Sulfation of biomolecules, which is widely observed from bacteria to humans, plays critical roles in many biological processes. All sulfation reactions in all organisms require activated sulfate, 3’-phosphoadenosine 5’-phosphosulfate (PAPS), as a universal donor. In animals, PAPS is synthesized from ATP and inorganic sulfate by the bifunctional enzyme, PAPS synthase. In mammals, genetic defects in PAPS synthase 2, one of two PAPS synthase isozymes, cause dwarfism disorder, but little is known about the consequences of the complete loss of PAPS synthase. To define the developmental role of sulfation, we cloned a Caenorhabditis elegans PAPS synthase homologous gene, pps-1, and depleted expression of its product by isolating the deletion mutant and by RNA-mediated interference. PPS-1 protein exhibits specific activity to form PAPS in vitro, and disruption of the pps-1 gene by RNAi causes pleiotropic developmental defects in muscle patterning and epithelial cell shape changes with a decrease in glycosaminoglycan sulfation. Additionally, the pps-1 null mutant exhibits larval lethality. These data suggest that sulfation is essential for normal growth and integrity of epidermis in C. elegans. Furthermore, reporter analysis showed that pps-1 is expressed in the epidermis and several gland cells but not in neurons and muscles, indicating that PAPS in the neurons and muscles is provided by other cells.

Body and tissue morphologies are generated by orchestrated events of cell movements and cell shape changes at an appropriate time and place. In the nematode Caenorhabditis elegans, which is an ideal model organism for the study of morphogenesis, embryos undergo a 4-fold increase in length and a 3-fold decrease in circumference without cell division after the cell proliferation phase (1). The elongation process requires proper patterning and shape change of the epidermis (hypoderms) and body wall muscle cells adjacent to the hypoderms in C. elegans (2, 3). In the patterning and shape change of the hypoderms and the body wall muscle, both the basement membrane, which is positioned between them, and dynamic cytoskeletal changes play an important role (2, 3). On the other hand, the extracellular cuticle that surrounds the hypoderms contributes to the maintenance of the final shape of the worms after the elongation (1). Sulfation, a common chemical modification of biomolecules, is critical for many biological processes. For example, sulfation of lipopolysaccharide signals determines the symbiosis between the bacteria Rhizobium meliloti and alfalfa (4). In vertebrates, tyrosine sulfation of chemokine receptor CCR5 facilitates HIV entry (5), and terminal SO_4^-4GalNAc of the pituitary hormone lutropin, which plays a critical role in the expression of hormone activity, modulates the circulatory half-life of the hormone (6). Proper sulfation of glycosaminoglycans (GAGs) is required for interactions with several extracellular signaling molecules; therefore, disrupting the genes that encode enzymes mediating sulfation reactions causes dorsal/ventral or segment polarity defects in Drosophila (7, 8) and induces abnormal mast cells (9, 10) or renal agenesis in mice (11). Furthermore, sulfation of several carbohydrates also plays important roles in nervous system development, particularly in axon guidance (12).

All sulfation reactions in all organisms require 3’-phosphoadenosine 5’-phosphosulfate (PAPS) as a donor. The formation of PAPS involves two catalytic reactions (13). The first reaction is carried out by ATP-sulfurylase (EC 2.7.7.4), resulting in the formation of adenosine 5’-phosphosulfate (APS). The second reaction is carried out by APS kinase (EC 2.7.1.25), resulting in the formation of PAPS. Synthesized PAPS is translocated into the Golgi apparatus by the PAPS transporter (14, 15); then several sulfotransferases transfer sulfate onto proteins and carbohydrates from PAPS.

In animals, ATP-sulfurylase and APS kinase activity are performed by the bifunctional enzyme, PAPS synthase (16, 17), although in plants and in simple organisms, including bacteria, fungi, and yeast, the two enzymes are present on separate peptides. In higher organisms, including humans, mice, guinea pigs, and chickens, PAPS synthase exists as two isozymes, PAPSS1 and PAPSS2. However, there is only a single PAPS synthase in lower animals. Mutations in the PAPSS2 gene cause developmental dwarfism disorders: autosomal recessive spondyloepiphyseal dysplasia in humans, brachymorphism in mice (18, 19). The disorder in brachymorphic (bm) mice is due to a glycine 79 to arginine (G79R) mutation in the APS kinase domain of PAPSS2, which fails to synthesize PAPS (19, 20). A decrease in the formation of PAPS in cartilage in bm mice induces undersulfation of chondroitin sulfate, a
major component of extracellular matrix in cartilage; chondroitin sulfate interacts with many extracellular matrix molecules, such as type II collagen. Subsequently, bm mice exhibit abnormalities in limb and axial skeletal length (21, 22) as well as in blood clotting and bleeding time (23, 24). Mutation in PAPSS2 does not cause lethality in mice, although PAPSS2 is inactive for PAPS synthesis in bm mice; in addition, expression of PAPSS1 is more ubiquitous than that of PAPSS2 (25–27). These facts indicate that PAPSS1 is the major PAPS synthase in mice; however, to date there have been no reports of genetic deficiencies in the PAPSS1 gene or in PAPSS1 and PAPSS2 knock-out mice. Also unknown are the exact cell types and molecular mechanisms of deficiencies resulted from PAPS synthesis disruption.

Gene knock-out studies of PAPS synthases will provide valuable information about the physiological roles of sulfation. However, as observed in bm and spondyloepimetaphyseal dysplasia, inactivation of PAPS synthesis would mainly cause defects in cartilage-containing chondroitin sulfate, one of the most abundant sulfated molecules, making it difficult to elucidate the physiological roles of other sulfated molecules in vertebrates (18, 19). Importantly, although chondroitin proteoglycans are the primary extracellular matrix components in C. elegans, as in higher organisms (28), sulfated forms of chondroitin GAGs have not been detected by biochemical analysis (29, 30). On the other hand, as in vertebrates, heparan sulfate (HS) proteoglycans are present in C. elegans, although they are not the major proteoglycans in the nematode (29, 30). The C. elegans genome contains a set of sulfotransferases (lst-1, lst-2, lst-3, and lst-6) involved in sulfation of HS in contrast to that of chondroitin sulfate. Although several reports have suggested that sulfation of HS plays a role in cell migration and axon guidance in C. elegans (31, 32), little is known about how PAPS synthesis affects developmental processes.

In this study, we show that a single C. elegans ortholog of the PAPS synthase gene pps-1 is present in the nematode genome and is expressed in the epidermis and several gland cells but not in neurons and muscles. Disruption of the pps-1 gene caused pleiotropic developmental defects and abnormality in patterning of muscles and shape change of epidermis. Our results showed for the first time that PAPS synthase is indispensable for normal growth and development and is involved in pattern formation and cell shape change of epidermis in C. elegans.

C. elegans PAPS Synthase

C. elegans PAPS Synthase

EXPERIMENTAL PROCEDURES

Strains—Most of the nematode and Escherichia coli strains used were from the Caenorhabditis Genetic Center. The deletion mutant strain tm1109 was isolated from pools of worms mutagenized by the UV/TMP method (33). Primers used for PCR screening and genotyping of the deletion allele were as follows: T14G10#F1 (5’-CCGCTACCTACCTCTTGTAGTA-3’), T14G10#R1 (5’-CCGTGCCAATCCTCGCATCT-3’), T14G10#F2 (5’-TGCCACGATCATATGCTG-3’), and T14G10#R2 (5’-AGACGGATCAGTCTCAGCCTA-3’).

RNAi by Feeding—The protocol for RNAi by feeding was based on described methods (34, 35). The CDNA clone for T14G10#F1 (5’-CCGCTACCTACCTCTTGTAGTA-3’) and T14G10#R1 were amplified by PCR from total cDNA of N2 worms. Primers for the T14G10#R1 cDNA were 5’-TGCCTACCTCCAGGGATGAA-3’ and 5’-AGGTTTGAGGTTTGAGTTG-3’. A fragment of cDNA was cloned into the L4440 (pPD129.36) vector, and the cloned plasmids were transformed into E. coli HT115 (DE3). A single colony of HT115 (DE3) containing the plasmid was grown in LB culture medium for 8 h and seeded onto NGM agar plates (80 ml/plate), which were incubated at 37 °C overnight. Following the addition of 2 mM isopropyl-β-D-thiogalactoside (100 µl/plate), the cells were cultivated for 4 h to induce the expression of double-stranded RNA (dsRNA). HT115 harboring the plasmid pPD129.36 without any insert was used as control. For the assay of PAPS synthase activity, mixed stage hermaphrodites were used, and for phenotypic characterization and the assay of lethality, L4 or L3 hermaphrodites were transferred on the plates, and dsRNA was introduced into the nematode by feeding.

cDNA Cloning of C. elegans PAPS Synthase—The cDNA encoding the full open reading frame of C. elegans PAPS synthase (accession number NM_069456) was amplified by PCR from C. elegans cDNAs, which were prepared from large scale mixed stage culture of N2 worms (36). The oligonucleotide primers used were 5’-ttttgctgaATGCTACCTCAGG-3’ (forward primer) and 5’-ttaactctgATGTTGTTTGGTAG-3’ (reverse primer). The sequences shown by lowercase letters indicate appropriate restriction sites. The amplified cDNAs were digested with Sall and HindIII and cloned into pBluescript II SK(+) (Stratagene). The plasmids were sequenced using an Applied Biosystems Prism 310 Genetic Analyzer (PE Biosystems). The cDNA with the precise sequence was recloned into pQE-9 as a His Tag fusion and into pET28a (+) as an enterokinase cleavage site (DDDDK) using the QuikChange site-directed mutagenesis kit (Stratagene) and two oligonucleotides, 5’-CATCACCATGCAGTGACAGGATCA-3’ and 5’-GTTTTGTAGTGATTTGTAG-3’. The resulting pQE-9-EK-PAPS was transformed using the E. coli M15 strain (Qiagen). LB medium (80 ml) with 100 µg/ml ampicillin and 25 µg/ml kanamycin was inoculated with 0.8 ml of overnight culture of the transformants and then shaken at 30 °C until the absorbance at 600 nm reached 1.0. After the addition of isopropyl-β-D-thiogalactoside to a final concentration of 0.1 mM, the bacteria were cultured at 20 °C for 16 h and harvested by centrifugation. The pellet was suspended in 10 ml of 20 mM sodium phosphate, 0.3 M NaCl, and suspended in 10 mM imidazole (pH 8.0) (buffer A) and sonicated. A milliliter of 5% (v/v) Triton X-100 was added, and the mixture was kept on ice for 30 min. After centrifugation, the supernatant was applied to an Ni2+–imipol–tri-nitro-methyl acetic acid-agarose (Qiagen) (0.2 ml of 0.7 cm; equilibrated with buffer A) containing 0.5% (v/v) Triton X-100. After washing with buffer A, the recombinant protein was eluted with 0.25 M imidazole, 20 mM sodium phosphate, and 0.3 M NaCl (pH 8.0). Finally, 1.7 mg of the protein was obtained. Protein concentration was determined by a Bio-Rad protein assay dye reagent using bovine serum albumin as a standard.

Assay of PAPS Synthase Activity—Free [35S]sulfate was prepared from 2.5 MBq of adenosine 3’-phosphate 5’-phospho[35S]sulfate (PAPS, 71.2 GBq/mmol; PerkinElmer Life Sciences) by mild acid hydrolysis (0.5 ml of 0.02 N HCl, 100 °C, 15 min), followed by paper electrophoresis (pyridine/acetate acid/water = 3:1:387, pH 5.4). Adenosine 5’-phosphate 5’-phospho[35S]sulfate (APS) was prepared from 2.5 MBq of [35S]PAPS using 50 µl of alkaline phosphatase beads (derived from bovine intestinal mucosa; 1280 units/g acrylic beads; Sigma) in 200 µl of 50 mM Tris–HCl (pH 8.0) at 37 °C for 15 min, followed by paper electrophoresis.

APS kinase activity was assayed according to the methods of Keller et al. (37). Briefly, 10 µl of the solution containing 16 KBq of [35S]APS, 0.1 M HEPES–NaOH (pH 7.2), 40 mM MgCl2, and 20 mM disodium ATP (Grade II; Sigma) was mixed with 10 µl of enzyme suspension (lyophilized powders were suspended in 20 mM HEPES–NaOH (pH 7.2), 0.15 M NaCl) and incubated at 37 °C for 10 min. The reaction was stopped by the addition of 20 µl of ice-cold ethanol, and the mixture was immediately applied to paper electrophoresis with the same conditions as described above. The paper was scanned using a radiochromatogram scanner RITA Star (Straubenhardt, Germany). [35S]APS fractions were cut off and counted for the radioactivity. To determine APS kinase activities, mix staged pps-1 RNAi-untreated and -treated nematodes were harvested by washing and sucrose flotation as described (38). Three hundred L4-staged tm1109 heterozygous and wild-type nematodes were harvested by washing and sucrose flotation as described (38). Three hundred L4-staged tm1109 heterozygous and wild-type nematodes were harvested by washing and sucrose flotation as described (38).
nematodes were placed on NGM agar plates with a lawn of OP50 E. coli cells by picking. They were cultured at 20 °C for 24 h and then harvested by washes. Because of the small amount, we omitted the sucrose flotation step that could cause loss of collected nematodes. Total PAPS synthase activity was assayed with the same conditions for APS kinase described above, except that 8.0 kBq of [35S]sulfate was used as a substrate instead of [35S]APS.

Analysis of GAGs—GAGs were prepared from dried homogenates of wild-type or RNAi-treated nematode (dry weight 55 or 119 mg, respectively). GAG chains were released from the proteoglycan core proteins by the sodium borohydride treatment. It should be noted that the amount of HS in C. elegans was so small (28) that 100 µg of shark cartilage chondroitin 6-O-sulfate (Seikagaku Corp.), which contained a negligible proportion of nonsulfated disaccharides, was added as a carrier after the borohydride treatment but before the purification steps. The unsaturated disaccharides were produced by digestion with chondroitinase ABC or a mixture of heparitinases I and II, and then the digests were derivatized with 2-aminobenzamide and analyzed by high performance liquid chromatography as described previously (28, 29).

Phenotypic Analysis—We determined the extent of lethality of the pps-1 mutant by picking late L4 heterozygous strains to separate plates, allowing them to lay eggs for 24 h, and transferring them to a new plate daily for the next 3 days. Eggs unhatched after 20, 24, and 40 h were scored as embryonic lethal at 25, 20, and 15 °C, respectively. Larvae that failed to develop into L4 or adult stage after 40, 46,
and 96 h were scored as larval arrest/lethal at 25, 20, and 15 °C, respectively. To determine the embryonic phenotype of *pps-1* RNAi, embryos were dissected from the adult animals that had had dsRNA introduced for the indicated time. The embryos were cultured for an additional 18 h in M9, and various phenotypes were scored. To determine the larval phenotype of *pps-1* RNAi-treated animals, embryos were dissected from the adult animals that were fed with *pps-1* dsRNA for 40 h. The dissected embryos were cultured for an additional 18 h in M9 and placed onto the dsRNA-expressing bacteria, and developmental stages were scored after 40 h. Phalloidin staining was performed as described previously (39).

**RESULTS**

**C. elegans PAPS Synthase**

**TABLE 1**

| Parental genotype, temperature (°C) | Percentage of larval lethality | Percentage of embryonic lethality | Percentage of deformed larva | Percentage of normal adults |
|-------------------------------------|-------------------------------|---------------------------------|-----------------------------|-----------------------------|
| pps-1(1tm1109+/+) 15 °C (P0, 5; F1, 1114) | 18.3 ± 2.6 | 0.7 | 0.6 | 81 |
| pps-1(1tm1109+/+) 20 °C (P0, 8; F1, 2204) | 17.7 ± 3.0 | 0.5 | 0.1 | 81.8 |
| pps-1(1tm1109+/+) 25 °C (P0, 9; F1, 1209) | 16.9 ± 3.8 | 1.2 | 0.6 | 81.9 |
| pps-1(1tm1109+/+) 20 °C + Ex pps-1 | 8.9 ± 2.9 | 0.8 | 0.1 | 90.3 |
| pps-1(1tm1109+/+) 20 °C + Ex pps-1 | 5.9 ± 1.1 | 0.2 | < 0.1 | 93.9 |

* Data were obtained from extrachromosomal (Ex) lines. All data of larval lethality shown are averages ± S.D.

The pps-1 null phenotype is a larval lethality on chromosome IV. The predicted *pps-1* gene product consists of 652 amino acid residues and shows 57% identity both with the human PAPSS1 and PAPSS2a at the amino acid level. *Pps-1* has a putative nuclear localization signal sequence at the N terminus. The APS kinase domain (Pfam: PF01583) and ATP sulfurylase domain (Pfam: PF01747) occupy one-third of the N terminus and two-thirds of the C terminus of the sequence, respectively. The APS kinase domain contains a nucleotide-binding P-loop motif (GAXGXXGK(S/T)) that is a critical site in APS kinase activity of mammalian PAPS synthases. On the other hand, the ATP sulfurylase domain has another type of nucleotide binding motif, HIVG, required for ATP sulfurylase activity of mammalian PAPS synthases (Fig. 1A).

To assess biochemically whether the protein encoded in the PAPS synthase gene could synthesize PAPS from free sulfate and ATP, we isolated the cDNA and expressed the protein in *E. coli* M15 cells. SDS-PAGE analysis of the purified recombinant protein showed one band with a molecular mass of 73 kDa (Fig. 1B). Using [35S]sulfate and [35S]APS, we assayed total PAPS synthase and APS kinase activities, respectively. [35S]PAPS was produced in both assays (Fig. 1, C and D). Specific activities of the protein for total PAPS synthase and APS kinase were 0.80 nmol/min/mg protein and 63 nmol/min/mg protein, respectively. It should be noted that in the total PAPS synthase assay, an intermediate product, [35S]APS, was scarcely detected, suggesting that APS produced from sulfate and ATP was quickly converted to PAPS.

**pps-1 Reporter Constructs Are Widely Expressed in Epidermal Cells and Gland Cells, but Not in Muscle Cells and Neurons, and Are Predominantly Localized to the Nucleus**—To determine the expression pattern of *pps-1*, two different types of *pps-1* genomic fragments were cloned into a reporter gene vector. Because the PCR product that contained the 3-kb sequence from the predicted ATG was sufficient to rescue the lethality of the *pps-1* mutant (see below; Table 1), the same 3-kb region was cloned into the reporter vector as the promoter-reporter construct *pps-1p::EGFP*. On the other hand, the 5-kb sequence upstream from the predicted stop codon that contains both the promoter and open reading frame sequence was cloned into the reporter vector as a translational fusion construct, *pps-1p(FL)::EGFP* (Fig. 1E). The *pps-1p::EGFP* reporter is widely but tissue-specifically expressed in somatic cells, *pps-1p::EGFP* is strongly expressed in seam cells (Figs. 2, A, H, and H'), gland cells (Fig. 2B), and neuronal support cells (amphid sheath cells; Fig. 2, C–C') throughout development. Relatively weak expression was also detected in the hypodermis (Fig. 2D) and the phasmid support cells during larval development. Additionally, weak expression was observed in the intestine at the adult stage (Fig. 2, E and E'). Unexpectedly, no signal was found in the neurons and muscles.

Next, in order to investigate the subcellular distribution of the PPS-1 protein, the expression pattern of *pps-1p(FL)::EGFP* was examined. This fusion construct rescued the lethality of the *pps-1* mutant (see below;
Table 1), suggesting that its expression reflects endogenous localization of pps-1. Neither enhancer nor silencer seems to be present in the pps-1 introns; the tissues expressing pps-1(FL)::EGFP and the timing of its expression were almost identical to those expressing pps-1p::EGFP. pps-1(FL)::EGFP is dominantly localized in nuclei of all expressing cells (Fig. 2, E–I).

Because some anterior daughters of seam cells fuse to hypodermal syncytium hyp? during larval development, we could not determine whether the pps-1p::EGFP expression in the hypodermis was derived from seam cells or hyp? cells. The signal of pps-1(FL)::EGFP was also detected both in nuclei of hypodermis that are derived from seam cells and in original hyp? cells (Fig. 2, F and G). Again, no signal was observed in the neurons and muscles, suggesting that PAPS is not synthesized in these cells.

Isolation of a pps-1 Deletion Mutant Exhibiting Larval Lethality and Molting Defects—Because only one orthologous gene of human PAPS synthase is present in the C. elegans genome, we expected that its function would be easily disrupted by using deletion mutagenesis and RNAi.

To address the function of C. elegans PAPSS, by using the null mutant allele, trimethylsoralen- and ultraviolet-treated deletion libraries (33) of the nematode were screened, and one recessive pps-1 deletion mutant allele, tm1109, was isolated from pools of mutagenized worms (Fig. 1E). Because the isolated mutant had a lethal phenotype (see below), it was balanced with mec-3 (u297). Since tm1109 lacked the exons containing the ATG translational initiation codon, tm1109 is predicted to be a molecular null allele of pps-1. Homozygous null mutant worms of pps-1(tm1109) segregating from heterozygous mothers hatched and initiated growth and development. However, they were arrested at the second (L2) or third (L3) larval stage and unhealthily survived for 1–2 days at 20 °C (Fig. 3, C and D). To estimate the penetrance of the lethal phenotype in pps-1(tm1109) homozygotes, visible phenotypes of 138 F1 animals segregating from one pps-1 heterozygous mother were scored, and single-worm PCR analysis of each DNA isolated from 113 animals was performed with the primers to identify the genotypes of the pps-1 locus. After 40 h, 114 of 138 embryos were grown normally, 23 were arrested at the L2 or L3 stage, and one was dead at the L2 stage. PCR analysis showed that all 23 of the arrested larvae showed only a –/– genotype, and all of the tested 90 normal larvae showed a +/+ or +/+ genotype but not –/– (Fig. 3A), suggesting that pps-1 homozygosity resulted in lethality with 100% penetrance. Table 1 shows the lethality of F1 progenies derived from heterozygous mothers at 15, 20, and 25 °C. Only 16–18% of larvae from tm1109 heterozygous hermaphrodites showed an arrested or lethal phenotype at any temperature, whereas no obvious embryonic lethality was observed as in RNAi (see below). These observations also indicate that tm1109 homozygotes from tm1109 heterozygous parents are less than one-fourth of progeny. Thus, inactivation of the pps-1 gene should cause partial sterility.

Occasionally, pps-1(tm1109) mutant animals exhibited the Mlt phenotype (Fig. 3E). Nomarski observations revealed an accumulation of refractile material in the rectum in those animals that had not shed the old cuticle (Fig. 3, F and G). These observations indicate that pps-1(tm1109) mutant animals form abnormal cuticle. The lethal phenotype of pps-1(tm1109) was rescued by the introduction of a genomic DNA fragment containing the wild-type pps-1 gene (Table 1).

RNAi of pps-1 Resulted in Pleiotropic Phenotypes—For pps-1 RNAi experiments, almost full-length pps-1 cDNA was used to synthesize dsRNA. The dsRNA was introduced into worms using the feeding method to create pps-1(RNAi) worms.

To assess the effect of pps-1 gene inactivation, pps-1(FL)::egfp and pps-1p::egfp transgenic worms that express a PPS-1 full-length protein-EGFP fusion or only EGFP under the control of pps-1 promoter, respectively, were fed with the dsRNA. In pps-1(FL)::egfp but not in pps-1p::egfp transgenic worms, substantial reduction of fluorescence in the epidermis of pps-1(RNAi) animals was observed. However, in several gland cells and neuronal support cells, no remarkable changes were detected. Presumably, these cells were resistant to interference under the tested conditions in this study. These observations indicate that PPS-1 synthesis was successfully knocked down in the RNAi-treated animals, at least in epidermis. To ensure the successful silencing of the pps-1 gene by RNAi, we also assayed APS kinase activity in whole extracts of control and pps-1 RNAi-treated worms. RNAi of pps-1 caused a 42% decrease in PAPS synthesis...
activity (Table 2). To avoid contamination of lethal worms in the samples, we harvested pps-1(RNAi) worms before the RNAi effect reached its full potency. Thus, the residual PAPS synthesis activity may be attributed to the insufficient effect of RNAi and/or its incomplete penetrance. To determine whether disruption of pps-1 affects sulfation, we next examined GAG content and disaccharide components of HS of pps-1(RNAi) worms. The disaccharides of the HS from RNAi-treated worms had indeed been less sulfated (a 20% decrease) than those of control animals, although pps-1(RNAi) worms showed a 28% decrease in chondroitin and a 51% decrease in HS (Tables 3 and 4). As compared with the disaccharide components of control animals, substantial decrease in sulfation in pps-1(RNAi) was found for 6-O-sulfation (44%) and N-sulfation (76%) of N-acetylgulcosamine residues of monosulfated disaccharide units. Unexpectedly, the content of di-/trisulfated disaccharide units that are minor components in C. elegans was less affected. There could be carryover of endogenous sulfated components that have been synthesized before dsRNA was induced. These data indicate that synthesis of monosulfated disaccharide units would be more sensitive to deple-
tion of PAPS in vivo than that of di-/trisulfated disaccharide units, although we cannot exclude the possibility that RNAi-ineffective cells, such as amphid sheath cells, may contain di-/trisulfated disaccharide-rich HS.

The temperature-dependent effect of pps-1 RNAi was examined, because pps-1(RNAi) showed no obvious defects at 20 °C (not shown). The pps-1 RNAi worms showed pleiotropic phenotypes at 25 °C. This may be the result of a low efficiency of RNAi, because we observed that the reduction in pps-1(FL);::EGFP was more moderate at 20 °C than at 25 °C (not shown). Similar temperature dependence of RNAi effects is reported for other C. elegans genes (42).

At 25 °C, the following phenotypes were observed: shortened malformed tail, which we call Nob (no back end) because it was superficially similar to that of nob-1/phi3-3 and pal-1 mutants (43, 44) (Fig. 4B); abnormal rounded protuberance, which we call Vab (variable morphogenetic defects) (Fig. 4C); Mlt (molting defects); Bli (blister phenotype) (Fig. 4D); and sterility (significantly reduced brood size; pps-1 RNAi, 121 ± 15, n = 10; vector RNAi, 184 ± 21, n = 10, p < 0.001). In the F1 generations of pps-1(RNAi) worms, the most severe phenotype was the embryonic lethality (Emb) following 2-fold stage arrest (Fig. 4F). Percentages of Emb, Nob, and Vab phenotypes of F1 animals from N2 hermaphrodites fed with pps-1 dsRNA for 24 and 40 h are summarized in Table 5. About half of the F1 progeny collected from P0 animals treated with pps-1 RNAi for 40 h hatched normally. Nevertheless, only 8% of normally hatched animals (n = 62) survived to adulthood; 92% of hatched animals with Mlt and/or Bli phenotype were unhealthy and died but not those with Nob and Vab phenotypes. In addition, these RNAi-treated adult worms had a few eggs in their uteri. These observations suggest that Nob and Vab arise from an abnormality before hatching and that the pps-1 gene is essential for normal growth and reproductive system. Furthermore, pleiotropic defects indicate that pps-1 functions are required for several aspects of development.

![C. elegans PAPS Synthase](image_url)

**TABLE 5 Phenotypes of pps-1 RNAi animals**

| Phenotypes of pps-1 RNAi animals | Emb, embryonic lethality; Vab, variable abnormal; Nob, no back end. |
|----------------------------------|---------------------------------------------------------------|
|                                 | pps-1 RNAi 24 h (n = 198) | pps-1 RNAi 40 h (n = 176) | Vector RNAi 40 h (n = 325) |
| Emb (%)                         | 3.9                        | 9.7                        | 2.2                      |
| Vab in anterior (%)             | 2.6                        | 17.6                       | 0                        |
| Vab in posterior (%)            | 7.8                        | 15.9                       | 0                        |
| Nob (%)                        | 4                          | 9.9                        | 0                        |
| Normal (%)                     | 85.7                       | 56.8                       | 97.8                     |

*Animals with Nob and Vab in posterior were scored double.

Silencing of pps-1 Causes Defects in Cell Shape of Epidermis and Patterning of Body Wall Muscle Cells—To visualize internal actin-containing structures, we carried out phalloidin staining. In pps-1 RNAi-treated animals showing the Nob phenotype, a malformed and tortuous intestine seemed to push away body wall muscles and hypodermis (Fig. 5, A and B). This observation demonstrates that axial elongation of the intestine was normal, but elongation of the hypodermis and body wall muscles failed. Consistent with this notion, Nob animals derived from pps-1(RNAi) in jclsl1 (ajm-1::GFP) worms (45), which express GFP marker at cell junctions between the epidermal, pharyngeal, and intestinal cells, exhibited an abnormal shape of the lateral epidermis seam cells at the posterior region of the pharynx bulb (Fig. 5, C and D); however, there was no significant difference in pharynx length and seam cell shape at the anterior region of the pharynx bulb between RNAi and control animals (not shown).

To find out why impairment of pps-1 function led to Nob and Emb phenotypes, we next examined pps-1(RNAi) F1 embryos derived from wild-type N2 or from jclsl1 worms by using four-dimensional time lapse microscopy. In wild-type embryogenesis, whereas the two rows of dorsal hypodermal cells intercalate into a single row (dorsal intercalation) at the point of the proliferation phase, ventral hypodermal cells begin to enclose the embryo (ventral enclosure) and continuous seam cells elon-
C. elegans PAPS Synthase

FIGURE 5. pps-1(RNAi) F1 larvae display defects in hypodermal cell shapes and body wall muscle cell positions. A, fluorescence micrograph of actin-phalloidin staining of actin in vector (RNAi) control larva shortly after hatching. The arrows and arrowheads in A and B indicate the posterior bulb of pharynx and intestine, respectively. B, actin-phalloidin staining of pps-1(RNAi) larva that displays the Nob phenotype. Note that the intestine is malformed and tortuous. C, GFP fluorescence of the ajm-1::gfp transgene in a vector (RNAi) larva. The asterisks and arrowheads in C and D indicate the posterior bulb of pharynx and the boundaries between seam cells, respectively. D, GFP fluorescence of ajm-1::gfp transgene in a pps-1(RNAi) larva showing the Nob phenotype. Note that elongation of seam cells is arrested. E, GFP fluorescence of myo-3::egfp transgene in a vector (RNAi) L3 stage larva. F, DIC micrograph of ccls4251 in a pps-1(RNAi) L3 stage larva showing the Nob phenotype. G, the same animal as in F under GFP fluorescence. The arrowhead indicates a laterally branched muscle cell. H, Nomarski DIC micrograph of ccls4251 in a pps-1(RNAi) L3 stage larva showing the Nob phenotype. I, the same animal as in H under GFP fluorescence. Note that muscle cells are missing (arrow) and abnormally clustered (arrowhead). J, tail half of a vector (RNAi) L3 larva that carries the extrachromosomal dpy-7p::dsred transgene under a DIC micrograph. K, the same animal as in J under GFP fluorescence. L, DIC micrograph of pps-1(RNAi) L3 larva carrying the extrachromosomal dpy-7p::dsred transgene displaying the Nob phenotype. M, the same animal as in L under DsRed fluorescence. The arrowhead indicates bulged hypodermis at the lateral protuberance position. Scale bars, 10 μm (A–D) and 50 μm (E, F, H, K, and M). WT, wild type.

gate along the anterior/posterior axis, resulting in circumferential force (elongation). No significant alternations during the cell proliferation phase, in the ventral enclosure process, or in the dorsal intercalation step were observed in pps-1(RNAi) F1 embryos (n = 25 for the observation during cell proliferation phase; n > 50 for the observation in ventral enclosure or dorsal intercalation processes), although the generation of 9.9% Nob and 9.7% Emb animals was observed under the same experimental conditions (Table 5). These observations indicate that pps-1 is involved in the elongation process per se rather than in ventral enclosure and dorsal intercalation. Thus, Nob and Emb phenotypes seem to be result from the abnormality in cell shape changes of seam cells in the elongation phase.

Next, we examined overall muscle organization by using the ccls4251 strain (46), which expresses GFP in all body wall muscle cell nuclei and mitochondria. In C. elegans, the monounucleated body wall muscle cells form four parallel rows along the longitudinal axis. In wild-type animals, one row is positioned ventrally, and one is dorsal on each side (Fig. 5E). In Nob animals of pps-1(RNAi), muscles were often branching laterally at the malformed region (Fig. 5, F and G), suggesting that the pps-1 gene is involved in dorso-ventral muscle patterning. Furthermore, in some cases of Vab phenotype caused by pps-1 RNAi, muscle cells were clustered abnormally at the bulged region and missing at posterior to the bulged region (Fig. 5, H and J). This result suggests that the pps-1 gene is also involved in anterior-posterior muscle patterning. However, the protuberance seemed to be mainly caused by the unusual cell shape of hypodermis, because it was often the case that muscle rows were properly positioned. To examine hypodermal cell shape, pps-1 dsRNA was introduced by feeding into transgenic worms co-injected with myo-3p::EGFP and dpy-7p::DsRed to visualize muscles and hypodermis. In Vab animals, in which muscle cells were correctly arranged, the hypodermis bulged at the protuberance position (Fig. 5, L and M). Thus, protuberance in pps-1 RNAi worms seems to be the result of defects in proper hypodermal cell shape formation and in patterning of body wall muscle cells. In contrast to RNAi experiments, none of the morphogenetic defects in embryos, such as the Emb, Nob, and Vab phenotypes, were found in pps-1(tm1109) mutants. Early embryonic transcriptome and in situ hybridization experiments in C. elegans have indicated that pps-1 mRNAs are expressed maternally (47) (see, on the World Wide Web, NEXTDB, at nematode.lab.nig.ac.jp/). Therefore, in pps-1 homozygous embryos produced from pps-1 heterozygous hermaphrodites, maternally supplied pps-1 mRNA would make them develop normally. Alternatively, maternally produced PAPS and/or maternally derived sulfated biomolecules may have allowed embryos to develop normally.

Supporting this idea, rescued tm1109 homozygous lines containing multicopy arrays that would be silenced in germ cells (49) exhibit Emb, Nob, and Vab phenotypes, suggesting that these phenotypes are attributed by depletion of pps-1 products and/or its related molecules in maternal germ cells. Interestingly, the Emb phenotype of the rescued tm1109 homozygous line was often more severe than that of RNAi F1 embryos. About 60% of embryos could not develop into the comma stage (Fig. 6B), although about 20% exhibited the elongation defect that is reminiscent of RNAi F1 embryos (Fig. 6A), whose PAPS synthase activity would be partially knocked out as shown in Table 2. To further
investigate the embryonic phenotypes caused by complete loss of *pps-1* gene. *pps-1*(RNAi) F2 embryos in a jcls1 background were examined, although we could not determine their PAPS synthesis activity because of the lethality. In addition, we could not determine when the defects appear, because variable conditional *pps-1*(RNAi) F1 adults produced progenies that were too small. In these embryos, a row of seam cells was disturbed, and occasionally several seam cells lacked *ajm-1::gfp* as terminal phenotypes (Fig. 6, D and E). These observations suggest that more complete depletion of *pps-1* results in defects earlier than the elongation phase and may include several developmental events, such as gastrulation, differentiation, dorsal intercalation, and ventral enclosure.

**DISCUSSION**

In summary, we identified only one ortholog of human PAPSS1 and PAPSS2 in the nematode *C. elegans* and confirmed PAPS synthase activity in *vivo* and *in vitro*. We also showed that *pps-1* is involved in controlling several aspects of both embryonic and postembryonic development, including molting, changes in cell shape, and patterning of epithelial and muscle cells.

Reporter analysis revealed that PPS-1 is expressed in several specific tissues, such as epidermis, throughout development and is localized to nuclei. In mammalian cells, PAPSS1 is localized in nuclei, whereas PAPSS2 is in cytoplasm (50). In our study, the nematode PPS-1 showed nuclear localization. These data suggest that the physiological properties of PPS-1 are more similar to those of PAPSS1 than PAPSS2 and that the cellular localization of PAPS synthase is conserved throughout the animal kingdom. Inactivation of the *pps-1* gene by RNAi resulted in morphological defects in body wall muscles and epidermis. Expression of *pps-1* in seam cells and hypodermis supports the idea that *pps-1* has critical roles in the development and behavior of epidermis. In *C. elegans*, heparan-6-O-sulfotransferase *hst-6* and heparan-2-O-sulfotransferase *hst-2* are essential for axon guidance and expressed in several neurons and muscles (31, 32). Transgenic rescue experiments using a tissue-specific promoter strongly suggest that neuronal expression of *hst-6* is necessary for axon guidance (31). Therefore, HS sulfation occurs in neurons and perhaps in muscles. However, in this study, we failed to detect neuronal and muscle expression of *pps-1*. PPS-1 appears to synthesize PAPS in a cell-autonomous manner, because we saw no difference between *pps-1*::EGFP and *pps-1(FL)::EGFP expression. These findings indicate that PAPS in the neurons and muscle cells could be provided by other *pps-1*-expressing cells, such as glial amphid sheath cells, gland cells, and hypodermis in *C. elegans*. PAPS may be secreted from cells that are producing it through plasma membrane-associated PAPS transporters or microvesicle-mediated exocytosis. Neuronal and muscular cells may uptake secreted PAPS. Alternatively, PAPS may pass through the gap junctions that permit the exchange of molecules, such as CAMP, between attached cells. Interestingly, a mutant worm of the *inx-3* gene encoding a gap junction protein shows an epidermal elongation defect (51).

Larvae lacking *pps-1* exhibited an inability to shed old cuticle during molts. Consistent with this phenotype, Kim et al. (52) have reported that RNAi of tyrosylprotein sulfotransferase-A results in a molting defect in *C. elegans*. Hence, a possible explanation for the molting defects in *pps-1* knock-down/knock-out mutant lies in the inability of protein sulfation catalyzed by tyrosylprotein sulfotransferase-A, which results from the depletion of PAPS as its donor. Both *pps-1*(RNAi) and *pps-1(tm1109)* homozygosity resulted in larval lethality, suggesting that sulfation is necessary for development and viability in *C. elegans*. It is possible that sulfation is involved in detoxification of xenobiotics, such as bacterial endotoxin, and that sulfation may be involved in a metabolic pathway of hormones that are required for growth and/or molting. Analysis of nuclear hormonal receptors ntr-23 and ntr-25 strongly suggests that a hormonally regulated pathway would control embryogenesis and be essential for viability in *C. elegans* (53–56). Although in *C. elegans* there is no evidence for synthesis of ecdysone, the major sterol-derived hormone in insect molting, it is highly possible that ecdysone-related hormones are essential for development (57–59). *pps-1* RNAi and mutants share several phenotypes with mutants of nuclear hormone receptors and sterol-modifying enzyme genes and cholesterol-depleted worms in embryogenesis and molting. However, sulfated sterol-derived hormones in lower organisms have not been identified yet.

F1 progeny of *pps-1* RNAi worms exhibited defects in the elongation process, although there were no obvious defects in dorsal intercalation and ventral enclosure. In contrast to F1 animals, F2 progeny and the *tm1109* zygotic rescued line exhibited more severe phenotypes. Taken together, almost complete depletion of sulfation activity affects early events of embryogenesis, such as proliferation, gastrulation, and differentiation, whereas moderate inhibition causes a specific epidermal elongation defect. To date, a number of genes involved in the elongation process have been cloned (2, 3), indicating that the process is very complicated at the cellular and molecular levels. Two major factors are required for the process. First is a contraction of circumferential filamentous bundles of actins that are formed in seam cells after ventral enclosure; second is activation of muscle twitching, although the mechanistic relationship between twitching and elongation is unclear. Muscle twitching of 2-fold arrested embryos derived from RNAi and zygotic rescued *pps-1(tm1109)* homozygous adults seems to occur (not shown). Therefore, the elongation defect caused by inactivation of *pps-1* would result from the inability of circumferential filamentous bundles of actin contraction rather than twitching. Mutations in the HS proteoglycan perlecan, *unc-52*, result in a Pat (paralyzed, arrested elongation at two-fold) phenotype (60). HS sulfation of UNC-52 could be involved in the elongation process. In this respect, we confirmed that HS purified from *pps-1*(RNAi) worms showed substantial decrease in 6-O-sulfation and N-sulfation. We also observed in *pps-1* mutant worms phenotypes similar to those observed in HS-related gene deficient worms (not shown). Thus, it is highly probable that several phenotypes observed in this study are due to changes of sulfation patterns of HS. Various other sulfated molecules are supposed to be responsible for the phenotypes, and further mosaic analysis of *pps-1* genes (at a single cellular level) would help in resolving the roles of sulfation and sulfated molecules using this model organism.

During *C. elegans* early embryonic development, chondroitin is indispensable for cytokinesis (28, 40, 48). However, in the present study, the embryos of *pps-1* null mutant and RNAi-treated animals did not exhibit a cytokinesis defect (judged from terminal phenotypes for *pps-1* RNAi F2 embryos, not shown) as observed in Refs. 28 and 40, suggesting that sulfation is dispensable for cytokinesis. These results are confirmed by previous biochemical studies demonstrating that *C. elegans* contains nonsulfated chondroitin (28–30).

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