Molecular Cloning and Characterization of a Human Multisubstrate Specific Nucleotide-sugar Transporter Homologous to Drosophila fringe connection*

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Takeshi Suda¶¶, Shin Kamiyama¶¶¶, Masayuki Suzuki¶¶¶, Norihiro Kikuchi¶¶¶¶, Ken-ichi Nakayama¶¶, Hisashi Narimatsu¶¶, Yoshifumi Jigami¶¶¶, Tatsuya Aoki¶, and Shoko Nishihara¶¶¶¶

From the ¶Laboratory of Cell Biology, Department of Bioinformatics, Faculty of Engineering, Soka University, 1-236 Tangi-cho, Hachioji, Tokyo 192-8577, Japan, the ¶Department of Surgery, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan, **Seikagaku Corporation, Central Research Laboratories, 3-1253 Tateno, Higashiyamato, Tokyo 207-0021, Japan, §§Mitsui Knowledge Industry Co., Ltd., 1-32-2 Honcho, Nakano-ku, Tokyo 164-8721, Japan, the ¶¶Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki, 305-8586 Japan, and ¶¶¶Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Corporation (JST), Kawaguchi Center Building, 4-1-8, Hon-cho, Kawaguchi, Saitama 332-0012, Japan.

Nucleotide-sugar transporters are crucial components in the synthesis of glycoconjugates. We identified a novel human nucleotide-sugar transporter gene, hfrc1, which is homologous to Drosophila melanogaster fringe connection, Caenorhabditis elegans sqv-7, and human UGTrel7. HFRC1 was localized within the Golgi apparatus following its transient expression in HCT116 cells. In human tissues, hfrc1 and UGTrel7 exhibited similar tissue distributions, although hfrc1 transcripts showed a 10 times greater abundance than those of UGTrel7. The heterologous expression of HFRC1 in the yeast revealed the multisubstrate specific transport activity of HFRC1 (for UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-glucose (UDP-Glc), and GDP-mannose (GDP-Man), with apparent \( K_m \) values of 8.0, 2.1, and 0.14 \( \mu \)M, respectively). In the mammalian cells, HFRC1 transported UDP-GlcNAc and UDP-Glc, but not GDP-Man. Overexpression of the hfrc1 gene in HCT116 cells modulated the cell surface heparan sulfate expression status. These results suggest that HFRC1 takes part in the synthesis of heparan sulfate by regulating the level of UDP-GlcNAc, a donor substrate for the heparan sulfate synthases.

Nucleotide sugars, high-energy donor substrates for glycosyltransferases, are synthesized in the cytosol (or in the nucleus in the case of CMP-sialic acid). On the other hand, glycosylation reactions occur in the lumen of the endoplasmic reticulum (ER)\(^1\) and Golgi apparatus (1, 2). Translocation of nucleotide sugars from the cytosol into the lumen compartment is mediated by specific nucleotide-sugar transporters (NSTs) (1, 2). In several studies, NSTs have been mentioned as possible crucial players in the synthesis of glycoconjugates (for reviews, see Refs. 3–5).

Recent studies have demonstrated that some NSTs are implicated in growth factor signaling during development through the regulation of proteoglycan synthesis. In Caenorhabditis elegans, sqv-7 mutants show a defect in vulva formation, with epithelial invaginations, during ontogeny (6). SQV-7 has been identified as a transporter of UDP-glucuronic acid (UDP-GlcUA), UDP-galactose (UDP-Gal), and UDP-N-acetylgalactosamine (UDP-GalNAc) (7). Biochemical analysis of sqv-7 mutants has demonstrated defects in both chondroitin and heparan sulfate biosynthesis in vivo (8). Furthermore, Selva et al. (9) and Goto et al. (10) reported that the Drosophila melanogaster gene of fringe connection (frc) is involved in embryonic Wingless/Hedgehog and fibroblast growth factor signaling (9, 10). FrC encodes a multisubstrate-specific NST that transfers UDP-sugars into the Golgi apparatus (9, 10). UDP-GlcUA and UDP-N-acetylgalactosamine (UDP-GlcNAc) act as substrates for the synthesis of heparan sulfate. Embryos with a mutation of frC display severe segment polarity phenotypes (9).

Although sqv-7 and frc are considered to be orthologous to each other, it remains obscure which NSTs of humans have corresponding functions. In humans, the UGTrel7 protein has a similar multisubstrate specificity to those of FRC and SQV-7; namely, UDP-GlcUA/UDP-GalNAc (11). However, localization of UGTrel7 to the ER, not to the Golgi apparatus (11), leaves open the possibility that other transporters may be involved in proteoglycan synthesis in the Golgi apparatus.

The structural conservation present among NSTs enables the identification of many putative NST sequences from data bases. By a data base search, we identified a putative NST gene that is closely related to sqv-7, frc, and UGTrel7. HFRC1 had a multisubstrate specificity for UDP-GlcNAc/UDP-glucose (UDP-Glc/GDP-mannose) in the Golgi fraction of hemagglutinin epitope; frc, fringe connection; mAb, monoclonal antibody; NST, nucleotide-sugar transporter; GDP-Fuc, GDP-fucose; GDP-Man, GDP-mannose; sqv, squashed vulva; UDP-Gal, UDP-galactose; UDP-GalNAc, UDP-N-acetylgalactosamine; UDP-Glc, UDP-glucose; UDP-GlcNAc, UDP-N-acetyl-D-glucosamine; UDP-GlcUA, UDP-glucuronic acid; ORF, open reading frame; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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yeast cells expressing this gene. Furthermore, alteration in the expression of hfrc1 affected the heparan sulfate on the cell surface of mammalian cells. Thus, the present study raises the possibility that HFRC1 takes part in heparan sulfate synthesis by supplying UDP-GlcNAc.

EXPERIMENTAL PROCEDURES

Materials—UDP-[3H]mannose (15 Ci/mmol), UDP-[1-3H]glucose (15 Ci/mmol), UDP-N-acetyl-6[3H]-glucosamine (15 Ci/mmol), and UDP-[U-14C]glucuronic acid (15 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). GDP-[3H]-fucose (6.95 Ci/mmol), UDP-[4,5-3H]galactose (48.3 Ci/mmol), CMP-[9-3H]-inosine (80 Ci/mmol), and UDP-N-acetyl-6-[3H]-glucosamine (39.7 Ci/mmol) were purchased from PerkinElmer Life Sciences. Zymolyase 100T, heparitinase I, chondroitinase ABC, anti-heparan sulfate/ human (F58) monoclonal antibody (mAb), and anti-chondroitin 4-sulfate/rat (2H6) mAb were obtained from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Anti-ERp57 (MaP.ERp57) mAb and fluorescein isothiocyanate-conjugated anti-influenza hemagglutinin epitope (HA) mAb (HA-probe, F-7) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rhodamine-conjugated anti-mouse IgG mAb and horseradish peroxidase-conjugated anti-mouse IgG mAb were obtained from Bio-Rad. All other reagents were of the highest purity commercially available.

Isolation of a Novel Human NST cDNA and Construction of Expression Plasmids—A novel putative NST gene was cloned using the same procedures as previously described (12). Briefly, a TBLASTN search was performed for the amino acid sequence of the open reading frame (ORF) of the GDP-fucose (GDP-Fuc) transporter gene (13). We identified a cDNA sequence encoding a full-length ORF (GenBank® accession number XM_047286). To obtain this cDNA and create recombination sites for the GATEWAY® cloning system (Invitrogen), we used two steps of attB adaptor PCR for the preparation of attB-flanked PCR products. For the first gene-specific amplification, a forward template-specific primer with attB1 (5’-aaaaagctgggtcgctct-3’) and a reverse template-specific primer with attB2 (5’-agaagctggtggctctcttataaatataaatcc-3’) were used. PCR was performed using Platinum® Pfx DNA polymerase (Invitrogen) and a cDNA library derived from human colon tissue. The insertion of a complete attB adaptor and cloning into the pDONR® 201 vector to create an entry clone for the subsequent subcloning were performed according to the instruction manual. The ORF of the UGTrel7 cDNA was obtained by the same procedures as mentioned above using the forward template-specific primer 5’-aaaaagcgattgattcggagagattcgcggat-3’ and the reverse template-specific primer 5’-aggaagctggtggcttcttataaatataaatcc-3’. Expression vectors were inserted with three copies of the HA epitope tags (YFPYDVPDYA) at the position corresponding to the C terminus of the expressing protein and converted to a Gateway destination vector with the cloning vector pDONR® 201. Each entry clone containing the cDNA expression vectors using the Gateway Cloning System according to the instruction manual.

Transient Transfection and Immunofluorescence Microscopy—Transient transfection and immunofluorescence microscopy was performed as previously described (12). HCT116 cells were subcultured onto a 4-well Lab-Tek chamber slide (Nalge Nunc International, New York) in Dulbecco’s modified Eagle’s/Ham’s medium containing 0.1% fetal bovine serum. After 24 h, cells were transfected with 0.25 μg/well of pCXN2 (14) or pCXN2 inserted with HA-tagged hfrc1 using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s protocol. After 72 h, cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 30 min at 4 °C. Each entry clone containing the cDNA expression vectors using the Gateway Cloning System according to the instruction manual.

Stable Transfection—HCT116 cells were subcultured onto 6-cm dishes (Falcon) in Dulbecco’s modified Eagle’s/Ham’s (1:1) medium containing 0.1% fetal bovine serum. After 24 h, cells were transfected with 8 μg of pCXN2 vector or pCXN2 inserted with HA-tagged hfrc1 using LipofectAMINE 2000 reagent according to the manufacturer’s protocol. After 48 h, transfectants were selected by the addition of 600 μg/ml Genetin (Invitrogen) to the medium, and cultured for 1 month.

Flow Cytometric Analysis—Flow cytometric analysis was performed using EPICS ELITE flow cytometer (Beckman-Coulter). As the negative control, cells were treated with 10 units/ml of heparitinase I or 100 units/ml of chondroitinase ABC.
for 90 min at 37 °C before immunoblotting. Data were analyzed using WinMDI 2.8 software (The Scripps Research Institute Cytometry software page).

RESULTS

Cloning of Human Hfrc1 cDNA—Using the TBLASTN algorithm, we identified a cDNA sequence (GenBank™ accession number XM_047286) homologous to the GDP-Fuc transporter gene. We cloned the full-length ORF as described under “Experimental Procedures,” and named it hfrc1. The phylogenetic tree of the transporter genes in human, Drosophila, and C. elegans is shown in Fig. 1A. Hfrc1 is more closely related to UGTr7, frc, and sqv-7 than to the GDP-Fuc transporter gene. The phylogenetic tree also demonstrates that both hfr1 and UGTr7 are orthologs of Drosophila frc (Fig. 1A). An alignment of the amino acid sequences of HFRC1, SQV-7, FRC, and UGTr7 is shown in Fig. 1B. HFRC1 consists of 337 amino acids with a calculated mass of 38.7 kDa. Hydropathy analysis and predictions of the transmembrane helices of the amino acid sequence revealed that HFRC1 are Type III transmembrane proteins with seven transmembrane domains. HFRC1 showed 54.3, 46.6, and 46.6% identities to UGTr7, FRC, and SQV-7, respectively. There is one potential N-glycosylation site in the HFRC1 sequence (double underlined). The gene structures of hfr1 and UGTr7 are shown in Fig. 1C. The hfr1 gene is mapped on human chromosome 9q22.3, and the mRNA is composed of 12 exons. On the other hand, the UGTr7 gene is mapped on human chromosome 1p32-p31, and the mRNA is composed of 12 exons.

Subcellular Localization of HFRC1 in Mammalian Cells—Hfr1 has a significant similarity to human UGTr7 and Drosophila frc. FRC is primarily localized to the Golgi apparatus (10), whereas UGTr7 is localized to the ER (11). To investigate the subcellular localization of HFRC1, HA-tagged HFRC1 protein was transiently expressed in a mammalian cell line, and immunostained. A mammalian expression vector, pCXN2, was inserted with the ORF of hfr1 or UGTr7 with three copies of the HA epitope tag at the C terminus, and transfected transiently into HCT116 cells. The cells were double-immunostained with anti-HA mAb and anti-beta-GalT1 mAb or anti-ERp57 mAb, and the immunofluorescence was observed microscopically. As shown in Fig. 2, HFRC1-HA was co-localized with beta-GalT1, which is a typical protein of trans-Golgi localization (15). In contrast, UGTr7-HA was co-localized with ERp57, which is a typical protein of the ER (Fig. 2). These results indicate that HFRC1 is localized to the Golgi apparatus, unlike UGTr7.

Tissue Distribution of Hfr1 and UGTr7 Transcripts in Human Tissues—Although hfr1 and UGTr7 are closely related genes, the subcellular localization of HFRC1 is different from that of UGTr7. To investigate whether hfr1 and UGTr7 have similar tissue distributions, the expression levels of hfr1 and UGTr7 transcripts in human tissues were analyzed using real time PCR.

The gene expression profiles of these genes in various human tissues are shown in Fig. 3. All transcript levels are shown relative to that of GAPDH. Hfr1 and UGTr7 displayed similar tissue distributions, each showing high levels of expression in the colon, stomach, lung, and leukocyte. However, the expression level of hfr1 transcripts was 10 times that of UGTr7 (note the different scales in the two panels in Fig. 3). HFRC1 Is a Multisubstrate Specific Protein That Transports UDP-GlcNAc, UDP-Glc, and GDP-Man—The yeast S. cerevisiae is an organism that has been widely used for the analysis of NSTs by heterologous expression. To express HFRC1 protein in S. cerevisiae, the yeast expression vector YEp352GAP-II was inserted with the ORF of hfr1, and introduced into W303-1a

yeast. The expression status of HA-tagged HFRC1 protein in the yeast was analyzed by Western blotting using antibody against the HA epitope tag. HA-tagged HFRC1 protein was

![Fig. 1. Amino acid sequence analysis.](image-url)
successfully expressed in the Golgi-enriched P100 fraction as a 42-kDa protein (Fig. 4A). The transport activity of HFRC1 for nucleotide sugars into P100 fractions was examined using radiolabeled substrates. As shown in Fig. 4B, the incorporation into the P100 fraction prepared from yeast cells (those expressing HFRC1 over that shown for the mock) was 6.2 times for UDP-GlcNAc, 4.2 times for UDP-Glc, and 1.9 times for GDP-Man. No significant difference was observed between hfrc1 and the mock in the transport of other nucleotide sugars. The substrate concentration dependences of UDP-GlcNAc, UDP-Glc, and GDP-Man transport by HFRC1 are shown in Fig. 4, C–E. HFRC1 showed saturable transport activity with increases in substrate concentration, and the apparent $K_m$ values for UDP-GlcNAc, UDP-Glc, and GDP-Man were estimated to be 8.0, 2.1, and 0.14 M, respectively.

Because the level of endogenous transport of GDP-Man is extremely high in S. cerevisiae, we also tested the transport activity of HFRC1 in a mammalian cell line. HCT116 cells stably expressing either HA-tagged hfrc1 or mock vector (vector alone) were analyzed for the uptake of these substrates into the P100 fraction. The hfrc1 transfectant showed 5.4 times the hfrc1 transcript level of the mock transfectant (6.6 versus 1.2 GAPDH transcript). The P100 fraction of the hfrc1 transfectant showed 1.7 times the UDP-GlcNAc transport activity and 1.8 times the UDP-Glc transport activity of the mock transfectant (380.6 versus 228.8 pmol/5 min/mg of protein, and 910.7 versus 493.2 pmol/5 min/mg of protein, respectively). However, in contrast to the yeast expression data, a difference in GDP-Man transport activity was hardly detectable between the hfrc1 transfectant and mock transfectant (168.9 versus 175.9 pmol/5 min/mg of protein). These results indicate that...
HFRC1 transports UDP-GlcNAc and UDP-Glc in the mammalian cells, but transports GDP-Man alone in the yeast.

Hfrc1 Has a Role in Heparan Sulfate Synthesis—Heparan sulfate molecules are found on the cell surface as glucosaminoglycan chains covalently attached to a core protein. Heparan sulfate has a repeat of GlcUA and GalNAc, whereas chondroitin sulfate has a repeat of GlcUA and GlcNAc, whereas chondroitin sulfate has a repeat of GlcUA and GlcNAc, whereas chondroitin sulfate has a repeat of GlcUA and GlcNAc, whereas chondroitin sulfate has a repeat of GlcUA and GlcNAc, whereas chondroitin sulfate has a repeat of GlcUA and GlcNAc, whereas chondroitin sulfate has a repeat of GlcUA and GlcNAc, whereas chondroitin sulfate has a repeat of GlcUA and GlcNAc, whereas chondroitin sulfate has a repeat of GlcUA and GlcNAc. Because HFRC1 can transport UDP-GlcNAc, the possibility needed to be considered that HFRC1 might contribute to the elongation of heparan sulfate by supplying UDP-GlcNAc, a donor substrate for the heparan sulfate synthases.

**Fig. 4.** Characterization of the transport activity of HFRC1 by yeast expression. A, expression status of the HFRC1 protein. Protein (50 μg) of each P100 fraction prepared from yeast cells expressing either mock vector (lane 1) or HA-tagged hfrc1 (lane 2) was subjected to SDS-polyacrylamide electrophoresis, and Western blot analysis was performed using a monoclonal antibody against the HA epitope. Arrow indicates HA-tagged HFRC1. B, substrate specificities. Protein (200 μg) of each P100 fraction was incubated in 100 μl of reaction buffer containing different concentrations of [3H]UDP-GlcNAc or [3H]GDP-Man and the incorporated radioactivity was measured. The specific incorporation was calculated by subtracting the value for the mock transfection from each of the corresponding values. Lower panels show the double-reciprocal plots used to determine the respective \( K_m \) values.

**Fig. 5.** Flow cytometric analysis of HCT116 cells stably expressing hfrc1. HCT116 cells stably expressing either mock vector (upper panel) or HA-tagged hfrc1 (lower panel) were harvested and immunostained with antibodies against chondroitin 4-sulfate (A) or heparan sulfate (B). Fainter, dotted lines indicate the negative controls, which were treated with heparitinase 1 (for heparan sulfate) or chondroitinase ABC (for chondroitin sulfate) before immunoblotting.

**DISCUSSION**

In the present study, we reported the molecular cloning and functional characterization of a novel human multisubstrate-specific NST, which is homologous to Drosophila frc and *C. elegans sqv-7*. In humans, hfrc1 also exhibited a significant similarity to another NST, UGTrel7. The phylogenetic tree indicates that both hfrc1 and UGTrel7 are orthologs of Drosophila frc (Fig. 1A). In Drosophila, frc is the only gene corresponding to these two human NSTs. We could not find any putative NST sequence homologous to hfrc1 or UGTrel7 from *Drosophila* data bases, except frc. This raises the possibility that UGTrel7 and hfrc1 may share a common ancestral origin. In fact, these two genes have a similar exon-intron organization, both being composed of 12 exons (Fig. 1C). Furthermore, hfrc1 and UGTrel7 displayed the same expression profiles in human tissues (Fig. 3), suggesting that these two genes are under similar regulation. Hfrc1 and UGTrel7 were mapped on different chromosomes, 9q22.33 and 1p32-p31, respectively. These locations are near the regions of 9q33–34 and 1q21–25, respectively, which are paralogous to the major histocompatibility complex. Kasahara et al. (20) suggested in their report that the genes in the major histocompatibility complex region of jawed vertebrates have risen as a result of ancient chromosomal duplications. These data suggest that hfrc1 and UGTrel7 have evolved from a common ancestral gene through either segmental chromosome duplication or gene duplication.

As shown in Fig. 5, an alteration in the expression of hfrc1 affected heparan sulfate on the cell surface of mammalian cells. UDP-GlcNAc, a transport substrate for HFRC1, can be utilized by the enzymes that catalyze the elongation of heparan sulfate. Because the elongation of proteoglycan takes place within the Golgi apparatus (2, 21–23), the localization of HFRC1 to the Golgi apparatus would allow it to regulate the donor-substrate level for the enzymes. Heparan sulfate is known to be involved in a variety of biological phenomena, such as morphogenesis, development, angiogenesis, blood coagulation, cell adhesion,
and lipid metabolism (23, 24). It is also involved in a variety of signaling pathways, in particular those of fibroblast growth factor (25), Wnt/Wingless (26–28), Decapentaplegic (27), and Hedgehog (28). Selva et al. (9) reported that frc mutant embryos display defects in Wingless, Hedgehog, and fibroblast growth factor signaling (9). **Hfrc1** is expected to be involved in growth factor signaling through the regulation of heparan sulfate synthesis in a way similar to that observed for frc.

The substrate specificity of Hfrc1 also suggests the possibility that Hfrc1 may be able to modulate Notch activity in humans by supplying UDP-GlcNAc. The interaction of the Notch receptor with its ligands, Serrate and Delta, is modulated by the addition of GlcNAc to an O-linked Fuc on the Notch by FRINGE enzyme (30–33). In *Drosophila*, mutation of frc leads to a defect in Notch maturation (10). Here, Hfrc1 exhibited an apparent *Km* value for UDP-GlcNAc comparable with that of FRC (8 versus 7.8 μM, respectively). It would be interesting to investigate whether hfrc1 can rescue the defects in glycosylation and signaling caused by frc mutations.

We also observed that Hfrc1 has a UDP-Glc transport activity. Hfrc1 is the first UDP-Glc transporter to be reported in a mammal, although the physiological significance of UDP-Glc transport in the Golgi apparatus is obscure. Possibly, it is involved in some glycosylation process in the Golgi apparatus, such as the addition of O-linked glucose (34) or the glucosyltransferase reaction by β4GalT1 in the presence of α-lactalbumin (35). On the other hand, we found that Hfrc1 transported GDP-Man alone in the yeast, not in the mammalian cells. This difference is perhaps because of the abundance of antiport molecules for GDP-Man within the yeast Golgi apparatus. It has been suggested that GDP-Man is transported into the lumen of the Golgi apparatus of yeast and *Leishmania*, but not into that of mammals (3). Whether or not Hfrc1 can act as a real GDP-Man transporter in mammalian cells needs to be evaluated in future investigations.

In humans, another UDP-GlcNAc transporter exists within the Golgi apparatus (36). We did not investigate whether Hfrc1 and this UDP-GlcNAc transporter operate in a complementary fashion or in different ways within the Golgi apparatus. Identification of the glycoconjugates that are affected by these genes may hold the key to a clarification of their functions. Analysis using RNA interference of each gene may also help to elucidate the significance of these transporters in glycosylation.

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