Members of the Nucleotide-Sugar Transporter Family and Their Functions

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Contents

Abstract

Glycosylation and sulfation are important posttranslational modifications of proteins and are performed in the lumens of the endoplasmic reticulum (ER) or the Golgi apparatus by glycosyltransferases and sulfotransferases, respectively. However, the donor substrates of transferases, nucleotide sugars and 3′-phosphoadenosine 5′-phosphosulfate (PAPS), are synthesized in the cytosol or nucleus. Nucleotide-sugar transporters (NSTs) or PAPST transporters (PAPSTs) transport nucleotide sugar or PAPS into the lumen of the ER and the Golgi and therefore determine the glycosylation or sulfation status by supplying donor substrates. These transporters belong to the NST family, solute carrier 35 (SLC35). In this chapter, all transporters with known transport activities are described. In addition, the methods used for the measurement of their transferase activities and their functional analysis using RNA interference (RNAi) are described.

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Introduction

Glycosylation is one of the most important posttranslational modifications, as glycan structures on glycoproteins and glycolipids play key roles in central biological processes such as cell fate determination/proliferation, morphogenesis/organ development, viral and bacterial infections, immune response, and cancer invasion. Glycosylation is performed in the lumens of the endoplasmic reticulum (ER) and the Golgi apparatus by a sequence of glycosyltransferase reactions (Fig. 1a). Thus, glycosyltransferase enzymes determine the glycosylation status. Glycosyltransferases transfer sugar molecules to acceptor substrates from sugar donors called nucleotide sugars, which are monosaccharides that are activated by the addition of a nucleoside mono- or diphosphate (UDP, GDP, or CMP). Most nucleotide sugars are synthesized in the cytosol; however, CMP-sialic acid is the one exception that is synthesized in the nucleus instead. After being synthesized, nucleotide sugars are transported from the cytosol into the lumen compartment by specific nucleotide-sugar transporters (NSTs). NSTs are multiple membrane-spanning proteins that transport nucleotide sugars in a coupled byproduct of a glycosyltransferase reaction and subsequent luminal nucleoside diphosphatase (NDPase) reaction inside the lumen (Fig. 1a). Therefore, NSTs also determine the glycosylation status as the supplier of donor sugars used in the glycosyltransferase reactions.

Sulfation is also an important posttranslational modification of proteins. Sulfated proteins and sulfated glycans play important roles in various biological processes. For example, heparan sulfate is indispensable to signaling pathways such as the wingless, hedgehog, bone morphogenetic protein, and fibroblast growth factor (FGF) pathways, during the developmental process. Sulfations are carried out for proteins and glycans in the lumen of the Golgi apparatus by various sulfotransferases (Fig. 1b). Thus, sulfotransferases determine the sulfation status, in the same vein as glycosyltransferases act as the main driver of glycosylation. An activated form of sulfate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), is a common sulfate donor. Sulfotransferases transfer sulfate from PAPS to a defined position on a target sugar or tyrosine (Tyr) residue. PAPS is synthesized in the cytosol and then transported by PAPS transporters (PAPSTs) into the lumen of the Golgi apparatus, where sulfation is carried out. PAPSTs are multiple membrane-spanning proteins that transport PAPS by a coupled antiport of adenosine 3′, 5′-diphosphate (PAP) (Fig. 1b). Therefore, PAPSTs also determine the sulfation status, as they supply the sulfate groups necessary as a substrate for sulfotransferase enzymes.

Both NSTs and PAPSTs are members of the solute carrier 35 (SLC35) transporter family. Nearly 200 different glycosyltransferases and 40 different sulfotransferases
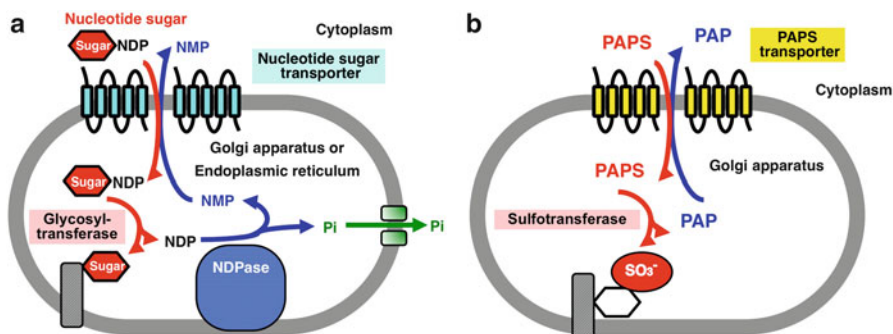
Fig. 1 Nucleotide-sugar transporters (a) or PAPS transporters (b) transport nucleotide-sugars and PAPS from the cytosol into the ER/Golgi lumens, the location where glycosylation or sulfation takes place. Nucleotide-sugars and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) are synthesized in the cytosol or the nucleus. Nucleotide-sugar transporters and PAPS transporters transport these molecules from the cytosol into the ER/Golgi lumens. Glycosyltransferases and sulfotransferases are then responsible for the transfer of sugars and sulfate molecules to acceptor substrates from these donor nucleotide sugars and PAPS, respectively. Released nucleoside diphosphates (NDPs) are hydrolyzed by nucleoside diphosphatase (NDPase) to form nucleoside monophosphates (NMPs) and inorganic phosphates (Pis). NMPs and released adenosine 3′, 5′-diphosphate (PAP) are exported in antiport with incoming nucleotide-sugars and PAPS. Pis exit via a phosphate transporter.

have been identified in humans, whereas only eight NSTs and two PAPSTs have been identified in humans (Fig. 2 and Table 1). This suggests that each of these transporters likely regulates a broader range of glycosylation or sulfation reactions, as compared to transferases, which appear to require more specificity.

Principles

Members of NST Family SLC35

NSTs and PAPSTs belong to the NST family. The SLC35 family was initially classified based on sequence data from human NSTs. However, recently, a large number of NSTs from many other species, including plants and worms, were cloned and their activities identified. Available information about all NSTs and PAPSTs whose NST or PAPST activity has been identified is presented in Table 1. Using the amino acid sequences of these transporter molecules, their phylogenetic tree was also constructed (Fig. 2). Given that the group of GDP-Man transporters was found near the subgroups, SLC35C and SLC35D, we proposed to add this group as a new subgroup to the SLC35 family, which has not been identified in humans. In addition, it is worth mentioning that the specific NST activity of each nucleotide sugar could not be easily estimated by the phylogenetic tree using similarity between amino acid sequences. For the determination and estimation of the physiological function of each member, the identification of its transporter activity is essential.
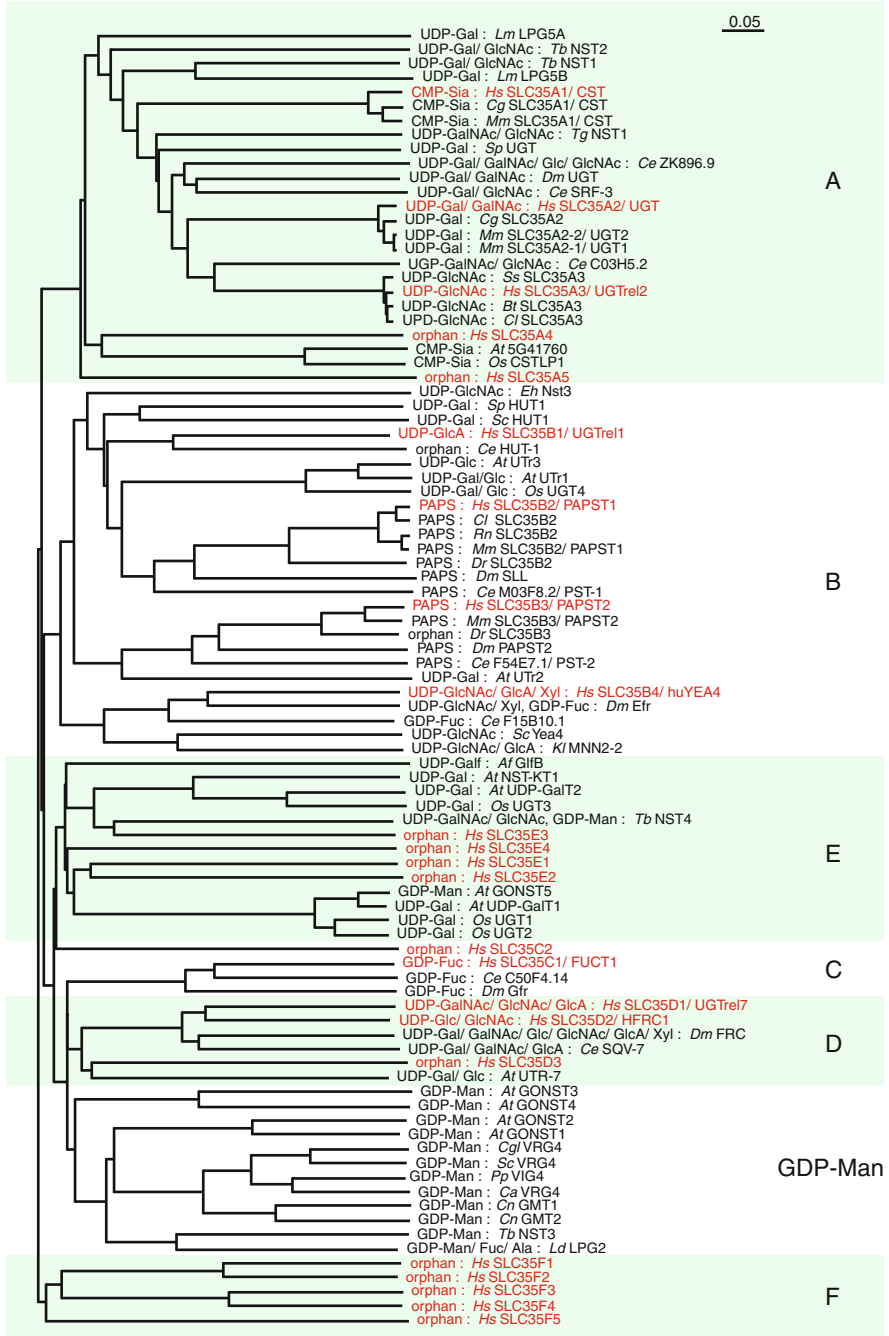
0.05

UDP-Gal : *Lm* LPG5A
UDP-Gal/ GlcNAc : *Tb* NST2
UDP-Gal/ GlcNAc : *Tb* NST1
UDP-Gal : *Lm* LPG5B
CMP-Sia : *Hs* SLC35A1/ CST
CMP-Sia : *Cg* SLC35A1/ CST
CMP-Sia : *Mm* SLC35A1/ CST
UDP-GalNAc/ GlcNAc : *Tg* NST1
UDP-Gal : *Sp* UGT
UDP-Gal/ GalNAc/ Glc/ GlcNAc : *Ce* ZK896.9
UDP-Gal/ GalNAc : *Dm* UGT
UDP-Gal/ GlcNAc : *Ce* SRF-3
UDP-Gal/ GalNAc : *Hs* SLC35A2/ UGT
UDP-Gal : *Cg* SLC35A2
UDP-Gal : *Mm* SLC35A2-2/ UGT2
UDP-Gal : *Mm* SLC35A2-1/ UGT1
UGP-GalNAc/ GlcNAc : *Ce* C03H5.2
UDP-GlcNAc : *Ss* SLC35A3
UDP-GlcNAc : *Hs* SLC35A3/ UGTrel2
UDP-GlcNAc : *Bt* SLC35A3
UPD-GlcNAc : *Cl* SLC35A3
orphan : *Hs* SLC35A4
CMP-Sia : *At* 5G41760
CMP-Sia : *Os* CSTLP1
orphan : *Hs* SLC35A5

A

UDP-GlcNAc : *Eh* Nst3
UDP-Gal : *Sp* HUT1
UDP-Gal : *Sc* HUT1
UDP-GlcA : *Hs* SLC35B1/ UGTrel1
orphan : *Ce* HUT-1
UDP-Glc : *At* UTr3
UDP-Gal/Glc : *At* Utr1
UDP-Gal/ Glc : *Os* UGT4
PAPS : *Hs* SLC35B2/ PAPST1
PAPS : *Cl* SLC35B2
PAPS : *Rn* SLC35B2
PAPS : *Mm* SLC35B2/ PAPST1
PAPS : *Dr* SLC35B2
PAPS : *Dm* SLL
PAPS : *Ce* M03F8.2/ PST-1
PAPS : *Hs* SLC35B3/ PAPST2
PAPS : *Mm* SLC35B3/ PAPST2
orphan : *Dr* SLC35B3
PAPS : *Dm* PAPST2
PAPS : *Ce* F54E7.1/ PST-2
UDP-Gal : *At* UTr2
UDP-GlcNAc/ GlcA/ Xyl : *Hs* SLC35B4/ huYEA4
UDP-GlcNAc/ Xyl, GDP-Fuc : *Dm* Efr
GDP-Fuc : *Ce* F15B10.1
UDP-GlcNAc : *Sc* Yea4
UDP-GlcNAc/ GlcA : *Kl* MNN2-2

B

UDP-Galf : *Af* GlfB
UDP-Gal : *At* NST-KT1
UDP-Gal : *At* UDP-GalT2
UDP-Gal : *Os* UGT3
UDP-GalNAc/ GlcNAc, GDP-Man : *Tb* NST4
orphan : *Hs* SLC35E3
orphan : *Hs* SLC35E4
orphan : *Hs* SLC35E1
orphan : *Hs* SLC35E2
GDP-Man : *At* GONST5
UDP-Gal : *At* UDP-GalT1
UDP-Gal : *Os* UGT1
UDP-Gal : *Os* UGT2

E

orphan : *Hs* SLC35C2
GDP-Fuc : *Hs* SLC35C1/ FUCT1
GDP-Fuc : *Ce* C50F4.14
GDP-Fuc : *Dm* Gfr

C

UDP-GalNAc/ GlcNAc/ GlcA : *Hs* SLC35D1/ UGTrel7
UDP-Glc/ GlcNAc : *Hs* SLC35D2/ HFRC1
UDP-Gal/ GalNAc/ Glc/ GlcNAc/ GlcA/ Xyl : *Dm* FRC
UDP-Gal/ GalNAc/ GlcA : *Ce* SQV-7
orphan : *Hs* SLC35D3
UDP-Gal/ Glc : *At* UTR-7

D

GDP-Man : *At* GONST3
GDP-Man : *At* GONST4
GDP-Man : *At* GONST2
GDP-Man : *At* GONST1
GDP-Man : *Cgl* VRG4
GDP-Man : *Sc* VRG4
GDP-Man : *Pp* VIG4
GDP-Man : *Ca* VRG4
GDP-Man : *Cn* GMT1
GDP-Man : *Cn* GMT2
GDP-Man : *Tb* NST3
GDP-Man/ Fuc/ Ala : *Ld* LPG2

GDP-Man

orphan : *Hs* SLC35F1
orphan : *Hs* SLC35F2
orphan : *Hs* SLC35F3
orphan : *Hs* SLC35F4
orphan : *Hs* SLC35F5

F

Fig. 2 (continued)

Procedure

Identification of NST Activity

In general, two types of assays for identifying NST activity are used: a heterologous expression system (Kamiyama et al. 2003; Suda et al. 2004) and a proteoliposome system (Caffaro and Hirschberg 2006). The former method is discussed in this section. A heterologous expression system makes use of a yeast expression system for the expression of NSTs derived from various other species. This is done in order to obtain a large enough microsome fraction expressing the NST of interest to allow for the determination of its Km value. This is possible because the yeast microsome normally shows only low endogenous NST activity, except for GDP-Man transporter activity, which is highly present in yeast. The experimental procedure consists of (I) a subcellular fractionation of yeast expressing the NST of interest and (II) performing the NST activity assay.

(I) Preparation of subcellular fractionation of yeast (*Saccharomyces cerevisiae*) expressing the NST of interest (Kamiyama et al. 2003, 2006; Suda et al. 2004; Goda et al. 2006; Sasaki et al. 2009):

1. An NST coding region is inserted into the yeast expression vector YEp352GAP-II with three copies of HA epitope tags (YPYDVPDYA) at the position corresponding to the C terminus of the NST of interest. (An HA tag can be inserted at the position corresponding to the N terminus if the NST has an ER retention signal, a dilysine motif, at its C terminus.)
2. The yeast strain W303-1a (*MATa*, *ade2-1*, *ura3-1*, *his3-11*, *his15*, *trp1-1*, *leu2-3*, *leu112*, and *can1-100*) is transformed by the lithium acetate procedure using the yeast expression vector YEp352GAP-II.
3. In order to select the transformants, the transformed yeast cells are grown at 30 °C in a synthetic defined medium that lacks uracil.
4. The cells are harvested and then washed with ice-cold 10 mM NaN_3.
5. The cells are converted into spheroplasts by incubation at 37 °C for 30 min in spheroplast buffer (1.4 M sorbitol, 50 mM potassium phosphate

Fig. 2 Phylogenetic tree of the members of the nucleotide-sugar transporter family, solute carrier 35 (SLC35). The phylogenetic tree was created based on amino acid sequences by using the ClustalX program. Branch lengths indicate evolutionary distances between members. The scale at the *top* represents the evolutionary distance. The nucleotide sugars or PAPS that each transporter transfers from the cytosol to the ER or Golgi lumen are shown in Fig. 1. Members found in *Homo sapiens* are shown in *red*. At *Arabidopsis thaliana*, Af *Aspergillus fumigatus*, Bt *Bos taurus*, Ce *Caenorhabditis elegans*, Ca *Candida albicans*, Cgl *Candida glabrata*, Cl *Canis lupus familiaris*, Cg *Cricetulus griseus*, Cn *Cryptococcus neoformans*, Dr *Danio rerio*, Dm *Drosophila melanogaster*, Eh *Entamoeba histolytica*, Hs *Homo sapiens*, Kl *Kluyveromyces lactis*, Ld *Leishmania donovani*, Lm *Leishmania major*, Mm *Mus musculus*, Os *Oryza sativa Japonica*, Pp *Pichia pastoris*, Rn *Rattus norvegicus*, Sc *Saccharomyces cerevisiae*, Sp *Schizosaccharomyces pombe*, Ss *Sus scrofa*, Tg *Toxoplasma gondii*, Tb *Trypanosoma brucei*.

Table 1 The members of nucleotide sugar transporter family, SLC35

Subgroup	No.	Synonym	Substrate (human)	Subcellular localization	Gene locus (human)	Disease (human)	Cloning and identification of activity of human gene	Cloning and identification of activity of other vertebrate genes[a]	Cloning and identification of activity of Drosophila gene[a]	Cloning and identification of activity of C. elegans gene[a]	Cloning and identification of activity of plant gene[a]	Cloning and identification of activity of genes of other species[a]
SLC35A	1	CST	CMP-Sia	Golgi	6q15	CDG IIf	J Biochem 124: 171–178 (1998)	CMP-Sia: Mm SLC35A1/CST (PNAS 93: 7572–7526 (1996))				UDP-Gal: Lm LPG5A (JBC 282: 14006–14017 (2007)), UDP-Gal/GlcNAc: Tb NST1 (JBC 288: 10599–10615 (2013)), Tb NST2 (JBC 288: 10599–10615 (2013)), UDP-Gal: Lm LPG5B (JBC 282: 14006–14017 (2007)), UDP-GalNAc/GlcNAc: Tg NST1 (PLoS Pathog 9: e100331 (2013)), UDP-Gal: Sp UGT (FEBS Lett 451: 295–298 (1999))
	2	UGT	UDP-Gal UDP-GalNAc	Golgi/ER	Xp11.23	CDG IIm	J Biochem 120: 236–241 (1996) J Biochem 120: 1074–1078 (1996)	UDP-Gal: Mm SLC35A2 (J Biochem 126: 1107–1117 (1999)), UDP-Gal: Cg SLC35A2 (JBC 276: 26291–26300 (2001))	UDP-Gal/GalNAc: Dm UGT (Eur J Biochem 269: 128–131 (2002))	UDP-Gal/GalNAc/Glc/GlcNAc: Ce ZK896.9 (Biochemistry 47: 4337–4344 (2008)), UDP-Gal/GlcNAc: Ce SRF-3 (JBC 279: 30440–30448 (2004))		
	3	UGTrel2	UDP-GlcNAc	Golgi	1p21.2	Autism spectrum disorder (complex vertebral malformation: calf)	J Biochem 126: 68–77 (1999)	UDP-GlcNAc: Cl SLC35A3 (PNAS 95: 7888–7892 (1998)), UDP-GlcNAc: Bt SLC35A3 (Genome Res. 16: 97–105 (2006)), UDP-GlcNAc: Ss SLC35A3 (Mol Membr Biol. 24: 519–30 (2007))		UDP-GalNAc/GlcNAc: Ce C03H5.2 (PNAS 103: 16176–16181 (2006))	
	4		Orphan		5q31.3						CMP-Sia: At 5G41760 (Carbohydrate Res 343: 2148–2152 (2008)), CMP-Sia: Os CSTLP1 (Phytochemistry 70: 1973–1981 (2009))	
	5		Orphan		3q13.2							

Family	#	Name	Substrate	Location	Locus	Disease					
SLC35B	1	UGTrel1	UDP-GlcA	ER	17q21.33		Biochem J 400: 281–289 (2006)			UDP-Glc: *At* UTr3 (Plant J 61: 423–435 (2010)), UDP-Gal/Glc: *At* UTr1 (JBC 277: 32923–42929 (2002)), UDP-Gal/Glc: *Os* UGT4 (J Biochem 148: 35–46 (2010))	UDP-GlcNAc: *Eh* Nst3 (Mol Biochem Parasitol 159: 44–53 (2008)), UDP-Gal: *Sc* HUT1 (Yeast 18: 533–541 (2001)), UDP-Gal: *Sp* HUT (Yeast 18: 543–554 (2001))
	2	PAPST1	PAPS	Golgi	6p21.1		JBC 278: 25958–15963 (2003)	PAPS: *Cl* SLC35B2 (Glycobiology 18: 53–65 (2008)), PAPS: *Mm* SLC35B2/PAPST1 (PLoS One 4: e8262 (2009)),PAPS: *Dr* SLC35B2 (PLoS Genet 4: e1000136 (2008))	PAPS: *Dm* SLL (JBC 278: 25958–15963 (2003)/EMBO J 22: 3635–3644 (2003))	PAPS: *Ce* M03F8.2/PST-1 (JCS 122: 4492–4504 (2009)/JBC 285: 24717–24728 (2010))	
	3	PAPST2	PAPS	Golgi	6p24.3		JBC 281: 10945–10953 (2006)	PAPS: *Mm* SLC35B3/PAPST2 (PLoS One 4: e8262 (2009))	PAPS: *Dm* PAPST2 (JBC 281: 28508–28517 (2006))	PAPS: *Ce* F54E7.1/PST-2 (JCS 122: 4492–4504 (2009)/JBC 285: 24717–24728 (2010))	UDP-Gal: *At* UTr2 (Planta 222: 521–529 (2005))
	4	huYEA4	UDP-GlcNAc UDP-GlcA DP-Xyl	Golgi	7q33		JBC 280: 27230–27235 (2005)		UDP-GlcNAc/ Xyl, GDP-Fuc: *Dm* Efr (JBC 285: 4122–4129 (2010))	GDP-Fuc: *Ce* F15B10.1 (Nat Genet 28:69–72 (2001))	UDP-GlcNAc: *Sc* YEA4 (JBC 275: 13580–13587 (2000)), UDP-GlcNAc/ GlcA: *Kl* MNN2-2 (PNAS 93: 5963–5968 (1996))
SLC35C	1	FUCT1	GDP-Fuc	Golgi	11p11.2	CDG IIc/ Leukocyte adhesion deficiency II	Nat Genet 28: 69–72 (2001) / Nat Genet 28: 73–76 (2001)		GDP-Fuc: Dm Gfr (Exp Cell Res 301: 242–250 (2004)/ PNAS 102: 18532–418537 (2005))	GDP-Fuc: *Ce* C50F4.14 (Nat Genet 28: 69–72 (2001))	
	2	OVCOV1	Orphan		20q13.12		Mamm Genome 13: 619–624 (2002)				

(continued)

Table 1 (continued)

Subgroup	No.	Synonym	Substrate (human)	Subcellular localization	Gene locus (human)	Disease (human)	Cloning and identification of activity of human gene	Cloning and identification of activity of other vertebrate genes[a]	Cloning and identification of activity of Drosophila gene[a]	Cloning and identification of activity of C. elegans gene[a]	Cloning and identification of activity of plant gene[a]	Cloning and identification of activity of genes of other species[a]
SLC35D	**1**	UGTrel7	UDP-GalNAc UDP-GlcNAc UDP-GlcA	ER	1p31.3	Schneckenbecken dysplasia	FEBS Lett 495: 87–93 (2001)		UDP-Gal/GalNAc/Glc/GlcNAc:	UDP-Gal/GalNAc/GlcUA: Ce SQV-7 (PNAS 98: 3738–3743 (2001))		
	2	HFRC1	UDP-Glc UDP-GlcNAc	Golgi	9q22.32		JBC 279: 26469–26474 (2004)		GcUA/Xyl1: Dm FRC (Nat Cell Biol 3: 809–815 (2001)/Nat Cell Biol 3: 816–822 (2001))			
	3		Orphan		6q23.3						UDP-Gal/Glc: At UTR-7 (Mol Plant 5: 1263–1280 (2012))	
GDP-Man											GDP-Man: At GONST1 (Mol Genet Genomics 272: 397–410 (2004)), GDP-Man: At GONST2 (Mol Genet Genomics 272: 397–410 (2004)), GDP-Man: At GONST3 (Mol Genet Genomics 272: 397–410 (2004)), GDP-Man: At GONST4 (Mol Genet Genomics 272: 397–410 (2004))	GDP-Man: Cgl VRG4 (Yeast 19: 691–698 (2002)), GDP-Man: Sc VRG4 (JBC 272: 31908–31914 (1997)), GDP-Man: Pp VIG4 (J Gen Appl Microbiol 52: 137–145 (2006)), GDP-Man: Ca VRG4 (J Bacteriol 184: 29–42 (2002)), GDP-Man: Cn GMT1 (Eukaryot Cell 6: 776–785 (2007)), GDP-Man: Cn GMT2 (Eukaryot Cell 6: 776–785 (2007)), Tb NST3 (JBC 288: 10599–615 (2013)), GDP-Man/Fuc/Ala: Ld LPG2 (JBC 280: 2028–2035 (2005))

SLC35E	1		Orphan	19p13.11		
	2		Orphan	1p36.33	GDP-Man: *At* GONST5 (Mol Genet Genomics 272: 397–410 (2004)), UDP-Gal: *At* UDP-GalT1 (Glycobiology 15: 193–201 (2005)), UDP-Gal: *OS* UGT1 (J Biochem 148: 35–46 (2010)), UDP-Gal: *OS* UGT2 (J Biochem 148: 35–46 (2010))	
	3	BLOV1	Orphan	12q15	UDP-Gal: *At* NST-KT1 (FEBS Lett 580: 4246–4251 (2006)), UDP-Gal: *At* UDP-GalT2 (Glycobiology 15: 193–201 (2005)), UDP-Gal: *OS* UGT3 (J Biochem 148: 35–46 (2010))	UDP-GalNAc/ GlcNAc, GDP-Man: *Tb* NST4 (JBC 288: 10599–615 (2013)), UDP-Galf: *Af* GlfB (JBC 284: 33859–33868 (2009))
	4		Orphan	22q12.2		
SLC35F	1		Orphan	6q22.31		
	2	HSNOV1	Orphan	11q22.3		
	3		Orphan	1q42.2		
	4		Orphan	14q22.2		
	5		Orphan	2q14.1		

[a]Only the sugar nucleotide transporters, of which substrates have already been determined, are described with references (substrates: name of transporters)

Abbreviation: *At* Arabidopsis thaliana, *Af* Aspergillus fumigatus, *Bt* Bos taurus, *Ce* Caenorhabditis elegans, *Ca* Candida albicans, *Cgl* Candida glabrata, *Cl* Canis lupus familiaris, *Cg* Criculus griseus, *Cn* Cryptococcus neoformans, *Dr* Danio rerio, *Dm* Drosophila melanogaster, *Eh* Entamoeba histolytica, *Hs* Homo sapiens, *Kl* Kluyveromyces lactis, *Ld* Leishmania donovani, *Lm* Leishmania major, *Mm* Mus musculus, *Os* Oryza sativa Japonica, *Pp* Pichia pastoris, *Sc* Saccharomyces cerevisiae, *Sp* Schizosaccharomyces pombe, *Ss* Sus scrofa, *Tg* Toxoplasma gondii, *Tb* Trypanosoma brucei.

(pH 7.5), 10 mM NaN$_3$, 40 mM 2-mercaptoethanol, and 1 mg/g of cells of zymolyase 100T (Seikagaku Kogyo Co. Ltd.; Tokyo, Japan)).

6. The spheroplasts are centrifuged into a pellet at 4 °C and washed twice with 1.0 M ice-cold sorbitol to remove traces of zymolyase.

7. The spheroplasts are suspended in ice-cold lysis buffer (0.8 M sorbitol in 10 mM triethanolamine (pH 7.2), 5 µg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride) and homogenized using a Dounce homogenizer.

8. The lysate is centrifuged at 1,000 g for 10 min to remove unlysed cells and cell wall debris.

9. The supernatant is centrifuged at 10,000 g for 15 min at 4 °C to obtain the P10 membrane fraction pellet.

10. The supernatant is centrifuged further at 100,000 g to obtain the P100 membrane fraction pellet.

11. Each membrane fraction pellet is used for the NST activity assay.

The expression level of HA-tagged NST in each membrane fraction is determined by western blot analysis using an anti-HA mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA).

(II) The NST activity assay (Kamiyama et al. 2003, 2006; Suda et al. 2004; Goda et al. 2006; Sasaki et al. 2009):

1. Each of the pellets described above (100 µg protein) is incubated at appropriate temperature for 5 min in 100 µl of reaction buffer (20 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 5.0 mM MgCl$_2$, 1.0 mM MnCl$_2$, and 10 mM 2-mercaptoethanol) that contains 1 µM radiolabeled nucleotide sugars or PAPS substrate.

2. The reaction is stopped by adding 1 ml of stop buffer (20 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 5.0 mM MgCl$_2$).

3. The radioactivity incorporated in the microsomes is trapped by a 0.45-µm nitrocellulose filter. Then, the filter is washed with 10 ml of stop buffer and trapped radioactivity is measured by using a liquid scintillation counter. The amount of incorporated radioactivity is calculated as the difference from a background value obtained by the same assay at corresponding temperature at 0 min for each sample.

Functional Analysis of NSTs by RNA Interference

RNA interference (RNAi) is widely used for the functional analysis of a broad range of biologically important molecules. The introduction and expression of double-stranded RNAs into a cell results in the knockdown of expression of a targeted gene through interference of this exogenously introduced RNA molecule with the RNA molecule of the target gene (Kamiyama et al. 2003, 2011; Goda et al. 2006; Sasaki et al. 2009). *NST* genes are no exception and can be targeted with this same scheme. In embryonic stem cells, short hairpin RNAs (shRNAs) expressed using shRNA expression vectors and 21–25-nt synthetic small interfering RNAs (siRNAs) can be used with effective knockdown efficiency (Sasaki

et al. 2009). However, in mature mammalian cell lines, synthetic siRNAs are more useful than shRNAs expressed using expression vectors (Kamiyama et al. 2006, 2011). In order to eliminate off-target effects, at least two different siRNA sequences should be used. As long as the sequences used for knockdown are in the $3'$ noncoding regions, rescue experiments can be carried out by the over expression of knockdown genes. The siRNA-induced knockdown of *NSTs* had a delayed effect on glycosylation compared to knockdown of genes coding for core proteins and glycosyltransferases. Effect of synthetic siRNAs cannot be kept long. Thus, it is recommended that the transfection of synthetic siRNAs should be repeated three times. For detailed descriptions of experimental conditions, refer to the references (Kamiyama et al. 2003, 2006, 2011; Goda et al. 2006; Sasaki et al. 2009).

Results

Determination of Km Values

The Km values of human FRC1 (*Hs* SLC35D2/HFRC1) for nucleotide sugars were determined by using the methods described above (Suda et al. 2004). *Hs* SLC35D2/HFRC1 is the human ortholog of *Drosophila* Frc (*Dm* FRC) and *C. elegans* SQV-7, which showed multi-substrate specific NST activity (Fig. 2). *Hs* SLC35D2/HFRC1 showed transport activity for UDP-GlcNAc and UDP-Glc and their Km values were measured to be 8.0 and 2.1 μM, respectively. Km values of *Dm* FRC for UDP-GlcNAc were 3 μM or 7.8 μM, where *S. cerevisiae* or *Leishmania* microsomal vesicles was used for overexpressing HA-tagged *Dm* FRC, respectively (Selva et al. 2001; Muraoka et al. 2007). The Km values for UDP-GlcNAc were not very different between human and *Drosophila*.

The Km values of PAPST1 and PAPST2 from various species for PAPS were also determined by using the method described above. Apparent Km values of 0.8 μM (Kamiyama et al. 2003), 1.2 μM (Kamiyama et al. 2003), 1.54 μM (Sasaki et al. 2009), and 4.03 μM (Dejima et al. 2010) were determined for human PAPST1 (*Hs* SLC35B2/PAPST1), *Drosophila* SLL (*Dm* SLL), mouse PAPST1 (Mm SLC35B2/PAPST1), and *C. elegans* PST1 (*Ce* M03F8.2/PST-1), respectively. The Km values of PAPST1 for PAPS were not significantly different between species. The Km values of 2.2 μM (Kamiyama et al. 2006), 2.3 μM (Goda et al. 2006), and 1.49 μM (Sasaki et al. 2009) were measured for human PAPST2 (*Hs* SLC35B3/PAPST2), *Drosophila* PAPST2 (*Dm* PAPST2), and mouse PAPST2 (*Mm* SLC35B3/PAPST2), respectively. The PAPST2 of each species showed a similar Km value as its PAPST1.

Functional Analysis by RNAi

NSTs or PAPSTs determine the status of glycosylation or sulfation by supplying the donor substrates to glycosyltransferases or sulfotransferases, respectively. To study

the role of PAPSTs in colorectal cancer, synthetic siRNAs were used in colorectal cancer cell lines as a knockdown for human *PAPST1* and *PAPST2* (Kamiyama et al. 2011). In this study, the siRNAs were transfected three times, once every 3 days, in order to obtain optimal interference and inhibition. Knockdown of either of the two *PAPST* genes reduced the amount of sulfation of glycans on cellular proteins, FGF signaling, and also cellular proliferation. This result indicates that PSPTs play a role in the proliferation of colorectal cancer cells by controlling the sulfation state of their target molecules.

Comments

Many NSTs have been cloned to date, and their activity has also been identified (Fig. 2 and Table 1). Several NSTs can transport the same nucleotide sugar. For example, four types of human NSTs have been identified to be able to transport UDP-GlcNAc, namely, *Hs* SLC35A3/UGTrel2, *Hs* SLC35B4/huYEA4, *Hs* SLC35D1/UG, and *Hs* SLC35D2/HFRC1. *Hs* SLC35A3/UGTrel2, *Hs* SLC35B4/huYEA4, and *Hs* SLC35D2/HFRC1 are localized in the Golgi apparatus, while *Hs* SLC35D1/UGTrel7 is localized in the ER (Table 1). The purpose of such redundancy is not yet clear. One possibility is that specific glycosyltransferases bind to each of these NSTs and use them differently. Redundancy can also be seen in "PAPSTs," such as PAPST1 and PAPST2, both of which are localized in the Golgi apparatus of humans and other species (Table 1). In plants, there are many UDP-Gal transporters in different SLC35 subgroups, SLC35B, SLC35D, and SLC35E, which are yet other examples of redundancy for which the purpose is not clear (Fig. 2 and Table 1). This unsolved issue of redundancy might be important for our understanding of the basic mechanisms underlying the glycosylation and sulfation systems.

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