 cells.

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Fig. 4. Complete nucleotide and deduced amino acid sequence of the HNK-1-sulfotransferase cDNA clone. The putative transmembrane region in the translation product is underlined, and potential N-linked glycosylation sites are indicated by asterisks.
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