**Carbohydrate-modifying Sulftotransferases: Structure, Function, and Pathophysiology**

Published, JBC Papers in Press, October 3, 2001, DOI 10.1074/jbc.R100049200

Minoru Fukuda‡, Nobuyoshi Hiraoaka, Tomoya O. Akama, and Michiko N. Fukuda§

*From the Glycobiology Program, Cancer Research Center, The Burnham Institute, La Jolla, California 92037*

Sulfated oligosaccharides play diverse roles in development, differentiation, and homeostasis. For example, heparan sulfate or heparan sulfate-like glycans were shown to play roles in binding growth factors to receptors (1, 2) and adhesion of herpes simplex virus 1 to the cell surface (3). Abolition of sulfation in heparan sulfate synthesis results in neonatal death during mouse development (4) and abnormal development in Drosophila (5, 6). These sulfate groups are formed by sulfotransferases. Sulfotransferases specifically transfer a sulfate group from the sulfate donor substrate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS),1 to a specific position of a specific carbohydrate residue. Molecular cloning of these sulfotransferases was achieved initially by purifying a given enzyme and cDNA cloning based on the amino acid sequence of the purified enzyme. These pioneering clonings include heparan sulfate N-deacetylase/sulfotransferase (7, 8), chondroitin sulfate GalNAc 6-O-sulfotransferase (9), heparan sulfate GlcN 3-O-sulfotransferase (10), and galactosylceramide 3-O-sulfotransferase (11). These studies demonstrate that sulfotransferases also have the same type II membrane topology as other Golgi enzymes such as glycosyltransferases. The crystal structure of an estrogen sulfotransferase revealed the amino acid sequence motifs that correspond to the binding sites for 5′-phosphosulfate and 3′-phosphate groups of the donor substrate, PAPS (12). Comparison of the amino acid sequences of these domains shows that they are conserved among the sulfotransferases cloned to date (13).

Initial molecular cloning of a sulfotransferase was also achieved by expression cloning using antibodies specific to a given carbohydrate. These studies include cloning of HNK-1 sulfotransferase (HNK-1ST) (14, 15). In one of these studies, it was revealed that there is a conserved amino acid sequence motif ZZRDPXXZ among cloned sulfotransferases, where X and Z denote any amino acid and a hydrophobic amino acid, respectively (14). This motif turned out to correspond to a part of the binding site for the 3′-phosphate group of PAPS. Site-directed mutagenesis of these amino acids and crystal structure analysis, described above, showed that the arginine 189 and serine 197 in this region are involved in hydrogen bonding to the 3′-phosphate group, whereas the aspartic acid 190 and proline 191 residues reside in the core structure of the 3′-phosphate binding site forming a tight turn in the polypeptide (Fig. 1). The lysine 128 residue in the 5′-phosphosulfate binding site may also be involved in binding to an acceptor in which sulfation takes place at the 3′-OH (Fig. 1), because the K128R mutant enzyme showed lower affinity to the acceptor compared with the wild-type enzyme (16).

The presence of the weak but discernible similarity among different sulfotransferases suggested the possibility that other sulfotransferases may be identified by their similarity to sulfotransferases already cloned. Indeed, following the cloning of HNK-1ST, two sulfotransferases were cloned based on their similarity to HNK-1ST. Studies on the substrate specificity of these sulfotransferases, however, unexpectedly revealed that these sulfotransferases encode for chondroitin GalNAc 4-O-sulfotransferases (Ch4STs) (17, 18). This is rather striking, considering that HNK-1ST and Ch4ST catalyze very different reactions; HNK-1ST adds a sulfate to the 3-position of glucuronic acid, which is in turn attached to the 3-position of galactose in N-acetyllactosamine, whereas Ch4ST adds a sulfate to the 4-position of N-acetylgalactosamine, which is in turn attached to the 4-position of glucuronic acid. The hydroxyl groups in both C-3 of glucuronic acid and C-4 of N-acetylgalactosamine are projected above their respective pyranose rings. It is tempting to speculate that the active sites of HNK-1ST and Ch4ST may approach the acceptor from above the plane of the respective acceptor. These results suggest that seemingly unrelated sulfotransferases could be cloned by their similarity to the probe sulfotransferase (see also Fig. 2). On the other hand, sulfotransferases sharing a similar reaction and acceptor are often related to each other.

Following the cloning of Ch4ST, N-acetylgalactosamine 4-O-sulfotransferases (GalNAc4ST-1 and GalNAc4ST-2) that add a sulfate to the 4-position of N-acetylgalactosamine in GalNAcβ1→4GlcNAC→R were thus molecularly cloned based on their similarity (19–21). It has been demonstrated that N-acetylgalactosamine 4-O-sulfation in hormonal glycoproteins such as lutropin is essential for maintaining an effective half-life for the hormonal glycoproteins once they are released into the bloodstream. Unsulfated forms are quickly taken up by unsulfated forms survive too long in the bloodstream, potentially causing an over-response in target tissues (22). This hypothesis can be tested now by generating mutant mice with defective GalNAc4ST-1 and/or GalNAc4ST-2 by gene targeting.

**N-Acetylglucosamine 6-O-Sulfotransferase Gene Family: Function and Pathophysiology**

Lymphocyte homing and recirculation are important processes for detection of foreign antigens by the immune system.

*© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.*
Counter-receptors expressed on high endothelial venules (HEV) capture circulating lymphocytes through L-selectin-dependent adhesion that requires sulfation in O-linked oligosaccharides attached to the counter-receptors. Previous studies showed that these sulfated oligosaccharides are either 6-sulfosialyl Lewis x or 6'-sulfosialyl Lewis x in the 5'-phosphate and 3'-phosphate binding site can form hydrogen bonds with the 5'-phosphate and 3'-phosphate of 3'-phosphoadenosine, 5'-phosphate (PAP), respectively. Asp190 and Pro191 participate in a tight turn of 90° between a β-sheet and an α-helix (adapted from Ref. 16).

To determine the roles of sulfation in L-selectin ligands, a sulfotransferase was molecularly cloned by screening the expressed sequence tag data base using cDNAs encoding Ch6ST (9) and keratan sulfate Gal 6-O-sulfotransferase (KS6ST) (24) as probes. This strategy was chosen because Gal- and GalNAc-6-O-sulfotransferases were assumed to be homologous to other 6-O-sulfotransferases. Two independent studies cloned this GlcNAc 6-O-sulfotransferase (GlcNAc6ST-2) from mouse and human, and they were called L-selectin ligand sulfotransferase (LSST) and HEC-GlcNAc6ST, respectively (25, 26). The mouse and human sequences share 73% identical amino acid residues and thus are regarded as orthologs to each other. In parallel, Uchimura et al. (27) earlier cloned a cDNA encoding another GlcNAc 6-O-sulfotransferase, GlcNAc6ST-1, by its similarity with mouse Ch6ST. The amino acid sequence of LSST shares 35.6, 35.4, and 32.3% identity with those of mouse GlcNAc6ST-1, human KS6ST, and chicken Ch6ST, respectively. Transfection studies using cDNAs encoding GlcNAc6ST-2, core2GlcNAcT-1, α1,3-fucosyltransferase VII, and CD34 showed that both mouse and human LSST form 6-sulfosialyl Lewis x predominantly in core2 branched O-glycans (25, 26). Chinese hamster ovary cell expressing these O-glycans enhance L-selectin-mediated adhesion compared with Chinese hamster ovary cells expressing non-sulfated sialyl Lewis x. By contrast, GlcNAc6ST-1 preferentially adds a sulfate to N-glycans, showing no enhanced L-selectin-mediated adhesion (25).

Human and mouse LSST were also shown to add a sulfate to extended core1 O-glycans, forming Galβ1→4(sulf0→6)-GlcNAcβ1→3Galβ1→3GalNAca1→Ser/Thr (28). This structure is the minimum epitope for MECA-79 antibody, which decorates the luminal surface of HEV and inhibits lymphocyte attachment to HEV (29). Moreover, biantennary O-glycans containing both core2 branch and extended core1 structures can have twin 6-sulfosialyl Lewis x.

Such O-glycans apparently have superior L-selectin ligand activity compared with those containing a core2 branch or extended core1 alone (28).

For L-selectin ligands, it has been reported that 6'-sulfosialyl Lewis x may also be present (23). α1,3-Fucosyltransferase VII, however, cannot add a fucose once sialyl N-acetyllactosamine contains 6'-sulfogalactose, whereas KS6ST cannot add a sulfate to 6'-galactose in sialyl Lewis x (30, 31). If 6'-sulfosialyl Lewis x is actually present in L-selectin ligand oligosaccharides, a novel sulfotransferase or a novel α1,3-fucosyltransferase must be present. Before attempting to clone such an enzyme, it is critical to determine whether 6'-sulfosialyl Lewis x actually exists in HEV and other organs.

The GlcNAc6ST gene family includes a GlcNAc 6-O-sulfotransferase that forms keratan sulfate together with KS6ST. Molecular cloning of this GlcNAc6ST in humans was achieved by an entirely different approach. It has been reported that impaired GlcNAc 6-O-sulfation in corneal keratan sulfate causes macular corneal dystrophy (MCD) in patients, with a symptom of opaque cornea (32, 33). By genetic linkage analysis, the critical region for MCD has been mapped to chromosome 16q22, a region flanked by D16S3115 and D16S3083 markers (34). Using two cell hybrid methods, one cDNA encoding this putative GlcNAc6ST (GlcNAc6ST, CHST6, or GlcNAc6ST-5) was found to reside within this region. Moreover, genomic analysis of MCD patients revealed that mutations are found in either its coding region or its promoter region (35). These two mutations then provide systemic absence of keratan sulfate (type I) or its absence only in the cornea (type II). Significantly, the mutation in the promoter region took place apparently because of homologous recombination between two tandemly duplicated genes, CGlcNAc6ST and intestinal GlcNAc6ST, (35) which was reported also by Lee et al. (36). By replacing a promoter region of CGlcnac6ST with that of IGlcNAc6ST, the mutated CGlcnac6ST is no longer expressed in cornea, leading to MCD type II (35).

A most recent study revealed that mouse ortholog (mouse IGlcNAc6ST) of human GlcNAc6ST is slightly closer to human IGlcNAc6ST in the amino acid sequence than human GlcNAc6ST, yet mouse IGlcNAc6ST can also form keratan sulfate (37). Mouse IGlcNAc6ST was shown to be present in Peyer’s patches (38), suggesting that both mouse and human IGlcNAc6ST may be involved in selectin ligand synthesis. It is tempting to speculate that diversion into CgIglcNAc6ST and IGlcNAc6ST in the human may represent sophistication of sulfotransferases in the human, having two different enzymes with diversified functions and tissue distribution.

### Each Sulfotransferase Has Unique Specificity

One of the main issues regarding sulfotransferases and glycosyltransferases is that there tends to be more than one enzyme that has identical or similar activities. Why do we humans, in particular, need such an apparent redundancy? In certain cases, we seem to have an answer by analyzing acceptor specificity in detail. Galactosylceramide can be sulfated at the 3-position of galactose by ceramide Gal-3-O-sulfotransferase (Gal3ST-1). After molecular cloning of Gal3ST-1 (11), three additional sulfotransferases that add a sulfate to the 3-position...
of galactose were cloned (39–42). It turned out that all of these additional sulfotransferases (Gal3ST-2, -3, and -4) differ in acceptor specificity. Gal3ST-2 can add a sulfate to both type 1 Galβ1→3GlcNAc and type 2 (N-acetyllactosamine) Galβ1→4GlcNAc oligosaccharide (39), whereas Gal3ST-3 adds a sulfate to N-acetyllactosamine (40, 42). Gal3ST-3 can also add a sulfate to 6-sulfo-N-acetyllactosamine, forming sulf−→3Galβ1→4[6-sulf−→6](GlcNAcβ1→R, whereas Gal3ST-2 cannot (40). By contrast, Gal3ST-4 adds a sulfate predominantly to core1 O-glycans, Galβ1→3GlcNAcα1→R, forming sulf−→3Galβ1→3-GlcNAcα1→R. These enzymes represent a clear example that seemingly similar enzymes may differ in acceptor specificity, achieving a unique reaction for each enzyme.

Another example can be seen in heparan sulfate GlcNSO₃⁻⁶-sulfotransferases (HS6ST). In mouse, three HS6STs have been cloned, and strikingly they differ in their requirement for specific acceptor structures; HS6ST-1 prefers iduronyl N-sulfoglucosamine, whereas HS6ST-2 prefers glucuronyl N-sulfoglucosamine and HS6ST-3 acts on both substrates (43). The acceptor specificity of HS6ST-2 results in the formation of heparan sulfate and GlcAβ1→4(sulf→6)(GlcNSO₃⁻⁶) structure in heparin. By contrast, HS6ST-1 and HS6ST-3 are likely involved in heparin biosynthesis. HS6ST-1, -2, and -3 are differentially expressed in different tissues, suggesting that different heparin and heparan sulfate structures are required in different tissues (43).

On the other hand, there are certain cases where one enzyme appears to be responsible for two seemingly unrelated reactions. This can be found in GlcNAc6ST-4/chondroitin sulfate GlcNAc 6-O-sulfotransferase (Ch6ST-2). Although the enzyme was originally cloned as the second chondroitin sulfate 6-O-sulfotransferase (Ch6ST-2) (44), entirely independent studies showed that this enzyme also acts as a GlcNAc6ST and thus is termed GlcNAc6ST-4/Ch6ST-2 (45, 46). Interestingly, Ch6ST-2 is only remotely related to Ch6ST-1 based on a phylogenetic analysis (Fig. 2). The results shown in Fig. 2 also illustrate that each subfamily of sulfotransferases can be recognized by this phylogenetic analysis.

We have emphasized so far that each sulfotransferase catalyzes a highly specific reaction, requiring restricted numbers of oligosaccharide acceptors unique to each sulfotransferase. This brings a second important concept of carbohydrate sulfation. Because sulfotransferases require specific acceptor structures, sulfation is largely dependent on glycosyltransferases that form precursor carbohydrates. This is particularly notable in the sulfation by GalNAc4ST-1 and GalNAc4ST-2. These sulfotransferases can transfer a sulfate equally well to acceptors mimicking a portion of tetraantennary and biantennary N-glycans and core2 branched O-glycans (22). This finding is consistent with the results obtained on carbohydrate structure and enzymatic property (47, 48). Moreover, a β1,4-N-acetylgalactosaminyltransferase recognizes the specific peptide region of glycoprotein hormones, forming a GalNAcβ1→4GlcNAcβ1→structure exclusively in a restricted set of glycoproteins such as pituitary hormones (49). These results indicate that the specificity of sulfation is achieved in a 2-fold fashion. Acceptor glycans are first synthesized in a specific manner, and in certain cases only a specific set of glycoproteins expresses the acceptor glycans. Second, the sulfotransferase itself will prefer one acceptor glycan over another as described for LSST, Gal3STs, and HS6ST.

**Future Perspectives**

Because sulfotransferases have only recently been molecularly cloned, studies on determining the roles of these sulfotransferases have just begun. One of the major research efforts has been to express a given sulfotransferase and assay the functions. This line of experiments was described above for the roles of LSST (GlcNAc6ST-2) in L-selectin-mediated adhesion. Another line of experiments is to evaluate functionality after a sulfotransferase is inactivated by gene targeting. This approach was also taken for studying the in vivo roles of LSST. Interestingly, the results show that LSST plays a dominant role in L-selectin ligand biosynthesis yet also suggests that another sulfotransferase may be involved in L-selectin ligand biosynthesis because ~50% of lymphocyte homing is sustained.
in the LSST knockout mouse (50). These results indicate that gene knockout of a putatively key sulfotransferase might provide an indication that still unidentified structures and/or sulfotransferases that form the structures may also play a role in the functionality of concern.

Another example can be seen in heparan sulfate N-deacetylation/sulfotransferase-2 (NDST-2) (8) knockout mice (51, 52). In this case, the phenotype is highly restricted to mast cells; connective tissue-type mast cells had altered morphology containing reduced amounts of histamine and mast cell proteases. These results indicate that NDST-2 plays a major role in the synthesis of heparin in mast cells, and no other NDST plays a dominant role in mast cells. By contrast, gene target disruption of NDST-1 (7) resulted in neonatal lethality because of pulmonary hyperplasia and respiratory distress (53, 54). As we described at the beginning, systemic inactivation of heparan sulfate iduronyl 2-O-sulfotransferase resulted in neonatal death, most likely from a failure of ureteric mesenchymal condensation and branching morphogenesis (4). These results, as a whole, indicate that sulfotransferases play roles in multiple layers of hierarchy necessary for development, differentiation, and homeostasis. Gene inactivation of each sulfotransferase will allow us to dissect these different layers and obtain knowledge on the mechanisms underlying each biological phenomenon. We expect that these research efforts will be further strengthened in coming years.

Acknowledgments—We thank the members of our laboratories and our collaborators for useful discussion and for participating in our research projects and Joseph P. Henig for organizing the manuscript.

REFERENCES

1. Yayen, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) Cell 64, 841–848
2. Rapprager, A. C., Krutka, A., and Olwin, B. B. (1991) Science 252, 1705–1708
3. Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G. (1999) Cell 99, 13–22
4. Bullock, S. L., Fletter, J. M., Beddington, R. S., and Wilson, V. A. (1998) Gene Dev. 12, 1894–1906
5. Lin, X., and Perrimon, N. (1999) Nature 400, 281–284
6. Tsuda, M., Kaminuma, K., Nakato, H., Archer, M., Staatz, W., Fox, B., Humphrey, M., Olson, S., Futch, T., Kaluza, V., Siegfried, E., Stam, L., and Sekiya, S. (1999) Nature 400, 276–280
7. Hashimoto, Y., Orellana, A., Gil, G., and Hirschberg, C. B. (1992) J. Biol. Chem. 267, 15744–15750
8. Eriksson, I. L., Sandbeck, D., Ek, B., Lindahl, U., and Kjellén, B. (1994) J. Biol. Chem. 269, 10438–10443
9. Fukuta, M., Uchimura, K., Nakashima, K., Kato, M., Kimata, K., Shimomura, Y., and Habuchi, O. (1995) J. Biol. Chem. 270, 18575–18580
10. Shworak, N. W., Liu, J., Fritze, L. M., Schwartz, J. J., Zhang, L., Logeart, D., and Hemmerich, S. (2001) J. Biol. Chem. 276, 23488–23495
11. Honke, K., Tsuchiya, K., Miyajima, A., Akama, T. O., Fukuda, M. N., and Ohara, O. (1997) J. Biol. Chem. 272, 25697–25704
12. Nakazawa, K., Hassell, J. R., Hascall, V. C., Lohmander, L. S., Newsome, D. A., and Krachmer, J. (1984) J. Biol. Chem. 259, 13751–13757
13. Klintworth, G. K., Meyer, R., Dennis, B., Hewitt, A. T., Stock, E. L., Lenz, M. E., Hassell, J. R., Stark, W. J., Jr., Kuettner, K. E., and Thonar, E. J. (1986) Ophthalmic Plastic Reconstruct. Surg. 7, 139–145
14. Smith, P. L., and Baenziger, J. U. (1988) J. Biol. Chem. 263, 904–910
15. Shworak, N. W., Liu, J., Fritze, L. M., Schwartz, J. J., Zhang, L., Logeart, D., and Hemmerich, S. (2001) J. Biol. Chem. 276, 23488–23495
16. Seko, A., Haru-Kuge, S., and Yamashita, K. (2001) J. Biol. Chem. 276, 21075–21080
17. Fujita, H., Futterer, J., and Sugiura, Y. (2000) Science 29942–9033
18. Smith, P. L., and Baenziger, J. U. (1988) Science 240, 930–933
19. Hemmerich, S., Bisttrip, A., Singer, M. S., van Zante, A., Lee, J. K., Tsai, D., Peters, M., Carminati, J. L., Brennan, T. J., Carter-Moore, K., Leviten, M., Fuentes, M. E., Rudder, N. H., and Rosen, D. S. (2001) Immunity 15, 237–247
20. Humphries, D. E., Wang, G. W., Friend, S. D., Girish, M. F., Qiu, W. T., Huang, C., Sharpe, A. H., and Stevens, R. L. (1999) Nature 400, 769–772
21. Forsberg, E., Pejler, G., Ringvall, M., Lunderius, C., Tomasini-Johansson, B., and Ringvall, M. (1998) J. Biol. Chem. 273, 23704–23711
22. Yamauchi, S., Mita, S., Matsubara, T., Fukuta, M., Habuchi, H., and Kimata, K. (2000) J. Biol. Chem. 275, 10443–10448
23. Hemmerich, S., Leffler, H., and Rosen, S. D. (1995) J. Biol. Chem. 270, 13035–13047
24. Fukuta, M., Inazawa, J., Torii, T., Tsuzuki, K., Shimada, E., and Habuchi, O. (1997) J. Biol. Chem. 272, 32321–32325
25. Hiraoka, N., Petryniak, B., Nakayama, J., Tsuibo, S., Suzuki, M., Yeh, J. C., Iwao, D., Tanaka, T., Miyasaka, M., Lowe, J. B., and Fukuda, M. (1999) Nature 323, 218–225