*JB Minireview*—New Paradigm in Glycobiology *J. Biochem.* **143,** 719–724 (2008)
doi:10.1093/jb/mvm221

# Expression and Function of the HNK-1 Carbohydrate

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Received October 25, 2007; accepted November 8, 2007; published online November 20, 2007

**Glycosylation is a major post-translational protein modification, especially for cell surface proteins, which play important roles in a variety of cellular functions, including recognition and adhesion. Among them, we have been interested in HNK-1 (human natural killer-1) carbohydrate, which is characteristically expressed on a series of cell adhesion molecules in the nervous system. The HNK-1 carbohydrate has a unique structural feature, *i.e.* a sulfated glucuronic acid is attached to the non-reducing terminal of an *N*-acetyllactosamine residue ($HSO_3$-3GlcAβ1-3Galβ1-4GlcNAc-). We have cloned and characterized the biosynthetic enzymes (two glucuronyltransferases and a sulfotransferase), and also obtained evidence that the HNK-1 carbohydrate is involved in synaptic plasticity and memory formation. In this review, we describe recent findings regarding the expression mechanism and functional roles of this carbohydrate.**

**Key words: enzyme complex, glucuronyltransferase, HNK-1, long-term potentiation, substrate specificity.**

Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ASOR, asialo-orosomucoid; CA1, cornu ammonis 1; EPSP, excitatory post-synaptic potential; GlcAT, glucuronyltransferase; HNK-1, human natural killer-1; HNK-1ST, HNK-1 sulfotransferase; LTP, long-term potentiation; NCAM, neural cell adhesion molecule; PI, phosphatidylinositol; PN, perineuronal net; SGGL, sulfoglucuronyl glycolipid; SM, sphingomyelin; TRP, transient receptor potential.

In the nervous system, various types of cells recognize and interact with each other to form a precise neural network. During these processes, carbohydrates expressed on proteins cause the structural diversity of their carrier proteins, resulting in the regulation of cell–cell recognition, interaction and cell migration (*1*). Among these carbohydrates, the HNK-1 (human natural killer-1) carbohydrate is one of the most characteristic glycoepitopes in the nervous system (*2*). The HNK-1 carbohydrate has a unique structural feature, *i.e.* a sulfated glucuronic acid is attached to the non-reducing terminal of an *N*-acetyllactosamine residue (Fig. 1) (*3*). This carbohydrate is carried by limited kinds of molecules such as cell adhesion molecules (NCAM, L1, P0, *etc.*), extracellular matrix proteins (tenascin-R, phosphacan, *etc.*) and glycolipids (SGGL-1 and SGGL-2) (*4*, *5*). The expression of the HNK-1 carbohydrate is associated with neural crest cell migration, neuron to glial cell adhesion and the outgrowth of astrocytic processes (*6*). During the course of investigation of the HNK-1 carbohydrate biosynthetic pathway, we cloned three related enzymes, that is, two glucuronyltransferases (GlcAT-P and GlcAT-S) (*7*, *8*) and a sulfotransferase (HNK-1ST) (*9*). The gene cloning of these enzymes allowed us to manipulate them genetically and provided us with new knowledge about the functions of the HNK-1 carbohydrate.

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## ENZYMATIC PROPERTIES AND ACCEPTOR SPECIFICITIES OF GLcAT-P AND GLcAT-S

The biosynthesis of the HNK-1 carbohydrate is mainly regulated by two glucuronyltransferases (GlcAT-P and GlcAT-S) and a sulfotransferase (HNK-1ST) (Fig. 1). We purified GlcAT-P from 2-week post-natal rat forebrains, and cloned the GlcAT-P cDNAs from rat, mouse and man (*7*, *10*, *11*). The primary structure deduced from the mouse cDNA sequence predicted a type II transmembrane protein comprising 347 amino acid residues. Using this cDNA as a probe, we cloned the second glucuronyltransferase (GlcAT-S) cDNAs from rat and mouse (*8*, *12*). The predicted amino acid sequence of mouse GlcAT-S consists of 324 amino acid residues and is about 50% identical to that of mouse GlcAT-P, respectively. Human GlcAT-P and GlcAT-S are named B3GAT1 and 2, respectively.

To determine how the two distinct glucuronyltransferases are involved in the biosynthesis of the HNK-1 carbohydrate, we compared the enzymatic properties of the two enzymes (*13*). Both GlcAT-P and GlcAT-S transferred glucuronic acid (GlcA) not only to a glycoprotein acceptor, asialo-orosomucoid (ASOR), but also to a glycolipid acceptor, paragloboside. However, they showed different acceptor specificities. Thus, GlcAT-P strictly recognized the *N*-acetyllactosamine (Galβ1-4GlcNAc) structure at the terminals of acceptor substrates. In contrast, GlcAT-S recognized not only the terminal Galβ1-4GlcNAc structure but also the lacto-*N*-biose (Galβ1-3GlcNAc) structure, and showed the highest activity toward triantennary *N*-linked oligosaccharides (*13*). It should be noted that the activities of these

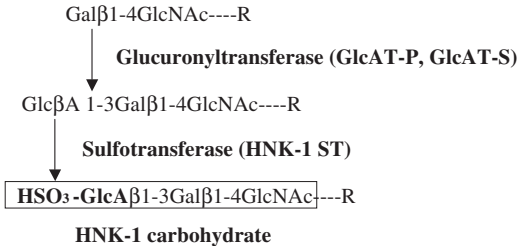



Fig. 1. **Biosynthesis and structural features of the HNK-1 carbohydrate.**

enzymes are affected by phospholipids. That is, the activity of GlcAT-P was enhanced in the presence of sphingomyelin (SM), but this effect was not observed for GlcAT-S. The activities of GlcAT-P and GlcAT-S towards a glycolipid acceptor substrate were only at detectable levels in the presence of phospholipids such as phosphatidylinositol (PI). These results suggest that the expression of the HNK-1 carbohydrate could be regulated not only by the expression level of the enzyme protein but also by the micro-environment around the enzyme, especially by the presence of SM and PI.

## CRYSTAL STRUCTURES OF GLcAT-P AND GLcAT-S

To elucidate how GlcAT-P and GlcAT-S recognize acceptor substrates at the atomic level, we determined the X-ray crystal structures of the recombinant soluble forms of human GlcAT-P and GlcAT-S (*14*, *15*). As a result, it was revealed that the structures of the overall apo-forms of GlcAT-P and GlcAT-S were very similar, and both of them formed a tight homodimer. This structure consists of 12 β-strands and 6 α-helices, and can be divided into two regions that constitute the donor and acceptor substrate-binding sites. The two regions are connected by a DXD motif, which is conserved in many UDP-sugar-dependent glycosyltransferases (*16*). All residues involved in donor substrate binding are conserved in GlcAT-P and GlcAT-S. On the other hand, there are marked differences in the manners they recognize their respective acceptor substrates. Phe245 is one of the most important GlcAT-P residues for the recognition of acceptor Galβ1–4GlcNAc. Upon acceptor binding, the side chain of Phe245 moves closer to GlcNAc and undergoes a parallel stacking interaction, but GlcAT-S has Trp234 at this position and the stacking interaction is tilted by 30 degrees. Computer-aided model building of the substrate–enzyme complexes revealed that the aromatic ring of Phe245 of GlcAT-P was stacked on the GlcNAc ring of Galβ1–3GlcNAc much more poorly than that of Galβ1–4GlcNAc. In addition, the C-terminal long loop of the neighbouring molecule serves as the key in the recognition of Galβ1–4GlcNAc. For example, Val320 and Asn321 interacted with the GlcNAc moiety through a hydrophobic interaction and a hydrogen bond, respectively, but these interactions are absent in the Galβ1–3GlcNAc bond model. These results suggested that Phe245, Val320 and Asn321 play important roles by distinguishing two different glycosidic linkages, β1-4 and β1-3. To support this, we performed the site-directed mutagenesis of GlcAT-S. Thus, the amino acid residues, *i.e*. Trp234 and Ala309, associated with the acceptor substrate recognition of GlcAT-S were doubly replaced with the corresponding amino acid residues, *i.e*. Phe245 and Val320, of GlcAT-P. The double mutant showed reduced activity towards a lacto-*N*-biose (Galβ1–3GlcNAc) substrate (*15*). These lines of evidence indicate that GlcAT-P and GlcAT-S have similar but significantly different acceptor specificities, suggesting that they may synthesize functionally and structurally different HNK-1 carbohydrates in the nervous system.

## FUNCTIONAL COMPLEX FORMATION OF HNK-1 CARBOHYDRATE-SYNTHESIZING ENZYMES

Almost all of the structures of HNK-1 carbohydrate so far reported are sulfated in the nervous system (*4*), suggesting that sulfation occurs as soon as the step of transfer of glucuronic acid to the non-reducing terminal of *N*-acetyllactosamine during biosynthesis. Besides, an increasing number of recent reports have revealed the existence of complexes of glycosyltransferases in the Golgi apparatus (*17*), and some of these complexes allow them to have higher catalytic activities than those of the single enzymes (*18*) or maintenance of the proper subcellular localization in the ER or Golgi apparatus (*19*). These facts led us to the hypothesis that a glucuronyltransferase (GlcAT-P or GlcAT-S) and the sulfotransferase (HNK-1ST) form an enzyme complex, followed by efficient biosynthesis of the HNK-1 carbohydrate epitope. Based on this assumption, we demonstrated by co-immunoprecipitation analyses that both GlcAT-P and GlcAT-S bind with HNK-1ST in cells using a transient expression system (*20*). Moreover, specific interaction of these HNK-1-related enzymes was shown by the finding that a sialyltransferase (ST3GalIV) or other sulfotransferase belonging to the HNK-1ST family (C4ST-1 or GalNAc4ST-1) was (were) not co-precipitated with HNK-1 carbohydrate-synthesizing enzymes in similar experiments. To our surprise, however, we found that GlcAT-I, which is known to be the glucuronyltransferase responsible for the biosynthesis of the linkage tetrasaccharide (GlcAβ1-3Galβ1-3Galβ1-4Xyl-R) of glycosaminoglycan (*21*), also interacted with HNK-1ST. We think that this interaction has some physiological significance because of the following two experimental facts. (i) HNK-1ST is expressed in various tissues where GlcATs (GlcAT-P and GlcAT-S) and the HNK-1 carbohydrate are not present. (ii) The sulfated linkage tetrasaccharide ($SO_4$-3GlcAβ1-3Galβ1-3(+/-Siaα2-6)Galβ1-4Xyl) has actually been found in human urine (*22*). Therefore, HNK-1ST maybe functions as a sulfotransferase that regulates the expression of the sugar chains of glycosaminoglycans such as part-time proteoglycans.

Next, we investigated the binding regions for these enzymes by means of pull-down assays using purified C-terminal catalytic domains. As a result, it was clearly demonstrated that each C-terminal region was sufficient for direct binding, while the formation of some glycosyltransferase-complexes was mediated by their transmembrane domains or the adjacent stalk region (*17*). Involvement of the catalytic domain in the GlcATs–HNK-1ST interaction suggested that their catalytic activities were influenced by the formation of complexes.

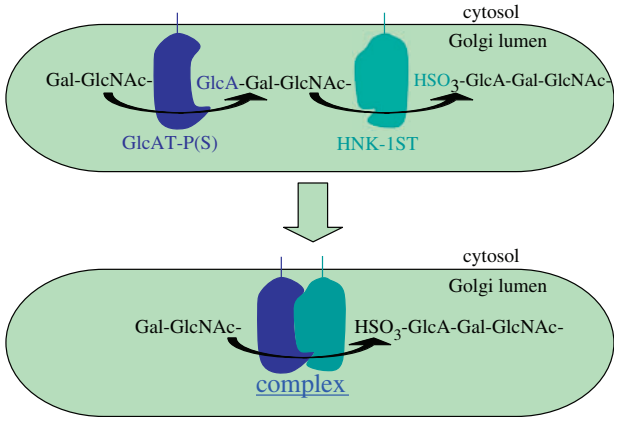


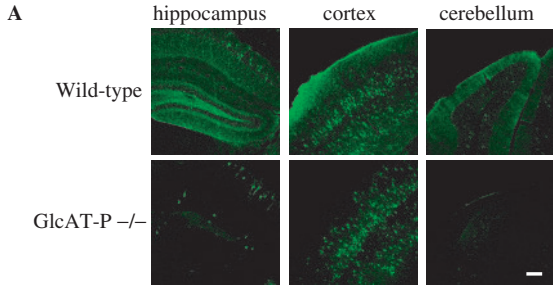

Fig. 2. **Schematic diagram of the enzyme complexes.** Glucuronyltransferases (GlcAT-P and GlcAT-S) and HNK-1ST form a complex in cells, resulting in efficient biosynthesis of the HNK-1 carbohydrate.

Actually, an *in vitro* activity assay revealed that the catalytic activity of HNK-1ST was up-regulated by the co-existence of either GlcAT-P or GlcAT-S, while glucuronyltransferase activities were unchanged even after the formation of complexes with HNK-1ST. These results indicated that efficient transfer of the sulfate group occurred soon after glucuronylation by GlcATs, which is consistent with our hypothesis described above (Fig. 2). Furthermore, for cells, we obtained results indicating that the acitivities of GlcATs are up-regulated by the co-existence of HNK-1ST, although the *in vitro* activities of GlcATs were not influenced by HNK-1ST. More complicated events probably occur during the carbohydrate biosynthetic process except for the complex formation from GlcATs and HNK-1ST. We expect that in future, other enzyme complexes will be discovered, leading to detailed clarification of the mechanism of carbohydrate biosynthesis.

## GLcAT-P GENE-DEFICIENT MICE

To elucidate the function of the HNK-1 carbohydrate *in vivo*, we generated and analysed mice with a targeted deletion of the *GlcAT-P* gene, GlcAT-P being a major glucuronyltransferase in the nervous system. The GlcAT-P-deficient mice exhibited normal birth and growth, and there was no significant difference in their appearance or body weight. Furthermore, no abnormal histological structures of the cerebral cortex, hippocampus and cerebellum were observed on Nissl staining. However, on western blot analysis and immunochemical staining, the expression of HNK-1 carbohydrate in GlcAT-P-deficient mice (–/–) was considerably reduced in the whole brain including the hippocampus (Fig. 3A) (*23*).

To examine the effect of the HNK-1 carbohydrate deficiency on synaptic plasticity, we analysed LTP (long-term potentiation) in the hippocampal CA1 region of GlcAT-P-deficient and wild-type mice, because the HNK-1 carbohydrate is commonly expressed on a series of cell adhesion molecules (CAMs) such as the neural cell




B

| Analyses | Phenotype (GlcAT-P deficient mice) |
|---|---|
| Long-term potentiation (LTP) in hippocampal CA1 region | Reduction of LTP (Synaptic plasticity ↓) |
| Morris water maze test | Longer escape latency (Learning and memory ↓) |
| Water-filled multiple T maze test | Longer escape latency (Learning and memory ↓) |
| Elevated plus maze test | Longer time in open arms (Anxiety ↓) |

Fig. 3. **Expression of the HNK-1 carbohydrate in mouse brain.** (A) Coronal brain sections from 11-week-old wild-type and GlcAT-P-deficient (–/–) mice were immunostained with HNK-1 mAb. Images of three regions (hippocampus, cortex and cerebellum) are shown. Scale bars, 100 μm. (B) Phenotypes of GlcAT-P-deficient mice.

adhesion molecule (NCAM), L1, telencephalin and tenascin-R (*1*, *4*, *5*). Moreover, these cell adhesion molecules are known to be involved in the formation and maintenance of the synapse structure or synaptic plasticity (*24*, *25*). We monitored excitatory post-synaptic potentials (EPSPs), which were evoked by stimulating afferent fibres in the stratum radiatum of the CA1 region, using the extracellular field potential recording technique. High-frequency stimulation of afferent fibres gave rise to LTP of excitatory synaptic transmission in the wild-type mice, while the magnitude of LTP in the GlcAT-P-deficient mice was significantly reduced compared with that in the wild-type ones. These results indicate that loss of the HNK-1 carbohydrate in the hippocampus decreases the synaptic plasticity (Fig. 3B). Furthermore, we examined the input–output relationship of the AMPA-mediated synaptic response in the CA1 region, but significant differences were not seen, suggesting that the basal synaptic transmission in GlcAT-P-deficient mice is not affected. In addition, the results of other experiments on pre-synaptic short-term plasticity, such as ones involving paired pulse facilitation and post-tetanic potentiation, suggested that the impairment of LTP in GlcAT-P-deficient mice is not due to pre-synaptic changes.

Next, considering the reduced LTP, two types of behavioural tests (Morris water maze test and water-filled multiple T-maze test) were carried out (*23*). The Morris water maze is well known as the most popular behavioural test that assesses spatial navigation and hippocampus-dependent memory formation. In the Morris

water maze test, the time taken to reach the hidden platform (escape latency) was significantly longer for GlcAT-P-deficient mice than wild-type ones during 4 days of training. In the water-filled multiple T-maze test, GlcAT-P-deficient mice also showed increased escape latencies as to the goal arm compared to wild-type mice (Fig. 3B). These lines of evidence indicate the involvement of the HNK-1 carbohydrate in higher order brain functions such as synaptic plasticity, learning and memory. This was confirmed by analyses of HNK-1 sulfotransferase (HNK-1ST)-deficient mice. Similar to GlcAT-P-deficient mice, the HNK-1ST-deficient mice showed no morphological defect in the brain, while impairment of LTP in the CA1 region of the hippocampus was observed (*26*). In addition, GlcAT-P-deficient mice spent a significantly longer time in open arms than the wild-type mice in the elevated plus maze test, suggesting reduction of anxiety-like behaviour (Fig. 3B).

As described earlier, the HNK-1 carbohydrate has almost completely disappeared in GlcAT-P-deficient mice. However, further studies revealed that a trace of HNK-1 immunoreactivity was present in some limited regions (Fig. 3A). The staining pattern of the remaining HNK-1 carbohydrate was similar to that of so called perineuronal nets (PNs), and we confirmed the co-localization of the remaining HNK-1 carbohydrate and the signal of WFA lectin, a well-known marker of the PNs structure. It is known that chondroitin sulfate proteoglycans, one of the major components of the PNs structure, are involved in reactivation of ocular dominance plasticity in the adult visual cortex (*27*), and the HNK-1 carbohydrate is also expressed with chondroitin sulfate proteoglycans such as neurocan and phosphacan (*28*). It is possible that this remaining HNK-1 carbohydrate could be associated with other types of neural plasticity. At present, it remains unclear which enzyme (including GlcAT-S) synthesizes the remaining HNK-1 carbohydrate.

A series of studies demonstrated that the HNK-1 carbohydrate regulates synaptic functions. So far, however, how the HNK-1 carbohydrate is involved in the nervous system and what role GlcAT-S in the brain plays remain unclear. We hope that the detailed molecular mechanisms by which the HNK-1 carbohydrate controls synaptic plasticity will be revealed in the near future.

## BIOSYNTHESIS OF NON-SULFATED TYPE OF HNK-1 EPITOPE BY GLcAT-S IN MOUSE KIDNEY

During the course of investigation on the intrinsic role of GlcAT-S different from that of GlcAT-P *in vivo*, we noticed that GlcAT-S mRNA was expressed more highly in mouse kidney than that in brain despite the highly neural specific expression of GlcAT-P mRNA (*29*). Moreover, the lack of expression of HNK-1ST in kidney forced us to consider that the non-sulfated HNK-1 carbohydrate was synthesized by GlcAT-S in kidney. In fact, we verified the expression of this carbohydrate epitope (GlcAβ1-3Galβ1-4-GlcNAc-) in kidney through immunochemical analyses with M6749 mAb, which recognizes the HNK-1 carbohydrate epitope with or without a terminal sulfate group at the 3-position of glucuronic acid. Two major carrier proteins were affinity-purified from a kidney membrane extract with M6749 mAb and identified as two metalloproteases (meprin-alpha and CD13/aminopeptidase-*N*) residing on the apical membranes of the proximal tubules in the renal cortex (*29*). In addition, mass-spectrometry analysis of *N*-glycans released from meprin-alpha clearly confirmed that the expected non-sulfated HNK-1 carbohydrate was actually expressed on complex type *N*-glycans in mouse kidney. As to the biosynthetic pathway for this carbohydrate in kidney, *in situ* hybridization analysis revealed that GlcAT-S mRNA expression had occurred in the renal cortex, which is consistent with the area of expression of the product carbohydrate, while GlcAT-P mRNA was not detected anywhere in kidney. These results strongly indicated that the non-sulfated HNK-1 carbohydrate in mouse kidney was biosynthesized not by GlcAT-P but by GlcAT-S. Immunohistochemical staining revealed that expression of the non-sulfated HNK-1 epitope had occurred on the proximal tubules in the cortex, where meprin-alpha and CD13/aminopeptidase-*N* are expressed. Curiously, however, the non-sulfated HNK-1 epitope was also detected in the thin ascending limb in the kidney inner medulla, where GlcAT-S and the two identified carrier molecules were not considered to be expressed. Although the details of the biosynthetic process and the carrier molecule(s) in this area remain unclear at present, recently there was a notable report that chondroitin- or heparan-sulfate proteoglycan in the thin-ascending loop of Henle was modified so as to have the HNK-1 carbohydrate in rat kidney (*30*). Although the same carrier proteoglycan may be expressed and may bear the non-sulfated HNK-1 carbohydrate in the thin ascending limb also in mouse kidney, further investigation is required for complete clarification.

More recently, Chang *et al*. (*31*) reported that klotho, known as a protein related to aging (*32*), hydrolysed the beta-glucuronic acid of *N*-glycans in a cation channel, TRPV5, to entrap this channel in the plasma membrane in kidney. They reported that the klotho–TRPV5 interaction regulates the $Ca^{2+}$ transport activity and permeability *via* the beta-glucuronic acid on *N*-glycans. Although our results indicated that the TRPV5 channel is not a major carrier protein of the non-sulfated HNK-1 carbohydrate epitope in mouse kidney, TRPV5 may express the non-sulfated HNK-1 carbohydrate (*i. e*. terminal glucuronic acid on *N*-glycans) to regulate calcium homoeostasis *in vivo*. The generation and analysis of GlcAT-S-deficient mice will shed light on this issue.

## FUTURE PERSPECTIVE

In this review, we described the expression mechanism and functional properties of the HNK-1 carbohydrate, focusing on biosynthetic enzymes GlcAT-P, GlcAT-S and HNK-1ST. However, there are some unsolved questions at present. In particular, it would be interesting but difficult to elucidate the molecular mechanism underlying the involvement of this carbohydrate in high-order brain functions such as synaptic plasticity, learning and memory. Besides, we are now trying to clarify how the non-sulfated HNK-1 carbohydrate synthesized by

GlcAT-S regulates its carrier protein's function. To resolve these unknown issues, further investigation is required.

This work was supported in part by the Protein 3000 Project, and by a Grant-in-Aid for Creative Scientific Research from the Ministry of Education, Culture, Sports and Technology.

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