Identification and Characterization of a Novel Drosophila 3′-Phosphoadenosine 5′-Phosphosulfate Transporter*

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Sulfation of macromolecules requires the translocation of a high energy form of nucleotide sulfate, i.e. 3′-phosphoadenosine 5′-phosphosulfate (PAPS), from the cytosol into the Golgi apparatus. In this study, we identified a novel Drosophila PAPS transporter gene dPAPST2 by conducting data base searches and screening the PAPS transport activity among the putative nucleotide sugar transporter genes in Drosophila. The amino acid sequence of dPAPST2 showed 50.5 and 21.5% homology to the human PAPST2 and SLALOM, respectively. The heterologous expression of dPAPST2 in yeast revealed that the dPAPST2 protein is a PAPS transporter with an apparent Km value of 2.3 μM. The RNA interference of dPAPST2 in cell line and flies showed that the dPAPST2 gene is essential for the sulfation of cellular proteins and the viability of the fly. In RNA interference flies, an analysis of the genetic interaction between dPAPST2 and genes that contribute to glycosaminoglycan synthesis suggested that dPAPST2 is involved in the glycosaminoglycan synthesis and the subsequent signaling. The dPAPST2 and sll genes showed a similar ubiquitous distribution. These results indicate that dPAPST2 may be involved in Hedgehog and Decapentaplegic signaling by controlling the sulfation of heparan sulfate.

Sulfation of proteins, proteoglycans, and lipids is involved in a variety of biological phenomena. It requires certain processes that provide a donor substrate for sulfotransferases. First, the free sulfate incorporated into the cell must be converted into a high energy form of nucleotide sulfate, namely 3′-phosphoadenosine 5′-phosphosulfate (PAPS), by PAPS synthase. Next, the PAPS must be translocated from the cytosol into the Golgi apparatus by PAPS transporters. These components that are involved in the PAPS providing pathways also play a crucial role in controlling the sulfation process; however, the sulfotransferases have long been believed to be the rate-limiting components (1).

A recent analysis of Drosophila mutants with defects in the sulfation pathway revealed the significance of heparan sulfate (HS) sulfation on growth factor signaling during development (for reviews see Refs. 1–3). A mutation in a gene encoding Drosophila N-deacetylase/N-sulfotransferase (sulfatase, sfl) causes defects in Wingless (4) and fibroblast growth factor signaling (5). Recently, we identified the PAPS transporter genes, human PAPST1 and the Drosophila ortholog slalom (sll) (6). Lüders et al. (7) demonstrated that sll is involved in growth factor signaling pathways during patterning and morphogenesis similar to sfl. The cell surface HS is involved in a variety of developmental signaling pathways, and the functions of HS are known to depend on its sulfation state (8, 9). A mutation in the sll gene resulted in defects in multiple signaling pathways, including those of Wingless and Hedgehog signaling (7). The sll gene is also required for the determination of the embryonic dorsal/ventral axis, possibly for the activation of the signaling cascade that is initiated by the product of the putative sulfotransferase gene pipe (7, 10).

In the Drosophila embryonic salivary glands, both sll and the PAPS synthase gene (papss) are required for the production of sulfated macromolecules (7, 11, 12). The Drosophila genome contains a single PAPS synthase gene, whereas two isoforms of this gene have been identified in mammals (13, 14). On the other hand, the Drosophila PAPS transporter gene sll belongs to a large family of nucleotide sugar transporters, which includes a number of putative genes. This study aimed at identifying a novel Drosophila gene involved in the sulfation pathway. We detected a gene, dPAPST2, by conducting data base searches and screening the transport activity among the putative nucleotide sugar transporter genes in Drosophila. The heterologous expression of this gene in yeast revealed that the dPAPST2 protein is a novel PAPS transporter. The dPAPST2

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§ The abbreviations used are: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; dHS6ST, Drosophila heparan sulfate 6-O-sulfotransferase; dHs3st-B, Drosophila heparan sulfate 3-O-sulfotransferase B; dOXT, Drosophila peptide O-sulfotransferase; dG4GalT7, Drosophila β1,4-galactosyltransferase 7; EGFP, enhanced green fluorescent protein; en, engrailed; GAG, glycosaminoglycan; GMR, glass multiple reporter; HA, influenza hemagglutinin epitope; HS, heparan sulfate; IR, inverted repeat; mAb, monoclonal antibody; papss, PAPS synthase; Rpl32, ribosomal protein L32; RNAi, RNA interference; S2, Schneider 2; sll, slalom; dsRNA, double-stranded RNA.
gene interacted genetically with the following genes that are involved in the synthesis of HS: the *Drosophila* genes encoding β1,4-galactosyltransferase 7 (dβ4GalIT7), heparan sulfate 6-O-sulfotransferase (dHS6ST), heparan sulfate 3-O-sulfotransferase B (dHS3st-B), and peptide O-xyllosyltransferase (dOXT) (15–19). In this study, we demonstrated the possibility that dPAPST2 plays a role in the sulfation of HS.

**EXPERIMENTAL PROCEDURES**

**Materials**—GDP-[2-3H]mannose (15 Ci/μmol), UDP-[1-3H]glucose (15 Ci/μmol), UDP-N-acetyl-[6-3H]galactosamine (15 Ci/μmol), UDP-[3H(U)]glucuronic acid (15 Ci/μmol), and carrier-free [35S]Na2SO4 (10 mCi/mi) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). GDP-[1-3H]fucose (6.95 Ci/μmol), UDP-[4,5-3H]galactose (48.3 Ci/μmol), CMP-[9-3H]sialic acid (33.6 Ci/μmol), UDP-N-acetyl-[6-3H]glucosamine (39.7 Ci/μmol), and [35S]-PAPS (1.82 Ci/μmol) were purchased from PerkinElmer Life Sciences. Zymolyase 100T was obtained from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). All other reagents were of the highest purity commercially available.

**Isolation of a Novel Drosophila PAPS Transporter cDNA**—A *Drosophila* putative nucleotide sugar transporter gene was identified and cloned using the same procedures as those described previously (6). We performed a TBLASTN search for the amino acid sequence of the *sll* open reading frame and identified a *Drosophila* gene (GenBank™ accession number, AY069640). In order to prepare attB-flanked PCR product, the recombination sites for the GATEWAY™ cloning system (Invitrogen) were created by performing two steps of attB adaptor PCR. The first gene-specific PCR used the expression sequence tag clone LD40702 as a template for the amino acid sequence of the coding sequence), and dHS6ST (nucleotide position 78–577 of the coding sequence), dβ4GalIT7 (nucleotide position 14–495 of the coding sequence), dHS6ST (nucleotide position 1–49 of the coding sequence), dOXT (nucleotide position 1052–1551 of the coding sequence of NM_139448), and paps (nucleotide position 139–638 of the coding sequence of NM_168824) were amplified by PCR using a cDNA library derived from *Drosophila melanogaster* or each expression sequence tag clone.

Each PCR fragment was inserted as an inverted repeat (IR) sequence into the pSC1 vector. The IR-containing fragments were then subcloned into the transformation vector pUAST, and these vectors were introduced into *Drosophila* embryos of the w1118 mutant stock that were used as hosts to construct UAS-IR fly lines according to the procedure reported by Spradling (23). Each line was mated with the appropriate driver fly lines, and the F1 progeny was raised at 28 °C to observe the phenotypes.

**Quantitative Analysis of the dPAPST2 Transcript by Real Time PCR**—RNA was extracted from the third instar larvae of each fly line by using the Trizol reagent (Invitrogen). First-strand cDNA was synthesized using a Superscript II first-strand synthesis kit (Invitrogen). Real time PCR was performed using qPCR Mastermix QuickGoldStar (Eurogentec, Seraing, Belgium) and the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster, CA). The gene-specific primer pairs and TaqMan probes that were used for each gene are as follows. For the quantitation of dPAPST2, 5′-CTCGCAGC-GGAATCCTC-3′ forward primer, 5′-AGGTCAAGGAAAGGATACGGA-3′ reverse primer, and the 5′-ACGT-CGGAATCTCCGCAGGAA-3′ probe were used. For the quantitation of *sll*, 5′-GGCCCAAGTTGTGTTTTACGAT-
AAT-3' forward primer, 5'-GGTAGATGAAAGCAGGAGA-GCATAT-3' reverse primer, and the 5'-CCACCCGCTG-ACCGAGTTGCT-3' probe were used for the quantitation of ribosomal protein L32 (Rpl32). 5'-GCAAGCCCAAGGGTATCG-3' forward primer, 5'-CATGTTGGGCT-CAGAATCTG-3' reverse primer, and the 5'-AAACAGAG-TGCGTCCGCGCT-3' probe were used. The probes were labeled with a reporter dye TAMRA and a quencher dye FAM and a quencher dye TAMRA at the 5'-ends and at the 3'-ends, respectively. Relative amounts of the dPAPST2 and sll transcripts were normalized to those of the Rpl32 transcripts in the same cDNA.

**RNAi of Cell Lines**—Double-stranded RNA (dsRNA) for each gene was synthesized by T7 transcription. The following sequences were selected as the template DNA for transcription: dPAPST2, nucleotide position 446–946 of the coding sequence; sll, nucleotide position 78–577 of the coding sequence; and enhanced green fluorescent protein (EGFP) sequences were selected as the template DNA for transcription: TCAGATACTG-3'.

**MEGASCRIPT T7 transcription kit** (Ambion, Austin, TX) amplified by PCR, and dsRNA was synthesized using a TATAGGGAGA) that was followed by a 20-nucleotide long primer sequence (GAATTAATACGACTCATATTGCGTCGCCGCTTCA-3') that was followed by a 20-nucleotide long primer sequence (GAATTAATACGACTCATATTGCGTCGCCGCTTCA-3'). The probes were extracted using the Trizol reagent (Invitrogen), and first-strand cDNA was synthesized using a MEGASCRIPT T7 transcription kit (Ambion, Austin, TX) according to the manufacturer's protocol.

Twenty-four hours prior to the transfection, Schneider 2 (S2) cells were subcultured in 10-cm dishes at a concentration of 1 × 10^7 cells/dish in Schneider's medium containing 10% fetal bovine serum. The cells were transfected with 10 μg/ml of each dsRNA by using the Cellfectin reagent (Invitrogen). RNA was extracted using the Trizol reagent (Invitrogen), and first-strand cDNA was synthesized using a Superscript II first-strand synthesis kit (Invitrogen).

**Metabolic Labeling**—Forty-eight hours after transfection and 48 h prior to analysis, the cells were subcultured in 10-cm dishes at a concentration of 1 × 10^7 cells/dish in inorganic sulfate-free Schneider's medium containing 10% fetal bovine serum and 100 μCi/ml carrier-free [35S]Na₂SO₄. The cells were rinsed twice with phosphate-buffered saline, suspended in 4 volumes of lysis buffer (10 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 1 mM EDTA, and 0.5% phenylmethylsulfonyl fluoride), and incubated on ice for 1 h. The solution was centrifuged at 15,000 x g for 30 min, and the supernatants were used as cell lysates. Fifty micrograms of the protein in each sample was precipitated with 10% trichloroacetic acid and washed with 5% trichloroacetic acid, followed by cold acetone. The precipitate was dried and dissolved in 0.5 N NaOH for scintillation counting.

**RESULTS**

**Identification of dPAPST2**—In order to identify a novel PAPS transporter gene, we performed a BLAST search of Drosophila data bases using the sll gene sequence as the query sequence. We selected four Drosophila candidate genes (CG7853, CG5802, CG14511, and CG5374) that showed high homology to sll (Fig. 1A), and we cloned the open reading frame of each cDNA. On screening the PAPS transport activity, a cDNA sequence (GenBankTM accession number, AY069640), possessing the symbol name CG7853 in Flybase, was identified as a second member of the PAPS transporter gene family (details described below). Because the sll gene is an ortholog of the human PAPST1 gene (6), we named the identified gene dPAPST2. We cloned the full-length open reading frame from the expression sequence tag clone LD40702, as described under “Experimental Procedures.” The dPAPST2 gene contains an open reading frame of 1188 bp encoding a protein of 396 amino acids. Very recently, we found that the putative transporter gene SLC35B3 encodes a PAPS transporter in humans, and we named it PAPST2 (24). The phylogenetic tree of Drosophila and human transporter genes (Fig. 1A) indicated that dPAPST2 is an ortholog of the human PAPST2. The alignment of the amino acid sequences of dPAPST2, SLL, human PAPST1, and human PAPST2 is shown in Fig. 1B. dPAPST2 showed 21.5 and 50.5% homology to SLL and PAPST2, respectively. A hydrophobicity analysis of the amino acid sequences predicted that dPAPST2 is a type III transmembrane protein with nine transmembrane domains (Fig. 1B).

**dPAPST2 Is a Novel Drosophila PAPS Transporter Gene**—The substrate specificity of the dPAPST2 protein was determined in a manner similar to that of SLL, as reported previously (6). HA-tagged dPAPST2 was inserted into the YEp352GAP-II yeast expression vector and expressed in S. cerevisiae. The HA-tagged dPAPST2 protein was detected in the yeast Golgi-enriched P100 membrane fraction (Fig. 2A).

The P100 membrane fraction was used to measure the transport activity of the dPAPST2 protein for nucleotide sugars and PAPS. As shown in Fig. 2B, the dPAPST2 protein exhibited PAPS transport activity (1.7 ± 0.2 versus 3.1 ± 0.3 pmol/mg protein, respectively, mean ± S.E. from six independent experiments; p < 0.05, Student’s t test). On the other hand, no significant difference was observed for other nucleotide sugars. These results suggest that the dPAPST2 protein is a PAPS-specific transporter similar to SLL. The saturated PAPS transport activity of dPAPST2 had an apparent Kₘ value that was estimated to be 2.3 μM (Fig. 2C).

**Spatiotemporal Expression Profiles of dPAPST2 and sll in the Fly**—The developmental expression profiles and tissue distributions of dPAPST2 and sll mRNAs were investigated by a

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**FIGURE 1. Amino acid sequence analysis of dPAPST2.** A phylogenetic tree of dPAPST2 and other transporters. The phylogenetic tree was constructed based on the amino acid sequences by using the ClustalX program. The Drosophila putative genes are identified from the Flybase symbol name. The branch length indicates the evolutionary distance between the members. The scale at the bottom represents the evolutionary distance. h, human; d, Drosophila; and Tp, transporter. B, alignment of amino acid sequences of PAPST1, SLL, PAPST2, and dPAPST2. The amino acid sequence analysis was performed using the ClustalX program. The introduced gaps are shown by hyphens. The asterisks indicate identical amino acids among all the proteins. The colons indicate fully conserved amino acids defined by a score of ≥0.5. The dots indicate fully conserved amino acids defined by a score of ≥0.5. The boldface letters indicate possible N-glycosylation sites in the sequences. The underlined amino acids are putative transmembrane regions obtained using the SOSui program developed by Mitsui Knowledge Industry Co., Ltd. (Tokyo, Japan).
quantitative analysis using real time PCR (Fig. 3, A and B). As shown in Fig. 3A, during development, the expressions of dPAPST2 and sll were altered in a similar manner. Both genes showed higher expression levels in the early embryonic stage than in the other stages. The tissue distributions of the dPAPST2 and sll mRNAs in third instar larvae and adult flies are shown in Fig. 3B. The dPAPST2 and sll genes showed a similar ubiquitous distribution. The expression of sll mRNA was several folds that of dPAPST2 mRNA in all the tested organs (note the different scales in the panels in Fig. 3).

dPAPST2 Is Involved in the Sulfation of Cellular Proteins—To confirm whether dPAPST2 serves as a PAPS transporter in Drosophila cells, we reduced dPAPST2 expression in the Drosophila cell line S2 by using RNAi. The S2 cells were transfected with a 500-nucleotide-long dsRNA that targets the dPAPST2 or sll gene. For the negative control, we used a dsRNA that targets the EGFP gene.

The cells that were treated with each dsRNA were analyzed 96 h after transfection. As shown in Fig. 4A, the treatment with dPAPST2 and sll dsRNAs reduced the level of the corresponding transcript by 77 and 57%, respectively. No alteration was observed in the level of the nontarget gene transcript (Fig. 4A). Both transcripts of dPAPST2 and sll decreased in the double-knockdown cells.

The PAPS transport activity in the Golgi-enriched fraction that was prepared from each knockdown cell culture is shown in Fig. 4B. By treatment with the dPAPST2 and sll dsRNAs, the activity in the Golgi-enriched fraction decreased to 69 and 44% that of the control, respectively. The total sulfate incorporation into cellulosic proteins was determined by metabolic labeling with [35S]PAPS, and the incorporated radioactivity was measured. The specific incorporation was calculated by subtracting the value obtained in the mock transfecction from each of the corresponding values. The right panel shows the double-reciprocal plots used to determine the \( K_m \) value.
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dPAPST2 Genetically Interacts with the Drosophila dβ4GalT7 Gene in the Wing.—We further investigated the biological functions of dPAPST2 in the fly. First, we crossed the UAS-dPAPST2-IR fly lines with seven GAL4-driver lines, and each of the driver lines had a different promoter that is expressed exclusively in the wing. However, no visible phenotype was detected in the wings of all tested flies. On the other hand, a pops RNAi fly line, en-GAL4/UAS-paps-IR showed the small-wing phenotype in the posterior compartment where en is exclusively expressed (Table 2 and Fig. 6, B and F). Interestingly, the dβ4GalT7 RNAi mutant fly line en-GAL4/+;UAS-dβ4GalT7-IR/+ also showed the small-wing phenotype in the posterior compartment (Table 2 and Fig. 6, C and F). Nakamura et al. (17) reported that the dβ4GalT7-knockdown fly showed the small-wing phenotype in the posterior compartment and reduced the HS and chondroitin sulfate biosynthesis. Human and Drosophila β4GalT7 proteins are involved in the synthesis of glycosaminoglycans (GAGs) by transferring a Gal to the Xyl-β-O-Ser in the tetrasaccharide structure of the protein-linkage region of GAGs. To investigate whether dPAPST2 is involved in the synthesis of GAGs, we

FIGURE 4. The dPAPST2 protein acts as a PAPS transporter in Drosophila S2 cells. Drosophila S2 cells were transfected with the dsRNA of EGFP, dPAPST2, or sll at a total concentration of 10 μg/ml. A, expression levels of the sll and dPAPST2 transcripts in the knockdown cells. The relative amounts of each transcript were normalized to those of the RpL32 transcript, which was measured in the same cDNA. The indicated values are the means ± S.E. obtained from three independent experiments. sll, left panel; dPAPST2, right panel. B, PAPS transport activity of Golgi-enriched fraction prepared from knockdown cells. Each Golgi-enriched fraction (50 μg of protein) was incubated at 30 °C for 5 min in 100 μl of the reaction buffer containing 1 μM [35S]PAPS, and the incorporated radioactivity was measured. Values shown are the mean ± S.E. obtained from four independent experiments.

FIGURE 5. Quantitative analysis of dPAPST2 and sll mRNAs in dPAPST2 RNAi mutant flies by real time PCR. The levels of the dPAPST2 (A) and sll (B) transcripts in Act5C-GAL4/+;UAS-dPAPST2-IR[1]/+ and Act5C-GAL4/UAS-dPAPST2-IR[2] RNAi fly lines were measured in the third instar larvae by using real time PCR. The relative amount of each transcript was divided by that in the wild type, namely the F1 progeny of w1118 crossed with the Act5C-GAL4 driver fly. Values shown are the mean ± S.E. obtained from three independent experiments.

TABLE 1

| Stock no. | Inserted chromosome   | Viability of F1 (CyO,GFP) | χ2   |
|-----------|-----------------------|---------------------------|------|
| [1]       | III                   | 0.32                      | 37.6' |
| [2]       | II                    | 0.28'                     | 44.4' |

* F1 progeny of each homozygous UAS-dPAPST2-IR fly crossed with the Act5C-GAL4/CyO,GFP fly.

† Number of adult Act5C-GAL4/+;UAS-dPAPST2-IR[1]/+ or Act5C-GAL4/UAS-dPAPST2-IR[2] flies was divided by that of CyO,GFP/+;UAS-dPAPST2-IR[1]/+ or CyO,GFP/UAS-dPAPST2-IR[2] flies, respectively; the later fly lines have no GAL4 driver. If all flies hatch normally, the ratio is expected to be 1.

p < 0.005.

cytoplasmic actin promoter. The F1 fly lines Act5C-GAL4/+;UAS-dPAPST2-IR[1]/+ and Act5C-GAL4/UAS-dPAPST2-IR[2], respectively, are expected to induce dPAPST2 gene silencing in all cells at all developmental stages of the fly.

As shown in Table 1, both the Act5C-GAL4/+;UAS-dPAPST2-IR[1]/+ and Act5C-GAL4/UAS-dPAPST2-IR[2] lines exhibited semi-lethality. The efficiency of gene silencing in the third instar larvae was determined by real time PCR. The level of the dPAPST2 transcript in the Act5C-GAL4/+;UAS-dPAPST2-IR[1]/+ and Act5C-GAL4/UAS-dPAPST2-IR[2] lines was only 16.6 and 19.5% that in the wild-type fly, respectively (Fig. 5A). On the other hand, the level of the sll transcript in each line was not affected (91.8 and 118.8%, respectively), thereby indicating the specific silencing of dPAPST2 gene expression (Fig. 5B). These results demonstrated that dPAPST2 is a PAPS transporter gene essential for the viability of the fly, and it is not an accessory factor for the functioning of the sll gene.
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TABLE 2
Genetic interaction between dPAPST2 and dß4GalT7

| Genotype                                      | Percentage of small-wing phenotype % (small wing/total wing) |
|-----------------------------------------------|-------------------------------------------------------------|
| en-GAL4/+;UAS-dß4GalT7-IR/+                  | 100 (56/56)*                                                |
| en-GAL4;UAS-papss-IR                         | 100 (268/268)                                               |
| en-GAL4/+;UAS-dPAPST2-IR[1]/+                | 0 (0/80)                                                    |
| en-GAL4/UAS-dPAPST2-IR[2]                    | 0 (0/92)                                                    |
| en-GAL4/UAS-lacZ;UAS-dß4GalT7-IR/+           | 0 (0/70)                                                    |
| en-GAL4/+;UAS-dß4GalT7-IR/UAS-dPAPST2-IR[1]  | 14.6 (6/41)                                                 |
| en-GAL4/UAS-dPAPST2-IR[2];UAS-dß4GalT7-IR+/+ | 5.9 (6/102)                                                 |

* Number of female wings that exhibited the small-wing phenotype in the posterior compartment.

FIGURE 6. Genetic interaction between dPAPST2 and dß4GalT7 in the wing. A–E show a representative wing of a fly with each genotype. A, wild type. B, en-GAL4/UAS-papss-IR. C, en-GAL4/+;UAS-dß4GalT7-IR/+ . D, en-GAL4/UAS-lacZ;UAS-dß4GalT7-IR/+ . E, en-GAL4/+;UAS-dß4GalT7-IR/UAS-dPAPST2-IR[1]. All lines were raised at 28 °C. Wings displayed in A and D had no aberrant phenotype. Wings displayed in B, C, and E exhibited the small-wing phenotype in the posterior compartment. The position of the anterior-posterior compartment boundary is indicated with dashed lines. F, relative area of the posterior compartment in the wing. The area of each posterior compartment was calculated using the public domain NIH image program (developed by the National Institutes of Health), and it was divided by the total area of the same wing. A–E on the x axis indicate each genotype described above. Values shown are the mean ± S.E. of the indicated number of wings.

DISCUSSION

This study identified a novel Drosophila gene involved in the sulfation of proteoglycans. This gene, dPAPST2, was identified to be a PAPS transporter gene that is essential for the viability of the fly. An analysis of the genetic interaction between dPAPST2 and the genes that are involved in the GAG synthesis revealed that the dPAPST2 gene is involved in the synthesis of HS.

In a previous study, we identified another PAPS transporter gene, sll, in Drosophila (6). The dPAPST2 and SLL proteins have similar functional properties, i.e., PAPS-specific transport activity with comparable K_m values (2.3 and 1.2 μM, respectively) and involvement in the sulfation of HS. It has been presumed that Drosophila has a relatively lesser number of nucleotide sugar transporter genes than mammals. For example, the Drosophila UDP-Gal/UDP-GalNAc transporter gene dmUGT is considered to be a single ortholog of three distinct human nucleotide sugar transporter genes, namely the UGT, UDP-GlcNAc transporter, and CMP-sialic acid transporter genes (Fig. 1A). Furthermore, Drosophila...
 fringe connection is the only gene that corresponds to the two human nucleotide sugar transporter genes hfre1 and UGTrel7 (21, 25). On the other hand, Drosophila sll and dPAPST2 correspond to each of the human orthologous genes PAPST1 and PAPST2, respectively (Fig. 1A); this suggests their early divergence in evolution. It is known that Drosophila possesses sulfated HS similar to vertebrates (26), and the sulfation of HS plays an important role in the development of Drosophila. There is a possibility that the existence of plural PAPS transporter genes in Drosophila may be reflected in the significance of diversity in sulfated HS.

The PAPS transport activity of the dPAPST2 protein was measured using a yeast expression system. The yeast S. cerevisiae has been widely used to examine the function of nucleotide sugar transporters because the Golgi-enriched fraction of this strain has limited endogenous nucleotide sugar transport activity, with the exception of GDP-Man transport activity. However, the yeast Golgi-enriched fraction had relatively high endogenous PAPS transport activity; therefore, the true activity and kinetics of the dPAPST2 protein, excluding other factors, remain uncertain. Alternatively, it may be suitable to use an artificial vesicle with zero background for the measurement of PAPS transport activity; however, such systems have certain disadvantages such as altered $K_m$ values (27). On the other hand, the silencing of the dPAPST2 gene in the S2 cells reduced the PAPS transport activity in the Golgi apparatus (Fig. 4B) and the total sulfate incorporation into cellular proteins (Fig. 4C).

Furthermore, the lethality of the dPAPST2-knockdown fly lines Act5C-GAL4/+;UAS-dPAPST2-IR[1]/+ and Act5C-GAL4/UAS-dPAPST2-IR[2] also indicates that dPAPST2 is an essential gene for the viability of the fly and is not a modulator of sll function. The details of the sulfation state of HS in dPAPST2-knockdown flies needs to be evaluated in future investigations.

Mutations in the genes involved in the synthesis of proteoglycans, including those encoding core proteins (4, 28, 29), nucleotide sugar transporter (30, 31), glycosyltransferases (32–34), and sulfotransferases (4, 5, 15), are responsible for the defects in the growth factor signaling function of the fly. Lüders et al. (7) reported that sll is involved in Wingless and Hedgehog signaling during development. On the other hand, we were unable to observe any specific phenotype of the growth factor signaling in the dPAPST2-knockdown flies. Because the expression of dPAPST2 is less than half that of sll in Drosophila tissues (Fig. 3), the reduction in the dPAPST2 level might be complemented by the function of SLL. On the other hand, dPAPST2 interacted genetically with db4GalT7, indicating the involvement of dPAPST2 in the synthesis of GAGs. In Drosophila, the db4GalT7 protein plays a role in normal Hedgehog signaling by
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FIGURE 8. Schematic representation of the functions of genes involved in HS sulfation. Developmental signaling functions of cell surface HS depends on its sulfation state. Sulfate is transferred from PAPS to defined positions on the sugar residues of the GAG of HS by sulfotransferases. PAPS is synthesized by PAPS synthase in the cytosol or nucleus in the presence of sulfate ions and ATP. Subsequently, PAPS is translocated from the cytosol into the Golgi lumen by PAPS transporters to supply the donor substrate for sulfotransferases. dOXT and dβ4GalT7 contribute to the synthesis of the linkage region of GAGs. Each Drosophila gene is identified based on its Flybase symbol name.

contributing to the synthesis of the linkage region of GAGs (17). A number of reports have mentioned that HS is required in Hedgehog and Decapentaplegic signaling (33–36). The small-wing phenotype of dβ4GalT7 RNAi flies is considered to occur following the impairment of Decapentaplegic signaling (17), which primarily regulates the growth of the wing along the anterior-posterior axis (37). In addition, dPAPST2 also interacted genetically with the dHS6ST, dHS3st-B, and dOXT genes in the developing eye. These genes have been characterized and are involved in the synthesis of sulfated GAGs in Drosophila (15, 16, 19). During photoreceptor differentiation, the Hedgehog protein controls the progression of the morphogenetic furrow, and Decapentaplegic signaling follows the Hedgehog signaling within and ahead of the morphogenetic furrow (38, 39). dPAPST2 is expressed in the wing and eye discs in the third instar larvae of Drosophila (Fig. 3B). Because the sulfation of HS is crucial for the signaling, dPAPST2 may be involved in Hedgehog and Decapentaplegic signaling by controlling HS sulfation (Fig. 8).

Drosophila has many sulfated molecules, including proteoglycans (26), glycoproteins, and glycolipids (40). The lethality of the fly lines Act5C-GAL4/++;UAS-dPAPST2-IR[1]/+ and Act5C-GAL4/++;UAS-dPAPST2-IR[2] also suggests the presence of dPAPST2-preferential tissue(s) or the distinct role of dPAPST2 from that of sll, although they exhibit similar ubiquitous distribution in the adult fly. The SLL, PAPSS, and PIPE proteins are involved in the production of an Alcian blue-positive sulfated macromolecule, which is not HS, in the embryonic salivary glands (12). The expression of dPAPST2 in the central nervous system may suggest the involvement of this gene in the synthesis of sulfated glycolipids or the HKN-1 epitope. Identifying the glycoconjugates that are influenced by dPAPST2 will provide clues to elucidate the role of the PAPS transporter gene in the fly.

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