Molecular Cloning and Identification of 3′-Phosphoadenosine 5′-Phosphosulfate Transporter*

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Nucleotide sulfate, namely 3′-phosphoadenosine 5′-phosphosulfate (PAPS), is a universal sulfuryl donor for sulfation. Although a specific PAPS transporter is present in Golgi membrane, no study has reported the corresponding gene. We have identified a novel human gene encoding a PAPS transporter, which we have named PAPST1, and the Drosophila melanogaster ortholog, slalom. The amino acid sequence of PAPST1 (432 amino acids) exhibited 48.1% identity with SLL (465 amino acids), and hydropathy analysis predicted the two to be type III transmembrane proteins. The transient expression of PAPST1 in SW480 cells showed a subcellular localization in Golgi membrane. The expression of PAPST1 and SLL in yeast Saccharomyces cerevisiae significantly increased the transport of PAPS into the Golgi membrane fraction. In human tissues, PAPST1 is highly expressed in the placenta and pancreas and present at lower levels in the colon and heart. An RNA interference fly of slalom produced with a GAL4-UAS system revealed the kinetic behavior of a PAPS-specific transport through an antiport mechanism (8–10); however, cloning of the transporter has not been reported.

Earlier studies had shown a saturable transport activity of PAPS using isolated Golgi vesicles (6) or reconstituted proteoliposome (7). To identify this transporter protein, the proteins responsible for PAPS translocating activity have been purified (8–10). The characterization of these purified proteins revealed the kinetic behavior of a PAPS-specific transport through an antiport mechanism (8–10); however, cloning of the transporter has not been reported.

Recently, several nucleotide-sugar transporters (NSTs) have been cloned and characterized in mammals, yeast, protozoa, and plants (11–13). Nucleotide-sugars are the donor substrates for glycosylation, which is catalyzed by glycosyltransferases. These NST proteins are highly hydrophobic Type III transmembrane proteins localized in the Golgi or endoplasmic reticulum membrane and provide a specific substrate for the glycosylation. The structural conservation among NSTs has contributed to the identification of novel NST-related sequences from existing databases, whereas the levels of the amino acids identified are not indicators of their substrate specificities (14). A large number of putative NST sequences from mammals, Drosophila, Caenorhabditis elegans, plants, and yeast are under investigation for substrate specificity and function.

By a BLAST search of the data base, we identified a putative

Glycosylation, phosphorylation, and sulfation are essential post-translational alterations of glycoproteins, proteoglycans, and glycolipids for normal growth and development. For sulfation, an activated form of sulfate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), 1 is used as a common sulfate donor (1). Sulfate is transferred from PAPS to a defined position on the sugar residue by sulfotransferases. Higher organisms, PAPS is synthesized in the cytosol (2, 3) by a bifunctional PAPS synthetase having both ATP-sulfurylase and adenosine-phosphosulfate kinase activities (4). Since sulfation occurs in the lumens of the endoplasmic reticulum and Golgi apparatus (5), PAPS must be translocated from the cytosol into the Golgi lumen through a specific transporter localized in the microsomal membrane.

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1 The abbreviations used are: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; HA, influenza hemagglutinin epitope; NST, nucleotide-sugar transporter; NF-BIE, nuclear factor of a light polypeptide gene enhancer in B-cells inhibitor epsilon; ENAI, RNA interference; UGTrel1, UDP-galactose transporter-related isozyme 1; mAb, monoclonal antibody; ORF, open reading frame.
Molecular Cloning of an Essential PAPS Transporter

NST gene homologous to human UGTrel1 (UDP-galactose transporter-related isozyme 1) (15). Unexpectedly, the heterologous expression of PAPST1 in yeast Saccharomyces cerevisiae did not result in any nucleotide-sugar transport activity but revealed PAPS transport activity. Furthermore, the Drosophila melanogaster orthologous gene, slalom (sll), had the same substrate specificity. When double-stranded RNA of sll was expressed under the control of a cytological promoter to induce the silencing of the sll gene, the RNAi fly showed marked lethality. Here we reported the functional properties of these novel PAPS transporters.

EXPERIMENTAL PROCEDURES

Materials—UDP-[3H]mannosine (15 Ci/mmol), UDP-[1-3H]glucosamine (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). [32P]PAPS (2.275 Ci/mmol), GDP-[1-3H]fucose (9.95 Ci/mmol), UDP-[4,5-3H]galactose (48.3 Ci/mmol), CMP-[9-3H]sialic acid (33.6 Ci/mmol), and UDP-N-acetyl-[6-3H]glucosamine (39.7 Ci/mmol) were purchased from PerkinElmer Life Sciences. Fluorescein isothiocyanate-conjugated anti-influenza hemagglutinin epitope (HA) monoclonal antibody (mAb) was obtained from Roche Applied Science. 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 Human and Drosophila PAPS Transporter cDNAs and Construction of Expression Plasmas—A TBLASTN search was performed for the amino acid sequence of the open reading frame (ORF) of UGTrel1 (15). We succeeded in identifying a candidate cDNA sequence encoding a full-length ORF. To obtain this cDNA and create recombinant 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′-aaaaagcaggcttcgcctggaccatggacgc-3′) and a reverse template-specific primer with attB2 (5′-aaaaagcaggctttcgcctgcgggtcatcactctttc-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™ vector to create an entry clone for the subsequent subcloning were performed according to the instruction manual. A Drosophila cDNA encoding the ORF of slalom (sll) was obtained from expressed sequence tag clone SD04658 (Invitrogen) and a cDNA library derived from human colon tissue. The insertion of a complete attB adaptor and cloning into the pDONR™ vector to create an entry clone for the subsequent subcloning were performed according to the instruction manual. A Drosophila cDNA encoding the ORF of slalom (sll) was obtained from expressed sequence tag clone SD04658 (Invitrogen) by PCR using a yeast expression vector, YEp352GAP-β II, kindly provided by Dr. K. Nakayama (National Institute of Advanced Industrial Science and Technology, Japan). Transformed yeast cells were grown at 30 °C in synthetic defined medium in which uracil was omitted for the selection of transformants. Subcellular fractionation and nucleotide-sugar transport assays were performed as described by Roy et al. (20). Yeast cells were transferred into spheroplasts and fractionated to yield a 10,000 × g membrane fraction (P10), a 100,000 × g membrane fraction (P100), and the supernatant of the cytosolic fraction (S100). Then 200 μg of protein of each fraction was incubated in 100 μl of reaction buffer (20 mM Tris-HCl, pH 7.5, 0.25 mM sucrose, 5.0 mM MgCl2, 1.0 mM MnCl2, and 10 mM 2-mercaptoethanol) containing 1 μM radiolabeled substrate at 30 °C for 5 min. After incubation, the radioactivity incorporated in the microsomes was trapped with a 0.45-μm nitrocellulose filter and measured by liquid scintillation. The amount of incorporated substrate was calculated as the difference with the background value obtained from the time 0 assay for each sample.

Western Blot Analysis—The indicated amounts of protein of samples were added to 3× SDS sample buffer (New England Biolabs Inc., Beverly, MA) and incubated at room temperature for 2 h. The samples were fractionated on a 5–20% gradient SDS-polyacrylamide gel using the XYL PANTÉLA electrophoresis system (DRC Corp., Tokyo, Japan). The separated proteins were electrotransferred onto polyvinylidene difluoride membrane. Anti-HA mouse mAb and horseradish peroxidase-conjugated anti-mouse IgG mAb were detected by ECL™ plus (Amersham Biosciences) according to the manufacturer’s instructions. Quantitative Analysis of the PAPST1 Transcript in Human Tissues by Real Time PCR—Total RNA was extracted from human tissues by the methods of Chomczynski and Sacchi (20). First-strand cDNA was synthesized using SuperScript™ first-strand synthesis kit (Invitrogen) according to the manufacturer’s instructions. Quantitation of PAPST1 expression in the different tissues was performed by real time PCR using the following primers: forward, 5′-gagagagccgaagctgtga-3′; reverse, 5′-tgggttctacatccttct-3′. The probe, which consisted of 5′-caagccgctcaggtcctcctg-3′, was labeled at the 5′-end with the reporter dye, FAM, and at the 3′-end with the quencher dye TAMRA (Applied Biosystems, Foster City, CA). Real time PCR was performed using a TaqMan Universal PCR Master Mix (Applied Biosystems) and ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The relative amount of PAPST1 transcript was normalized to an endogenous control, human glyceraldehyde-3-phosphate dehydrogenase transcript in the same cDNA.

Construction of sll RNAi Fly and Quantitative Analysis of the Transcript—A 500-bp cDNA fragment of sll was amplified by PCR (forward primer, 5′-acatctctcttcagcatctagc-3′; reverse primer, 5′-gacttggtcagagactc-3′) and inserted as an inverted repeat with a head-to-head orientation into a modified Bluescript vector, pSCL. The cloning of sll into the transformation vector pUAST was done as previously reported (21). The transformation of Drosophila embryos was carried out according to Spradling (22) with w1118 stock as a host to make four UAS-sll inverted repeat fly lines. Each line was mated with the Act5C-GAL4 fly line, and the F1 progeny was raised at 25 °C to determine the phenotype.

The quantitation of the sll transcript in third instar larvae was performed using real time PCR as described above. Sequences of used primers and probe are as follows: forward, 5′-ggatccgagcttgctgctg-3′; reverse, 5′-gatagagctcagagcagacta-3′; probe, 5′-caagccgctcaggtcctcctg-3′. The relative amount of sll transcript was normalized to an endogenous control, ribosomal protein L32 (RpL32) transcript in the same fly.

RESULTS

Cloning of Human PAPST1 and sll cDNA—We identified a cDNA sequence (GenBank™ accession number BC024288) homologous to UGTrel1 and cloned the full-length ORF as described under “Experimental Procedures.” We named it PAPST1. We also found a gene producing marked alignment with UGTrel1 in Drosophila, sll (GenBank™ accession number NM_079665). sll is known in Flybase as CG7623 (Flybase ac-
cession number FBgn0038524) and predicted to be an UDP-galactose transporter. An alignment of the amino acid sequences of PAPST1, SLL, and UGTrel1 is shown in Fig. 1A. PAPST1 consisted of 432 amino acids with a calculated mass of 47.5-kDa, whereas SLL has 465 amino acids with a calculated mass of 52.3-kDa. Hydrophobicity analysis and predictions of transmembrane helices of the amino acid sequence were carried out using the SOSui program developed by Mitsui Knowledge Industry Co., Ltd. The putative transmembrane domains are underlined. Possible N-glycosylation sites in the SLL sequence are double underlined. B, phylogenetic tree of human and Drosophila transporters. Dendrograms showing relationships for human and Drosophila transporters were constructed based on the amino acid sequences using the ClustalX program. Transporters are identified by the substrate specificity and the GenBank™ accession number. The branch length indicates the evolutionary distance between each member. The scale at the bottom represents evolutionary distance.

**Subcellular Localization of PAPST1 in Mammalian Cells**—The subcellular localization of transiently expressed PAPST1 protein in SW480 cells was investigated by immunofluorescence staining. A mammalian expression vector, pCXN2, was inserted with the ORF of PAPST1 with an HA epitope tag at the C terminus and transfected transiently into SW480 cells. The cells were double immunostained with anti-HA mAb and anti-βH9252,1,4-galactosyltransferase 1 mAb. The immunofluorescence microscopy of cells expressing HA-tagged PAPST1 is shown in Fig. 2A. PAPST1-HA showed partial co-localization with βH9252,1,4-galactosyltransferase 1, which is a typical protein of trans-Golgi localization (16). This indicates that PAPST1 is localized in the Golgi apparatus but not endoplasmic reticulum. Further support for this observation was derived from Western blotting of stable transfectants. Namalwa cells stably expressing HA-tagged PAPST1 or mock vector (vector alone) were

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**Fig. 1. Amino acid sequence analysis.** A, Clustal multiple sequence alignment of PAPST1, SLL, and UGTrel1. A multiple sequence alignment was performed using the ClustalX program. Introduced gaps are shown with hyphens. The asterisks indicate positions of amino acids identical among all proteins. The colons indicate fully conserved amino acids of “strong” groups defined by a score of >0.5. The dots indicate fully conserved amino acids of “weaker” groups defined by a score of ≤0.5. Hydropathy analysis and predictions of transmembrane helices of the amino acid sequence were carried out using the SOSui program developed by Mitsui Knowledge Industry Co., Ltd. The putative transmembrane domains are underlined. Possible N-glycosylation sites in the SLL sequence are double underlined. B, phylogenetic tree of human and Drosophila transporters. Dendrograms showing relationships for human and Drosophila transporters were constructed based on the amino acid sequences using the ClustalX program. Transporters are identified by the substrate specificity and the GenBank™ accession number. The branch length indicates the evolutionary distance between each member. The scale at the bottom represents evolutionary distance.

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**Table 1.**

| Protein | Exons | mRNA Composition |
|---------|-------|------------------|
| PAPST1  | 4     | Composed of four |
| SLL     | 5     | exons. The *sll* gene is mapped on Drosophila chromosome 3, and the mRNA is composed of five exons. **Subcellular Localization of PAPST1 in Mammalian Cells**—The subcellular localization of transiently expressed PAPST1 protein in SW480 cells was investigated by immunofluorescence staining. A mammalian expression vector, pCXN2, was inserted with the ORF of PAPST1 with an HA epitope tag at the C terminus and transfected transiently into SW480 cells. The cells were double immunostained with anti-HA mAb and anti-β1,4-galactosyltransferase 1 mAb. The immunofluorescence microscopy of cells expressing HA-tagged PAPST1 was shown in Fig. 2A. PAPST1-HA showed partial co-localization with β1,4-galactosyltransferase 1, which is a typical protein of trans-Golgi localization (16). This indicates that PAPST1 is localized in the Golgi apparatus but not endoplasmic reticulum. Further support for this observation was derived from Western blotting of stable transfectants. Namalwa cells stably expressing HA-tagged PAPST1 or mock vector (vector alone) were
fractionated into a 10,000 \times g \text{ fraction (P10)}, a 100,000 \times g \text{ microsomal fraction (P100), and the supernatant of the cytosolic fraction (S100). PAPST1 transfectant showed 16.3 times the PAPST1 transcript level of the mock transfectant (4.41 and 71.71 \times 10^{-3}/\text{glyceraldehyde-3-phosphate dehydrogenase transcript, respectively). As shown in Fig. 2B, PAPST1 protein was detected mainly in the P100 microsomal membrane fraction with a small amount in the P10 fraction by Western blotting. HA-tagged PAPST1 proteins migrated as a 48-kDa protein. These proteins were not detected in cells transfected with mock vector. These results indicate that HA-tagged PAPST1 protein is predominantly localized on Golgi membrane.

Substrate Specificities of PAPST1 and SLL Proteins Expressed in Yeast Cells—To investigate the functional properties of PAPST1 and SLL, we used a heterologous yeast expression system. Yeast strain S. cerevisiae is widely used, because the isolated microsomal vesicles have little nucleotide-sugar transport activity except for GDP-mannose. A yeast expression vector, YEp352GAP-II, was inserted with the ORF of PAPST1 or sl/ and introduced into W303-1a yeast. PAPST1 and SLL proteins were expressed in the yeast P100 membrane fraction (Fig. 3A). The substrate specificities of PAPST1 and SLL were examined using radiolabeled substrates. As shown in Fig. 3B, the transport activity of PAPS into the P100 fraction prepared from yeast cells expressing PAPST1 and SLL is significantly higher than that shown for the mock (2.5 and 4.9 times, respectively). No difference was observed among PAPST1, SLL, and mock in the transport of other nucleotide-sugars. This was confirmed in Namalwa cells stably transfected with PAPST1. The P100 fraction of the PAPST1 transfectant showed 4.3 times the PAPS transport activity of the mock transfectant (1.34 \pm 0.13 and 5.72 \pm 0.06 pmol/5 min/mg of protein, respectively). The substrate concentration dependences of PAPS transport by PAPST1 and SLL are shown in Fig. 3C. The apparent $K_m$ values of PAPST1 and SLL were estimated to be 0.8 and 1.2 $\mu$M, respectively.

Tissue Distribution of PAPST1 Transcripts in Human Tissues—The gene expression of PAPST1 in human tissues was analyzed using real time PCR. The distribution of PAPST1 transcripts in human tissues is shown in Fig. 4. The placenta had the most PAPST1 among the tissues tested. Relatively high levels of PAPST1 expression in the pancreas, mammary gland, and skeletal muscle were also observed. PAPST1 transcripts were hardly detectable at all in colon, heart, and prostate. All transcript levels are shown relative to that of glyceraldehyde-3-phosphate dehydrogenase.

Lethality of Inducible sll RNAi Flies—Proteoglycans, including heparan sulfate, chondroitin sulfate, and dermatan sulfate, are sulfated at various positions along their glycosaminoglycan chains. Since PAPS is the sole substrate for sulfation, the down-regulation of PAPS transport into Golgi lumen may display an abnormal biological phenotype. To elucidate the impor-
tance of the PAPS transporter to the viability of D. melanogaster, we made an inducible sll RNAi fly using the GAL4-UAS system (21, 23). First we made four UAS-sll inverted repeat fly lines, and then we used Act5C-GAL4 as a GAL4 driver to induce sll gene knock-down in all cells of the fly. In the F1 generations of the Act5C-GAL4 fly and the UAS-sll inverted repeat fly, double-stranded RNA of sll was expressed ubiquitously under the control of the cytoplasmic actin promoter to induce sll gene silencing.

The amount of sll transcript in the third instar larvae of each F1 is shown in Table I. All transcripts were analyzed by real time PCR and are shown as relative amounts to that of RpL32. The relative amount of sll transcript in the F1 of the UAS-sll inverted repeat fly crossed with the Act5C-GAL4 fly is reduced to approximately one-fifth of that in the F1 of w1118 crossed with Act5C-GAL4, Act5C-GAL4/+ , which corresponds to the wild type. All four lines of the F1 of the UAS-sll inverted repeat fly crossed with Act5C-GAL4 exhibited pupal lethality, and no fly developed into an adult (Table I). These results clearly demonstrated that sll is essential for the viability of flies.

DISCUSSION

We have identified and characterized a human PAPS transporter, PAPST1, and the Drosophila ortholog sll. PAPS is considered to be translocated from cytosol into Golgi lumen via a specific transporter. The present study is the first to achieve the cloning and molecular characterization of PAPS transporters.

To isolate these novel transporters, we used the cloning strategy of searching databases for cDNAs homologous to human UGTrel1. UGTrel1 is a gene of unknown function that has similarity with human UDP-galactose transporter genes (15). In humans, Muraoka et al. (24) identified a gene related to UGTrel1 and reported that the product transports both UDP-glucuronic acid and UDP-N-acetylglucosamine. The phylogenetic tree of these transporters indicated that PAPST1 is more closely related to UGTrel1 (Fig. 1B). PAPST1 was defined in GenBankTM as a nuclear factor of a light polypeptide gene enhancer in B-cell inhibitor epsilon (NFκBIE), although no evidence of this has been provided. Two distinct NFκBIE proteins were reported in 1997 independently by two laboratories (25, 26). In some data bases, there has been confusion over PAPST1 and two other NFκBIEs about the sequence and gene locus. Although we did not assess the effect of PAPST1 on nuclear factor κB DNA binding activity, we failed to find any

| Stack No. | Insertion chromosome | sll transcripta (percentage of wild type) | Lethality of F1 (lethal/total pupa) |
|-----------|----------------------|------------------------------------------|----------------------------------|
| 1         | 3                    | ND                                       | 20/20                            |
| 2         | 3                    | ND                                       | 5/5                              |
| 3         | 2                    | 20.7                                     | 56/56                            |
| 4         | 3                    | 24.2                                     | 22/22                            |

a The relative amount of each sll mRNA to RpL32 mRNA in F1 progeny of w1118 crossed with Act5C-GAL4, Act5C-GAL4/+, which corresponds to the wild type, was presented as 100. ND, not determined.

Mandon et al. (8) purified rat liver Golgi membrane transporter to a 75-kDa protein. The PAPS transport activity was characterized using phosphatidylincholine liposome and assessed to have an apparent Kₘ of 1.7 μM. Independently, Ozeran et al. (9, 10) purified and characterized a 230-kDa rat liver Golgi membrane translocase protein. From its kinetic properties, they characterized it as a specific transporter of PAPS, which acts through an antiport mechanism with adenosine 3',5'-biphosphate as the returning ligand. The kinetic behavior of PAPST1 resembles that of a rat protein (a saturable transporter of PAPS with an apparent Kₘ of 0.8 μM (Fig. 4B)); however, PAPST1 is different from these proteins in regard to its apparent molecular mass. The HA-tagged PAPST1 protein expressed in Namalwa cells showed a band of 48-kDa on Western blot analysis (Fig. 3). It is not clear whether these proteins are homo- or heteropolymers of PAPST1 or distinct PAPS transporters. Whether PAPST1 is the sole PAPS transporter or not should be evaluated in further investigations.

As shown in Table I, the RNAi fly of sll induced with the GALA-UAS system confirmed that sll is essential for viability. Recent studies on Drosophila demonstrated that the mutation of some genes required for proteoglycan biosynthesis, dally (27, 28), sugarless (29–32), and tout-velu (32, 33), resulted in defective signaling during development. A number of reports have suggested that heparan sulfate proteoglycans are involved in a variety of signaling pathways, in particular those of fibroblast growth factor (34), Wnt/Wingless (35), Decapentaplegic (27), and Hedgehog (36). Heparan sulfate proteoglycans are thought to be required for stabilizing the complex between a ligand and its receptors or restricting the extracellular diffusion of ligands (35). It is known that the developmental signaling functions of cell surface heparan sulfate proteoglycans are dependent on their sulfation states (35, 37). In Drosophila, the mutation of a gene encoding N-deacytelysl/N-sulfotransferase (sulfateless) caused defects in Wingless (27–31) and fibroblast growth factor signaling (38). Indeed, sll in the flybase is involved in signal transduction by growth factors of Wingless and Hedgehog during patterning and morphogenesis (39). We reported the requirement of Drosophila β1,4-galactosyltransferase I, which contributes to the synthesis of the linkage structure of proteoglycans, for viability (21). However, we did not investigate whether the lethality of the sll RNAi fly is caused by a reduction in the sulfation of proteoglycans or other sulfated glycoconjugates, such as sulfatides (40) and HNK-1 epitope (41). As shown in Fig. 2A, PAPST1 was only partially co-localized with trans-Golgi β1,4-galactosyltransferase 1. Thus, it might be involved in not only proteoglycan synthesis but also sulfatide synthesis, which occurs in the early Golgi compartment. Further clarification is necessary regarding the role of the PAPS.
transporter in proteoglycan synthesis and the signaling pathway.

Mutations of some genes related to PAPS synthesis are known to be responsible for human inherited disorders. The diastrophic dysplasia sulfate transporter gene was identified with the subsequent PAPS transport pathway. AChondrogenesis type IB, atelosteogenesis type II, dysplasia epiphysealis multiplex, and other diastrophic dysplasias is still unknown. In addition, Kurima et al. found that the PAPS synthase 2 gene is responsible for mouse brachymorphism characterized by a dome-shaped skull, short thick tail, and shortened limbs (43). Missense and nonsense mutations of PAPS synthase 2 were demonstrated in the human inherited disorder, spondylo-epimetapectase dysplasia (44). On the other hand, no genetic disorder has been associated with the subsequent PAPS transport pathway.

We also identified the Drosophila ortholog, slt. D. melanogaster is a well established model for genetic analysis. The analysis of the RNAi fly of all may help to elucidate the biological function of PAPS transport and its role in post-translational sulfation.

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Molecular Cloning and Identification of 3'-Phosphoadenosine 5'-Phosphosulfate Transporter

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