Physical and Functional Association of Glucuronyltransferases and Sulfotransferase Involved in HNK-1 Biosynthesis*

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HNK-1 carbohydrate expressed predominantly in the nervous system is considered to be involved in cell migration, recognition, adhesion, and synaptic plasticity. Human natural killer-1 (HNK-1) carbohydrate has a unique structure consisting of a sulfated trisaccharide (HSO3-3GlcAβ1–3Galβ1–4GlcNAc-) and is sequentially biosynthesized by one of two glucuronyltransferases (GlcAT-P or GlcAT-S) and a sulfotransferase (HNK-1ST). Considering that almost all the HNK-1 carbohydrate structures so far determined in the nervous system are sulfated, we hypothesized that GlcAT-P or GlcAT-S functionally associates with HNK-1ST, which results in efficient sequential biosynthesis of HNK-1 carbohydrate. In this study, we demonstrated that both GlcAT-P and GlcAT-S were co-immunoprecipitated with HNK-1ST with a transient expression system in Chinese hamster ovary cells. Immunofluorescence staining revealed that these enzymes are mainly co-localized in the Golgi apparatus. To determine which domain is involved in this interaction, we prepared the C-terminal catalytic domains of GlcAT-P, GlcAT-S, and HNK-1ST, and we then performed pulldown assays with the purified enzymes. As a result, we obtained evidence that mutual catalytic domains of GlcAT-P or GlcAT-S and HNK-1ST are important and sufficient for formation of an enzyme complex. With an in vitro assay system, the activity of HNK-1ST increased about 2-fold in the presence of GlcAT-P or GlcAT-S compared with that in its absence. These results suggest that the function of this enzyme complex is relevant to the efficient sequential biosynthesis of the HNK-1 carbohydrate.

Glycosylation is one of the major post-translational protein modifications, especially for cell surface proteins, that play important roles in a variety of cellular functions, including recognition and adhesion. Among them, human natural killer-1 (HNK-1) carbohydrate, which is recognized by HNK-1 monoclonal antibody, is predominantly expressed in the nervous system (1, 2). HNK-1 carbohydrate is expressed on several glycoproteins and glycolipids and is considered to be involved in cell migration, recognition, and adhesion. The unique structural feature of this carbohydrate is a sulfated trisaccharide (HSO3-3GlcAβ1–3Galβ1–4GlcNAc-) (3, 4). Recently, we cloned two glucuronyltransferases (GlcAT-P and GlcAT-S) (5–9) and one sulfotransferase (HNK-1ST) (10) that are involved in the biosynthesis of HNK-1 carbohydrate. To investigate the biological function of HNK-1 carbohydrate in vivo, we generated GlcAT-P gene-deficient mice and revealed that HNK-1 carbohydrate is involved in synaptic plasticity (11). More recently, we found a nonsulfated form of HNK-1 carbohydrate in mouse kidney (12), where only GlcAT-S was expressed and not HNK-1ST. In the nervous system, however, all the HNK-1 carbohydrate structures identified so far are sulfated forms because of the presence of glucuronyltransferases (GlcAT-P and GlcAT-S) and HNK-1ST (13, 14). The fact that only the existence of HNK-1ST is sufficient for the sulfation of HNK-1 carbohydrate and that a nonsulfated form has not been detected in brain leads us to hypothesize that GlcAT-P or GlcAT-S interacts with HNK-1ST, resulting in sequential and efficient biosynthesis of the HNK-1 carbohydrate.

Oligosaccharides expressed on glycoproteins and glycolipids are biosynthesized by glycosyltransferases in a stepwise manner. Recently, endoplasmic reticulum (ER) or Golgi resident glycosyltransferases and related proteins, such as epimerase, sulfotransferase, and sugar-nucleotide transporters, were found to associate physically and functionally both for retention in the ER or Golgi and for regulation of their enzymatic activities (15, 16). In many cases, the N-terminal cytoplasmic tail, membrane-spanning domain, or stem region, but not the C-terminal catalytic domain, is needed and sufficient for formation of a complex.

In this study, we investigated whether or not GlcAT-P or GlcAT-S interacts with HNK-1ST by using a transient expression system in CHO cells and by performing immunoprecipitation and immunofluorescence staining. Furthermore, we examined the effects of the interaction on their catalytic activities in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials

Monoclonal antibody (mAb) M6749 was a generous gift from Dr. H. Tanaka (Kumamoto University). Anti-HNK-1 mAb was purchased from the American Type Culture Collection. Mouse anti-FLAG M2 mAb and rabbit anti-FLAG polyclonal antibodies (pAb) were purchased from Sigma. Mouse anti-EGFP mAb and rabbit anti-EGFP pAb were purchased from Clontech. HRP-conjugated anti-mouse IgG and HRP-conjugated anti-mouse IgM were purchased from Zymed Laboratories Inc.. Alexa Fluor 568 anti-mouse IgG was purchased from Molecular

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The abbreviations used are: HNK-1, human natural killer-1; ASOR, asialo-orosomucoid; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein; GAG, glycosaminoglycan; GlcAT, glucuronyltransferase; HRP, horseradish peroxidase; mAb, monoclonal antibody; pAb, polyclonal antibodies; PBS, phosphate-buffered saline; ST, sulfotransferase; BiTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxyethyl)propane-1,3-diol; MES, 4-morpholineethanesulfonic acid; ER, endoplasmic reticulum.
Expression Plasmid

Construction of Full-length cDNAs in pIRES—Rat GlcAT-P cDNA was released from pCRII/GlcAT-P (5) with EcoRI and inserted into the EcoRI site of multicloning site A in pIRES (pIRES/P). Rat HNK-1ST cDNA was amplified by PCR with the primer pair listed below using pBlueScript/HNK-1ST as a template to create SalI sites at both the 5' and 3' ends of the coding sequence of HNK-1ST, and then cloned into the SacI site of multicloning site B in pIRES/P (pIRES/P+ST). Rat GlcAT-S cDNA was released from pEF-BOS/GlcAT-S (6) with XbaI and then inserted into the Nhel site of multicloning site A in pIRES (pIRES/S). HNK-1ST cDNA was then released with Sall and NotI from pBlueScript/HNK-1ST and cloned into multicloning site B in pIRES digested with the same restriction enzymes (pIRES/S+ST).

Construction of Epitope-tagged cDNAs in Expression Plasmid—Full-length cDNAs of the rat GlcAT-P, rat GlcAT-S, human GlcAT-I, and rat ST3GalV coding sequences were amplified by PCR using the primers listed below to create a 5'-EcoRI site (skipping initiation ATG codon) and a 3'-EcoRV site, and then cloned into p3XFLAG-CMV-10 (SIGMA) using the EcoRI and EcoRV sites. The rat HNK-1ST cDNA was released from Sall and NotI from pBlueScript/HNK-1ST and cloned into multicloning site B in pIRES/S digested with the same restriction enzymes (pIRES/S+ST).

Cell Culture and Transfection

CHO cells were cultured in α-minimum Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C until 50 – 80% confluency. For transfection, cells were plated on 100-mm tissue culture dishes, grown overnight, and then transfected with various expression constructs using FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. Briefly, 2.5-fold volume of FuGENE 6 and 4 μg of each DNA were incubated with 300 μl of α-minimum Eagle’s for 15 min at room temperature in a polystyrene tube, and then the mixture was added to the tissue culture dishes.

Cell Lysis and Immunoprecipitation of Transiently Expressed Proteins

Cells were collected 24 h after transfection and lysed with lysis buffer consisting of 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and protease inhibitor mixture (Nacalai Tesque). After centrifugation, the clarified lysate was incubated with anti-FLAG rabbit pAb (Sigma) or anti-EGFP rabbit pAb (Clontech) for 1 h. The mixture was then incubated with protein G-Sepharose TM4 Fast Flow (Amersham Biosciences) for 2 h with gentle shaking. The beads were precipitated by centrifugation (500 × g for 3 min) and then washed three times with an excess volume of wash buffer consisting of 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Triton X-100. Proteins bound to the Sepharose beads were eluted by boiling in Laemmli sample buffer.

Immunostaining of CHO Cells

24 h post-transfection, the cells were rinsed with PBS, fixed with ice-cold methanol, and then incubated in PBS with 3% bovine serum albumin. The cells were incubated with anti-FLAG M2 mouse mAb (Sigma) followed by incubation with Alexa Fluor 568 anti-mouse IgG antibodies (Molecular Probes). The cells were then visualized and digitized with a Fluoview laser confocal microscope system (Olympus, Japan).

SDS-PAGE and Western Blot Analysis

Proteins were separated by 5–20% gradient SDS-PAGE with the buffer system of Laemmli and then transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in PBS containing 0.05% Tween 20, the membranes were incubated with primary antibodies, followed by HRP-conjugated secondary antibodies, and then protein bands were detected with ECL (Pierce) using a Luminogram Analyzer LAS-3000 (Fuji).

Preparation of Protein A and Protein A-fused HNK-1ST (prot.A-STcat)

COS-1 cells plated on 100-mm tissue culture dishes were transfected with pEF-BOS-prot.A or pEF-BOS-prot.A-HNK-1STcat (10 μg each) using FuGENE 6 transfection reagent. After 6 h of incubation, the culture medium was replaced with serum-free Opti-MEM I (Invitrogen), followed by incubation for a total of 3 days. Culture medium containing secreted proteins was applied to a normal human IgG-Sepharose column (Amersham Biosciences). Unbound proteins were washed out with more than 10 column volumes of wash buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and sequentially with 2 column volumes of 10 mM glycine–HCl, pH 15.0. Bound proteins were eluted with 100 mM glycine–HCl, pH 3.4, and then immediately neutralized with 1 M Tris–HCl, pH 8.0.

Pulldown Assay

The catalytic domains of human GlcAT-P and human GlcAT-S were expressed in and purified from Escherichia coli as described previously.
Association of HNK-1 Carbohydrate Synthetic Enzymes

(17). Prot.A-STcAt and protein A were purified from COS-1 cell culture media as described above. An aliquot (125 ng) of each protein was added to 250 µl of buffer (100 mM MES, pH 6.5, 0.2% Nonidet P-40, 5 mM MnCl₂), and then the mixture was incubated for 1 h at 37 °C. A fraction of the mixture was recovered for Western blot analysis, and normal IgG-Sepharose was added to the rest of the mixture followed by incubation for 2 h. The beads were precipitated by centrifugation (500 × g for 3 min) and then washed three times with an excess volume of buffer. Proteins bound to beads were eluted by boiling in Laemmli sample buffer, followed by SDS-PAGE and Western blot analysis with anti-FLAG M2 mAb.

Glucuronyltransferase Assay

Glucuronyltransferase activity toward asialo-orosomucoid (ASOR) was measured essentially as described previously (18) with slight modification. Before adding to the reaction mixture, FLAG-Pcat or FLAG-Scat was incubated with or without an equivalent amount of prot.A-STcAt at 37 °C for 30 min for enzyme complex formation. Then the preincubated enzyme solution was added to the assay reaction mixture as described previously (18).

Sulfotransferase Assay

For this assay, we prepared an acceptor substrate, GlcA-ASOR, by transferring glucuronic acid to ASOR with GlcAT-P and GlcAT-S. FLAG-Pcat and FLAG-Scat (750 ng each) were added to 3 ml of the reaction mixture (100 mM MES, pH 6.5, 0.2% Nonidet P-40, 2.5 mM ATP, 20 mM MnCl₂, 3 mg of ASOR, 1.2 µmol of UDP-GlcA), followed by incubation at 37 °C for 6 h. 375 ng of FLAG-Pcat and FLAG-Scat were then added to the mixture, followed by incubation at 37 °C overnight. The prepared mixture was dialyzed against an excess volume of sterile water. FLAG-Pcat and FLAG-Scat were then removed with a Blue-Sepharose column (Amersham Biosciences). Under these conditions, about 9.1 mol of GlcA was transferred to 1 mol of ASOR. The purified prot.A-STcAt was incubated with or without an equivalent amount of FLAG-Pcat or FLAG-Scat at 37 °C for 30 min for enzyme complex formation. The preincubated enzyme solution was then incubated at 37 °C for 1 h in a reaction mixture with a final volume of 50 µl consisting of 20 mM BisTris-HCl, pH 6.6, 0.1% Triton X-100, 10 mM MnCl₂, 2.5 mM ATP, 20 µg of GlcA-ASOR, and 100 µM [35S]Adenosine 3′-phosphate, 5′-phosphosulfate (300,000 dpm). After incubation, the assay mixture was spotted onto a 2.5-cm Whatman No.1 disk. The disk was washed with a 10% (v/v) trichloroacetic acid solution three times, followed by ethanol/ether (2:1, v/v), and then with ether. The disk was air-dried, and then the radioactivity of [35S]HSO₃⁻GlcA-ASOR was counted with a liquid scintillation counter (Beckman LS-6000).

RESULTS

Co-immunoprecipitation of GlcAT-P or GlcAT-S and HNK-1ST—HNK-1 carbohydrate is highly expressed in the nervous system. The HNK-1 carbohydrate structures on P0 and NCAM, which are major carrier glycoproteins in the peripheral and central nervous systems, respectively, have been determined (13, 14). All the HNK-1 carbohydrate structures so far determined are sulfated, i.e. sulfoglucuronic acid attached to N-acetyllactosamine, a nonsulfated form of HNK-1 carbohydrate not having been found on such molecules. These results indicate that most of the HNK-1 carbohydrate in the nervous system is expressed as a sulfated form. These results suggest the possibility that the HNK-1 carbohydrate biosynthetic key enzymes, glucuronyltransferases (GlcAT-P and GlcAT-S) and sulfotransferase (HNK-1ST), associate with each other, which results in efficient sequential biosynthesis. To investigate the interaction between the glucuronyltransferases (GlcAT-P and GlcAT-S) and HNK-1ST, FLAG-tagged glucuronyltransferases (FLAG tag fused at N terminus of each glucuronyltransferase, FLAG-P or FLAG-S) and EGFP-tagged HNK-1ST (EGFP fused at C terminus of HNK-1ST, ST-EGFP) were transiently expressed in CHO cells. Western blot analysis using the cell lysates revealed that each enzyme was detected at around the position of the expected molecular weight with anti-FLAG mAb (40–50 kDa) and anti-EGFP mAb (70 kDa), respectively (Fig. 1A). However, one major band and a few minor bands were detected for each enzyme. The major band corresponded to a fully glycosylated enzyme and the minor bands to partially glycosylated enzymes because they converged as one band with N-glycosidase F digestion (data not shown). As shown in Fig. 1A, bottom panel, HNK-1 carbohydrate was expressed on several glycoproteins in CHO cells co-expressing both FLAG-P or FLAG-S and ST-EGFP (lanes 4 and 5), indicating that these fusion enzymes have enzymatic activity. However, the immunoreactive bands for the cells expressing FLAG-S (Fig. 1A, bottom panel, lane 5) were weaker than for those expressing FLAG-P (lane 4), suggesting that the activity of FLAG-P is greater than that of FLAG-S and/or that the N-terminal FLAG tag may have a little effect on the GlcAT-S activity. Next, these enzymes in CHO cell lysates were immunoprecipitated with anti-FLAG rabbit pAb or anti-EGFP rabbit pAb, and then Western blot analyses were performed with anti-FLAG mouse mAb and anti-EGFP mouse mAb, respectively. As shown in Fig. 1B, both FLAG-P and FLAG-S were co-immunoprecipitated with ST-EGFP (upper panel, lanes 7 and 9) and vice versa (lower panel, lanes 8 and 10), indicating that each glucuronyltransferase bound with HNK-1ST and formed an enzyme complex in CHO cells.

Co-localization of Glucuronyltransferases and HNK-1ST in CHO Cells—To investigate co-localization of glucuronyltransferases and HNK-1ST in CHO cells, we examined the immunofluorescence staining of the CHO cells. FLAG-P and FLAG-S were stained with anti-FLAG mAb (Fig. 2, A and D), and ST-EGFP was visualized according to its own EGFP fluorescence (Fig. 2, B and E). As shown in Fig. 2, C and F, both FLAG-P and FLAG-S were co-localized with HNK-1ST in CHO cells. These enzymes were mainly localized in the Golgi apparatus.
because they were co-localized with a Golgi marker, wheat germ agglutinin (data not shown).

**Binding Specificities as to Formation of These Enzyme Complexes**—To investigate the binding specificities of these interactions, we performed immunoprecipitation experiments using other glycosyltransferases instead of GlcAT-P or GlcAT-S. We used FLAG-tagged GlcAT-I (Fig. 3, A and B) involved in the biosynthesis of the linkage region of glycosaminoglycans, and GlcAT-I expresses amino acid sequence similarity with GlcAT-P and GlcAT-S (19). We expected that GlcAT-I would not bind to HNK-1ST, but FLAG-GlcAT-I (FLAG-I) was detected in the anti-EGFP pAb immunoprecipitate (Fig. 3B, upper panel, lane 5), and ST-EGFP was detected in anti-FLAG pAb immunoprecipitate (Fig. 3B, lower panel, lane 6), indicating that GlcAT-I also bound with HNK-1ST, as in the case of GlcAT-P and GlcAT-S. Next we used FLAG-tagged ST3GalIV (Fig. 3, C and D), which is a sialyltransferase and transfers sialic acid to the nonreducing terminal of N-acetyllactosaminyl residues on glycoproteins (20). This enzyme is thought to compete with GlcAT-P or GlcAT-S for the acceptor substrates, i.e. the nonreducing terminal of N-acetyllactosaminyl residues. As shown in Fig. 3D (upper panel, lane 5, and lower panel, lane 6), the interaction of ST3GalIV with HNK-1ST was almost negligible compared with that of GlcAT-P or GlcAT-S with HNK-1ST, indicating that ST3GalIV did not form a complex with HNK-1ST. These results suggest that GlcAT-P and GlcAT-S bind specifically with HNK-1ST and that the interaction of GlcAT-I with HNK-1ST may have some biological significance in vivo.

To examine the binding specificity of sulfotransferases, we used two sulfotransferases, CAST1 and GalNAc4ST1, both of which belong to the HNK-1ST family (21, 22). An EGFP tag was fused with these sulfotransferases at their C termini, and then they were co-expressed with FLAG-tagged GlcAT-P in CHO cells. By using these cell lysates, immunoprecipitation and Western blot analyses were carried out (Fig. 4, A and B). As expected, FLAG-P was not detected in the anti-EGFP pAb immunoprecipitate (Fig. 4B, upper panel, lanes 3 and 5), and both CAST1-EGFP and GalNAc4ST1-EGFP were barely detected in the anti-FLAG immunoprecipitate (Fig. 4B, lower panel, lanes 4 and 6), suggesting that the interactions of both CAST1 and GalNAc4ST1 with GlcAT-P are considerably weaker than that of HNK-1ST with GlcAT-P. We also confirmed that GlcAT-S was not co-immunoprecipitated with either CAST1 or GalNAc4ST1 (data not shown).

As described above, ST3GalIV, CAST1, and GalNAc4ST1 did not bind or barely bound with HNK-1 biosynthetic enzymes (GlcAT-P and GlcAT-S and HNK-1ST). However, it is possible that these overexpressed enzymes showed the abnormal subcellular localization because of their fused tags; therefore, they may not have had the opportunity to encounter their counterparts in CHO cells. To exclude this possibility, we examined their intracellular localization by means of immunofluorescence staining (Fig. 5). FLAG-I and FLAG-ST3GalIV were stained with anti-FLAG mAb (Fig. 5, A and D). CAST1-EGFP and GalNAc4ST1-EGFP were visualized according to their own EGFP fluorescence (Fig. 5, H and K). As shown in Fig. 5, A, D, H, and K, they were mainly localized in the Golgi apparatus, suggesting that the fused tags had no influence on their intracellular transport. Even if they were considerably co-localized with their counterparts as shown in Fig. 5, C, F, J, and L, they would not interact with each other (Figs. 3D and 4B). These lines of evidence indicate the specific interaction of GlcAT-P or GlcAT-S with HNK-1ST. GlcAT-P (or GlcAT-S) and HNK-1ST Form a Complex through Their Catalytic Domains—As shown in Fig. 3, GlcAT-I as well as GlcAT-P and GlcAT-S interacted with HNK-1ST. These three glucuronotrans-
ferases have a type II membrane topology as in the case of other glycosyltransferases, i.e. an N-terminal short cytoplasmic tail, a transmembrane region, a stem region, and a catalytic region. GlcAT-I exhibits amino acid sequence similarity with GlcAT-P and GlcAT-S, the highest homology being seen in the catalytic domain. Thus, we hypothesized that the C-terminal catalytic domain of these enzymes is involved in the interaction with HNK-1ST. To examine this hypothesis, we prepared truncated enzymes only consisting of their catalytic domains. The FLAG-tagged human GlcAT-P catalytic domain (FLAG-Pcat, T58 to C terminus) and FLAG-tagged human GlcAT-S catalytic domain (FLAG-Scat, A50 to C terminus) were expressed in COS-1 cells and purified from the culture medium on a normal protein A column. We then performed pulldown assays using FLAG-Pcat or FLAG-Scat and prot.A-STcat in the presence or absence of FLAG-Pcat or FLAG-Scat (Fig. 7A, lane 1). FLAG-Scat (lane 2), prot.A-STcat (lane 3), both prot.A-STcat and FLAG-Pcat (lane 4), both prot.A-STcat and FLAG-Scat (lane 5), only protein A (lane 6), both protein A and FLAG-Pcat (lane 7), or both protein A and FLAG-Scat (lane 8) were incubated at 37 °C and then subjected to SDS-PAGE and Western blot (WB) analysis with HRP-conjugated normal rabbit IgG (upper panel) or anti-FLAG mAb (middle panel). Lower panel, a preincubated mixture was incubated with normal IgG-Sepharose beads for pull down of prot.A-STcat, protein A, and their binding proteins. Proteins bound to the beads were then subjected to SDS-PAGE and Western blot analysis with anti-FLAG mAb.

Effect of the Interaction on the Glucuronyltransferase and Sulfotransferase Activities in Vivo—Because the catalytic domains are involved in the formation of a complex, it is suggested that their catalytic activities might be regulated by the interaction. To examine the influence of the interaction on the catalytic activity, we measured the glucuronyltransferase activities of the purified recombinant FLAG-Pcat and FLAG-Scat prepared from the E. coli expression system in the presence or absence of prot.A-STcat (Fig. 7, A and B). ASOR was used as an acceptor substrate to measure the glucuronyltransferase activity, and the assay was carried out as described previously (18). Under these assay conditions, the bacterially expressed FLAG-Pcat and FLAG-Scat exhibit the specific activities of about 1,100 and 450 nmol/min/mg protein, respectively (17). The specific activities were comparable with that of GlcAT-P purified from rat brain (9). As shown in Fig. 7, A and B, the activities of FLAG-Pcat and FLAG-Scat were hardly affected by the addition of prot.A-STcat. We also measured the sulfotransferase activity of the purified prot.A-STcat in the presence or absence of FLAG-Pcat or FLAG-Scat (Fig. 7B). As an acceptor substrate, we prepared GlcA-ASOR as described under “Experimental Procedures.” The sulfotransferase activity of prot.A-STcat increased with the addition of FLAG-Pcat or FLAG-Scat to a similar degree (about 2-fold). These results indicate that the formation of an enzyme complex through the catalytic domain up-regulates HNK-1ST activity.

Effect of the Interaction on the Enzyme Activities in Vivo—We found that the formation of an enzyme complex regulated sulfotransferase activity in vitro. Next, we investigated whether or not the interaction also regulated enzymatic catalysis in vivo. As shown in Fig. 1A, bottom panel, the HNK-1 biosynthesis ability of FLAG-tagged GlcAT-S is lower than that of FLAG tagged GlcAT-P. This may be because of the fused tag at its N terminus. To eliminate the effect of fused tags, full-length cDNAs were subcloned into the pIRES vector to express no-tagged enzymes. The expression vector pIRES has an internal ribosome entry site that allows two kinds of cDNAs to be expressed. We prepared four kinds of vectors that encoded GlcAT-P (pIRES/P), GlcAT-S (pIRES/S),

![FIGURE 5. Intracellular Localization of GlcAT-I, ST3GalIV, C4ST1, and GalNAc4ST1.](Image 70x569 to 280x734)

![FIGURE 6. In vitro pulldown assays using FLAG-Pcat or FLAG-Scat and prot.A-STcat.](Image 361x554 to 517x733)
a novel mechanism may regulate glucuronyltransferase activity. The transferase activities of these enzymes toward glycoprotein substrates were measured. Taking the activity in the absence of a binding partner as 100%, the activity in the presence of the partner is shown as the relative percentage. All experiments were carried out three times independently. A, the glucuronyltransferase activity of FLAG-Scat toward ASOR was measured with (right bar) or without (left bar) prot.A-STcat. B, the glucuronyltransferase activity of FLAG-Scat toward ASOR was measured with (right bar) or without (left bar) prot.A-STcat. C, the sulfotransferase activity of prot.A-STcat toward GlcA-ASOR was measured without glucuronyltransferase (left bar), with FLAG-Scat (middle bar), or with FLAG-Scat (right bar).

FIGURE 7. In vitro transferase activity of GlcAT-P, GlcAT-S, and HNK-1ST with or without binding counterparts. The transferase activities of these enzymes toward glycoprotein substrates were measured. Taking the activity in the absence of a binding partner as 100%, the activity in the presence of the partner is shown as the relative percentage. All experiments were carried out three times independently. A, the glucuronyltransferase activity of FLAG-Scat toward ASOR was measured with (right bar) or without (left bar) prot.A-STcat. B, the glucuronyltransferase activity of FLAG-Scat toward ASOR was measured with (right bar) or without (left bar) prot.A-STcat. C, the sulfotransferase activity of prot.A-STcat toward GlcA-ASOR was measured without glucuronyltransferase (left bar), with FLAG-Scat (middle bar), or with FLAG-Scat (right bar).

FIGURE 8. Effect of the interaction on glucuronyltransferase activity in CHO cells. CHO cells were transfected with the empty vector (lane 1), pIREs/P (GlcAT-P) (lane 2), pIREs/P + ST (GlcAT-P + HNK-1ST) (lane 3), pIREs/S (GlcAT-S) (lane 4), and pIREs/S + ST (GlcAT-S + HNK-1ST) (lane 5). The cell lysates were subjected to SDS-PAGE and Western blot analysis with M6749 mAb (A), HNK-1 mAb (B), anti-GlcAT-P pAb (C), or anti-GlcAT-S pAb (D).

both GlcAT-P and HNK-1ST (pIREs/P + ST), and both GlcAT-S and HNK-1ST (pIREs/S + ST). These four plasmids were transfected in CHO cells, and then we performed Western blot analysis using cell lysates with HNK-1 mAb or M6749 mAb (Fig. 8). HNK-1 mAb only recognizes sulfated glucuronic acid attached to N-acetyllactosamine residues, although M6749 mAb recognizes both sulfated and nonsulfated forms (12). As shown in Fig. 8B, several bands were detected with HNK-1 antibody only when GlcAT-P or GlcAT-S and HNK-1ST were co-expressed (lanes 3 and 5), whereas both sulfated and nonsulfated forms of HNK-1 carbohydrate were detected with M6749 mAb in Fig. 8A, lanes 2–5. The immunoreactive bands with M6749 antibody when both GlcAT-P and HNK-1ST were expressed (Fig. 8A, lane 3) were more intense compared with those when only GlcAT-P was expressed (Fig. 8A, lane 2). However, the expression level of GlcAT-P when only GlcAT-P was expressed (Fig. 8C, lane 2) was higher than when both GlcAT-P and HNK-1ST were expressed (Fig. 8C, lane 3). Therefore, the relative intensity of immunoreactive bands with M6749 antibody versus the amount of GlcAT-P was more increased by HNK-1ST. In the case of GlcAT-S, although the enzyme itself was expressed at a similar level regardless of the existence of HNK-1ST (Fig. 8D), the immunoreactive bands with M6749 antibody when GlcAT-S and HNK-1ST were co-expressed were greater than those when only GlcAT-S was expressed (Fig. 8A, lanes 4 and 5). These results suggest that the amount of product synthesized by GlcAT-P and GlcAT-S increases with the co-existence with HNK-1ST. However, as shown in Fig. 7, glucuronyltransferase activity was not affected by HNK-1ST in our in vitro assay system. We are not able to explain this difference under the present conditions, but a novel mechanism may regulate glucuronyltransferase activity in vivo.

DISCUSSION

In this study, we demonstrated that glucuronyltransferases (GlcAT-P and GlcAT-S) and HNK-1ST, all of which are involved in HNK-1 biosynthesis, directly interact with each other through their catalytic domains on immunoprecipitation and pulldown assays. Moreover, we showed that this interaction enhanced the enzymatic activity of HNK-1ST in vitro. As for the mode of interaction, the bacterially expressed GlcAT-P and GlcAT-S lacking of glycosylation were used for the pull-down assays (Fig. 6). Thus, their interaction with HNK-1ST could not be due to the binding of GlcAT’s glycans, suggesting that they presumably associate through protein–protein interaction rather than protein–carbohydrate interaction.

As for the physiological significance of this interaction, there are two possibilities. First, the HNK-1 carbohydrate attaches to limited kinds of glycoproteins, such as NCAM, L1, P0, tenascin-R, phosphacan, etc., in the nervous system. The association could be involved in regulation of the acceptor glycoprotein specificity of GlcAT-P and GlcAT-S. By associating with HNK-1ST, GlcAT-P (or GlcAT-S) may reach a limited region in the Golgi apparatus or there may be a change in the conformation of their catalytic domains resulting in the recognition of limited kinds of substrate. However, overexpression in CHO cells, the intracellular localization of GlcAT-P, GlcAT-S, and HNK-1ST did not change whether or not the binding partner was expressed (data not shown). Moreover, as shown in Fig. 8A, GlcAT-P (or GlcAT-S) transferred glucuronic acid to similar kinds of proteins regardless of the presence of HNK-1ST. These results indicate that the substrate specificities of GlcAT-P and GlcAT-S are not likely to be affected by the interaction with HNK-1ST. Second, this association could be involved in the efficient sequential biosynthesis of HNK-1 carbohydrate. This is supported by the fact that the almost all the HNK-1 carbohydrate found in the nervous system was expressed as a sulfated form. Furthermore, we recently generated GlcAT-P gene-deficient mice (11) and another group analyzed HNK-1ST gene-deficient mice (23). These two types of mutant mice showed very similar phenotypes, i.e. abnormal synaptic plasticity, suggesting that HNK-1 carbohydrate normally functions in the nervous system only when sequential sulfation occurs as soon as glucuronic acid is transferred. This was coincident with the finding that the association had an effect on the enhancement of the sulfotransferase activity but not on the glucuronosyltransferase activity (Fig. 7). These lines of evidence indicate that GlcAT-P and GlcAT-S associate with HNK-1ST, resulting in efficient biosynthesis of HNK-1 carbohydrate in the nervous system.

Over the past few years, many groups have reported that several proteins related to carbohydrate biosynthesis form heterocomplexes.
The first report was about a heterocomplex of N-acetylgalcosaminyltransferase I and mannosidase II (24). These two enzymes are both relevant to N-glycan biosynthesis and associate through their stem regions. Heterocomplexes of glycosyltransferases involved in glycolipid biosynthesis have also been reported. Giraudo et al. (25) revealed that N-acetylgalactosaminyltransferase and galactosyltransferase 2, both of which are involved in GM1 biosynthesis, associate through their N-terminal cytosolic tails. They also reported an association among three enzymes, galactosyltransferase 1, sialyltransferase 1, and sialyltransferase 2, which are involved in the biosynthesis of ganglioside precursors (26). These three enzymes also interact through their N-terminal domains. In the case of GAG biosynthesis, McCormick et al. (27) reported that EXT1 and EXT2 associate, resulting in a change in their intracellular localization from the ER to the Golgi apparatus. EXT1 and EXT2 are involved in the polymerization of heparan sulfate, and both of them exhibit even polymerization activity alone. However, the heterocomplex possesses substantially higher glycosyltransferase activity than EXT1 or EXT2 alone. This association is thought to occur through their C-terminal catalytic domains, but there is no direct evidence of this. Heterocomplexes also occur among glycosyltransferases and other classes of proteins that are involved in glcan biosynthesis. Sprod et al. (28) reported that UDP-galactose transporter associates with UDP-galactose:cereamide galactosyltransferase. The intracellular localization of UDP-galactose transporter significantly changes from the Golgi to the ER on interaction with UDP-galactose:cereamide galactosyltransferase. The intramolecular domains needed for their association remain unidentified. More recently, it was reported that the recombinant soluble C-terminal catalytic domains of β3Gn-T2 and β3Gn-T8 associate and that the association enhances their enzymatic activities (16). However, they did not demonstrate whether or not these two enzymes interact in cells. The biological significance of the interaction also remains unclear. Therefore, this is the first report that glucuronoyltransferases and sulfotransferase involved in HK-1 biosynthesis form a complex through their catalytic domains. However, we cannot exclude the possibility that the N-terminal regions of GlcAT-P, GlcAT-S, and HK-1ST are involved in association with other glycosylation-synthesis related proteins. Glycosyltransferases and other related proteins may associate with each other through their several domains, so glycosylation should be strictly regulated at accurate times in proper regions. Therefore, a number of novel heterocomplexes will probably be found in the future.

In this study, we obtained the evidence that GlcAT-I involved in glycosaminoglycan biosynthesis interacts with HK-1ST (Fig. 3B). The physiological meaning of this interaction remains unclear at present. It is unlikely that the interaction of GlcAT-I with HK-1ST is responsible for the HK-1 biosynthesis because GlcAT-I exhibits no transferase activity toward the N-acetyllactosamine structure in vitro (29). Besides, even transiently overexpressed GlcAT-I produced only a negligible level of HK-1 carbohydrate compared with GlcAT-P and GlcAT-S, as judged on Western blot analysis (data not shown). Thus, we suggest that the interaction of GlcAT-I with HK-1ST may regulate the native function of GlcAT-I, that is linkage region biosynthesis of glycosaminoglycans. This hypothesis can explain the contradiction that HK-1ST is expressed where no GlcAT-P or GlcAT-S but only GlcAT-I is expressed. It should be noted that a sulfated GAG linkage region (HSO₂⁻3GlcAβ1⁻3Galβ1⁻3(Siaα2⁻6)Galβ1⁻4Xyl⁻) has been detected in sugar chains derived from human urinary thrombomodulin (30), suggesting that HK-1ST may be involved in the transfer of a sulfate group to the GAG linkage region. Moreover, it is known that some proteoglycan molecules are expressed as part-time proteoglycans such as thrombomodulin (30) and amyloid precursor protein (31). A part-time proteoglycan is a molecule that is expressed in two forms as to glycosylation. One bears long disaccharide repeats attached to the linkage region, the same as normal proteoglycan, and the other has only a linkage region that is not elongated with disaccharide repeats. The mechanism underlying this phenomenon is unknown, but if HK-1ST regulates the GlcAT-I function and transfers a sulfate group to GlcA of the linkage region, HK-1ST may be related to the formation of a part-time proteoglycan.

Recently, we succeeded in the crystallization of the catalytic domain of human GlcAT-P with N-acetyllactosamine, and we revealed the structural basis of the catalytic reaction mechanism at the atomic level (32). Now we are trying to crystallize GlcAT-S and HK-1ST in a similar way. Finally, if co-crystallization of GlcAT-P or GlcAT-S and HK-1ST is achieved, we will probably be able to elucidate the interaction mechanism and the structural change on the formation of a complex at the atomic level.

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