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Molecules and Cells

Tyrosylprotein Sulfotransferase Regulates Collagen Secretion in *Caenorhabditis elegans*

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The sulfation of tyrosine residues is an important post-translational modification involved in the regulation of protein function. We examined the activity of worm tyrosylprotein sulfotransferase (TPST-1) on a typical cuticle collagen, ROL-6, in *C. elegans*. We verified that TPST-1 sulfates three tyrosine residues of ROL-6 *in vitro*. We found that these tyrosine residues are important for the secretion of ROL-6::GFP. Mutant ROL-6::GFP proteins that contain more than two substitutions of the target tyrosine residues are severely deficient in cuticle localization. Consistently, knock down of *tpst-1* blocked the cuticle localization of ROL-6::GFP. Therefore, the sulfation of ROL-6 by TPST-1 is critical for the proper localization of ROL-6. We also confirmed that worm TPST-1 is localized to the *trans*-Golgi network (TGN). Our results indicate that TPST-1 regulates cuticle organization by promoting the transport of ROL-6 from the TGN to the cuticle.

INTRODUCTION

Post-translational modifications are important mediators of protein function. Recently, protein sulfation has been recognized as a widespread post-translational modification in multicellular organisms. The sulfation of tyrosine residues occurs almost exclusively on secreted and transmembrane spanning proteins (Huttner, 1988). This modification occurs on the luminal side of the *trans*-Golgi network (TGN). Two tyrosylprotein sulfotransferases (TPST1 and TPST2), which catalyze tyrosine sulfation, have been identified in humans. Both human TPST enzymes contain a highly conserved sulfotransferase domain (Marchler-Bauer and Bryant, 2004).

Tyrosine sulfation is a determinant of protein-protein interactions and is involved in leukocyte adhesion, hemostasis, and chemokine signaling (Kehoe and Bertozzi, 2000). Tyrosine sulfation has been studied in secretory proteins, such as gastrin, cholecystokinin (CCK), and neuropeptides, which are regulated by multiple post-translational modifications. Progastrin is partially sulfated at the TGN, and the tyrosine sulfation promotes the proteolytic process (Bundgaard et al., 1995). Pro-CCK also passes through the regulated secretory pathway and undergoes tyrosine sulfation (Beinfeld, 2003). Recently, functional studies of TPST at the organism level have been conducted in mice. TPST1-deficient mice exhibit reduced body weight and increased frequencies of post-implantation fetal death (Ouyang et al., 2002), while TPST2-deficient mice exhibit male infertility (Borghei et al., 2006). A mutation in TPST2 causes the dwarfism associated with hypothyroidism (Sasaki et al., 2007) and a double deficiency of TPST1 and TPST2 results in early postnatal pulmonary failure and primary hypothyroidism in mice (Westmuckett et al., 2008). Thus, tyrosine sulfation facilitates a myriad of diverse biological processes.

To further elucidate the biological roles of TPST and protein sulfation, we employed *Caenorhabditis elegans*. The major phenotype of *tpst* knock down by RNA*i* in *C. elegans* is a molting defect (Kim et al., 2005). Since *C. elegans* cuticle formation involves many post-translational modifications of collagens and *tpst-1* RNA*i* suppressed the Rol phenotype of the *rol-6* mutant, we hypothesized that ROL-6 is a likely target of protein sulfation by TPST-1. In the present study, we demonstrate that ROL-6 sulfation by TPST-1is important for cuticle formation, as it regulates the secretion and function of ROL-6.

MATERIALS AND METHODS

Analysis of protein sulfations by *C. elegans* TPST-1

A TPST-1 cDNA excluding the transmembrane region was cloned into the pcDNA3.1 (+) vector. The following primers were used to amplify *tpst-1* cDNA: 5′-GGAATTCGACGACCCGT ACTACAGTAAA-3′ and 5′-ATCTAGACTATAATTTCGATACTTCATTATC-3′. His-TPST-1 fusion proteins were expressed in human embryonic kidney (HEK) 293 cells and purified on Ni-NTA agarose beads (NEB, USA). The lysate of untransfected HEK 293 cells was used as a control. Western blot analysis using anti-His antibodies produced a single band of approximately 50 kDa. Treating the purified protein with *N*-glycosidase-F resulted in a band of 47 kDa, which indicated

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that ER-Golgi targeting normally occurs. We assumed that the post-translational modification of the purified protein was normal and proceeded with the enzyme assays. Three peptides that contained tyrosine residues in ROL-6 were synthesized from Takara Korea Biomedical Inc. The peptides are named R6-N1 (^{67}NRVRRQQYGGYGATG^{81}), R6-N2 (^{86}APTPNPYGGYGATG^{99}), and R6-C1 (^{321}GKDAEYCmKCmPGREGD^{335}). 'm' indicates the methylated R6-C1 peptide. Two cysteine residues of the R6-C1 peptide were methylated using iodoacetic acid (IAA) to prevent the formation of a disulfide bond (Hammermeister et al., 2000). Synthetic peptide (100 μM) was incubated with 20 μg recombinant TPST-1 in a reaction buffer (50 mM HEPES, pH 7.0; 1 mM 5′-AMP; 5 mM $MnCl_2$; 50 mM NaF; 0.1% Triton X-100; and 2 μM PAPS), and the volume of each reaction was 50 μl, as described by Hanai et al. (2000). After incubation (30 min at 37°C), reaction mixtures were concentrated and desalted using an Oasis® HLB column (Waters, USA). The sulfated peptide mass was determined by TOF mass spectrometry (mass range: 100-2000 m/z), and the sulfated peptide could then be dissociated by tandem collision induced dissociation (CID) analysis. The mass spectrometer was operated with a capillary voltage at 1000 V and the collision energy (CE) varied from 10 to 60 eV. All samples were analyzed using a Q-STAR Pulsar Quadruple TOF mass spectrometer (Applied Biosystems, USA) equipped with nano-electrospray ion sources (Protana, Denmark). The method for opening the Protana emitters has been described by Nemeth et al. (Nemeth et al., 2001).

C. elegans strains, cultures, and RNA interference

Wild-type N2 and mutant strains were cultured as described by Brenner (1974). Feeding RNA*i* was carried out as described by Kamath et al. (2001).

Construction and transformation of ROL-6 reporter gene

The *rol-6::gfp* reporter construct was generated by PCR of genomic DNA, which included the promoter and full-length coding sequence of the *rol-6* gene. The following primers were used to amplify *rol-6* genomic DNA: 5′-ATCTGCAGTTATCATCTTCGGTTTTG-3′ and 5′-TATCTAGACAATTGGAATTTGCGATGACG-3′. The PCR products were ligated into the PstI and BamHI sites of the GFP vector (pPD95.77, Dr. Andrew Fire). To observe the localization of ROL-6::GFP, this reporter construct was injected with *ttx-3*::*gfp* as a transgenic marker, which is expressed exclusively in the AIY interneuron pair (Hobert et al., 1997). The concentration of total injected DNA including 75 μg/ml *ttx-3::gfp* was 150 μg/ml. The integration line, *jgIs5* [ROL-6::GFP;TTX-3::GFP] was made by UV irradiation.

Mutagenesis of *rol-6::gfp*

QuikChange site-directed mutagenesis (Stratagene) of *rol-6* cDNA was performed to introduce a single mutation that changed a tyrosine codon to phenylalanine. The following primers were used for mutagenesis: Y74F (5′-AGATGTCAACAATTTGGAGGATATGGAGCC-3′), Y74R (5′-GGCTCCATATCCTCCAAATTGTTGACATCT-3′), Y77F (5′-CAATATGGAGGATTTGG AGCCACTGGTGTT-3′), Y77R (5′-AACACCAGTGGCTCCAAATCCTCCATATTG-3′), Y326F (5′-GGAAAAGATGCCGAATTCTGCAAATGCCCA-3′), and Y326R (5′-TGGGCATTTGCAGAATTCGGCATCTTTTCC-3′). The tyrosine-substituted sites of ROL-6 are underlined, and the mutation sites were at Y74, Y77, and Y326. For multiple mutant constructs, 2 or 3 tyrosine residues were replaced by phenylalanines. These mutant cDNAs were cloned into pPD95.77, and mutant ROL-6::GFP constructs were injected into N2 and *rol-6* mutant worms.

Subcellular localization of TPST-1

To express TPST-1::GFP in *C. elegans*, PCR products obtained from the *tpst-1* cDNA clone (yk166c1) were cloned into pPD95.77. The following primers were used: 5′-ATTCTAGAATGAGAAAAAATCGAGAGTTGC-3′ and 5′-ATCCCGGGCCTAATTTCGATA CTTCATTA-3′. Next, the promoter of TPST-1 (279bp) was amplified by PCR and ligated into the TPST-1::GFP construct. The following primers were used: 5′-ATGTCGACCGAAAAATAATTCCTAATC-3′ and 5′-ATTCTAGATATAATATTCGCCTTTCTC-3′. Transgenic worms expressing TPST-1::GFP were harvested and incubated in Hoechst 33342 (10 μg/ml; Sigma, UK) for 15 min. To express TPST-1::GFP in HeLa cells, PCR products of a full-length *tpst-1* cDNA were cloned into the XhoI and EcoRI sites of the pEGFP-N1 expression vector (Clontech Ltd). The following primers were used: 5′-ATCTCGAGATGAGAAAAAATCGAGAGTTGC-3′ and 5′-TGAATTCACTAATTTCGATACTTCATTATC-3′. HeLa cells were transfected with TPST-1::GFP constructs using lipofectamine (Lipofectamine 2000™; Invitrogen). Cells grown on glass coverslips were fixed, permeabilized, and sequentially incubated with anti-Golgi 58K antibody (Sigma) or anti-TGN38 antibody (Santa Cruz Biotechnology), followed by anti-mouse or -goat secondary antibodies conjugated with rhodamine (R) (Santa Cruz Biotechnology). After antibody staining, cells were examined by fluorescence microscopy. All microscopic images were captured and processed using an AxioCam HRc digital camera attached to an Imager M1 fluorescence microscope and Axiovision Rel. 4.6 software (Zeiss).

RESULTS AND DISCUSSION

TPST-1 can sulfate three tyrosine residues of ROL-6

C. elegans TPST was originally named TPST-A by Ouyang and Moore (Ouyang and Moore, 1998). However, *tpst-a* does not follow the official nomenclature for *C. elegans* genes, so we corrected it to *tpst-1*. TPST-1 knock down by RNA*i* results in the suppression of several Rol mutants (Kim et al., 2005). In order to determine the molecular mechanism underlying Rol suppression and *tpst-1* function, we examined whether TPST-1 directly sulfates collagens. We selected a typical cuticle collagen, ROL-6, which is named for the rolling phenotype of the *rol-6* mutant. ROL-6 belongs to type IV collagens, and these collagens play important functions *in vivo* such as monocyte attachment and migration (Kostidou et al., 2008). ROL-6 contains three conserved regions with tyrosine residues that could potentially be sulfated (Yang and Kramer, 1994). We expressed His-tagged TPST-1 in HEK293 cells, and the purified TPST-1 protein is normally N-glycosylated (Fig. 1A). Three peptides containing five tyrosine residues of ROL-6 were synthesized and used in *in vitro* sulfation analysis (Fig. 1B). At first, the sulfated peptides were observed by TOF mass spectrometry and could be dissociated in tandem collision induced dissociation (CID) analysis. The sulfated peptides were also confirmed by sequencing. As a result, sulfations of tyrosine residues were detected in the R6-N1 [Y74 and Y77] and R6-C1 [Y326] peptides (Figs. 1C and 1E), but not in the R6-N2 peptide (Fig. 1D). The facile loss of 80 Da (Fig. 1C) or 40 Da (Fig. 1E) from the peptides indicated the existence of tyrosine sulfations. The ions of the sulfated R6-N2 peptide were not observed in either the TOF mass spectrum (Fig. 1D) or in the MS/MS spectrum for a putative mass (doubly charged ions; m/z, 738.7679 +40 or +80 Da) (data not shown). Some tyrosine residues of SQT-1 peptides were also sulfated by TPST-1 using the same method (data not shown). SQT-1 is closely related to ROL-6, and its Rol mutant phenotype is also suppressed by *tpst-1* RNA*i* (Kim et al.,

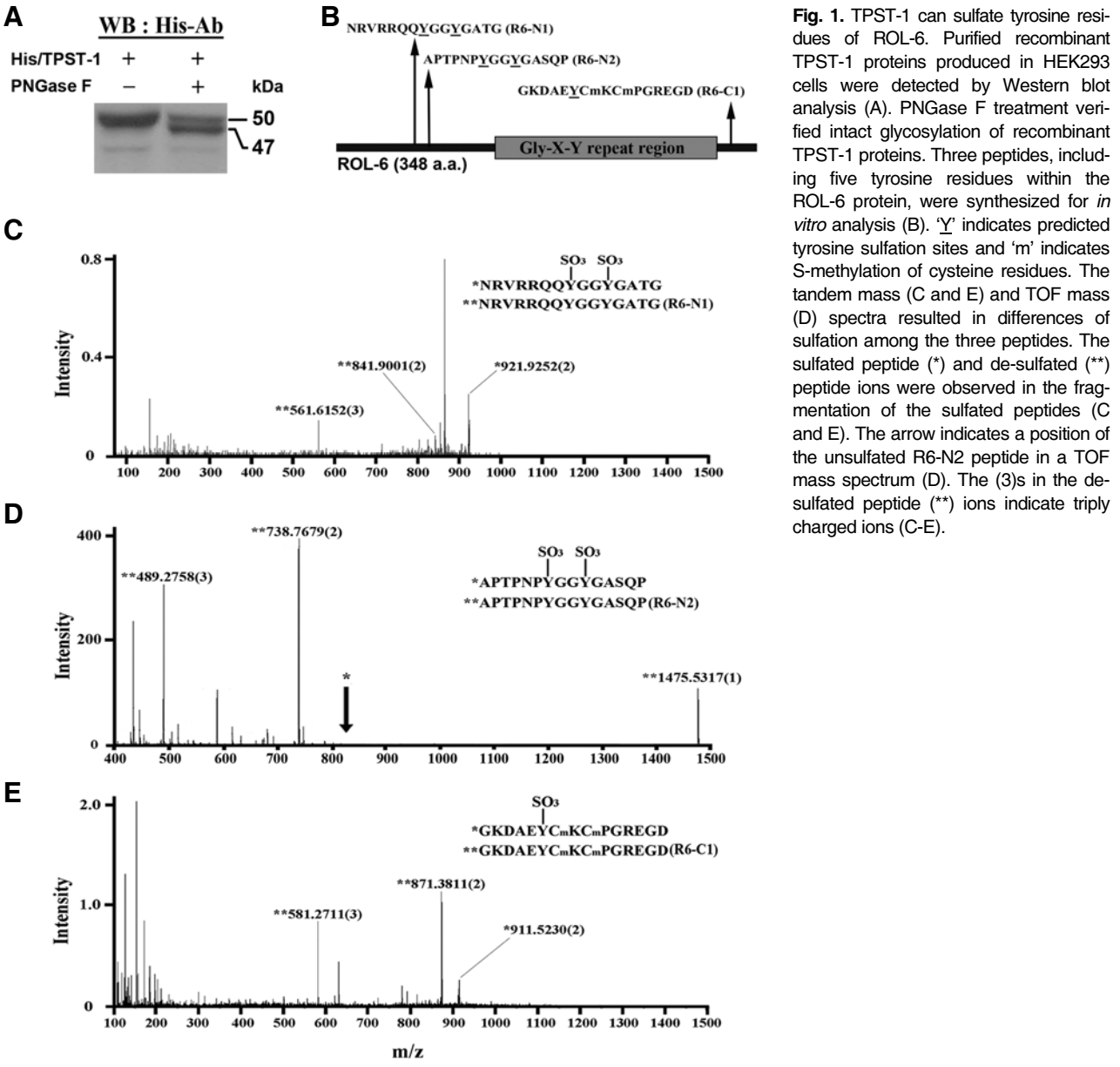

Fig. 1. TPST-1 can sulfate tyrosine residues of ROL-6. Purified recombinant TPST-1 proteins produced in HEK293 cells were detected by Western blot analysis (A). PNGase F treatment verified intact glycosylation of recombinant TPST-1 proteins. Three peptides, including five tyrosine residues within the ROL-6 protein, were synthesized for *in vitro* analysis (B). 'Y' indicates predicted tyrosine sulfation sites and 'm' indicates S-methylation of cysteine residues. The tandem mass (C and E) and TOF mass (D) spectra resulted in differences of sulfation among the three peptides. The sulfated peptide (*) and de-sulfated (**) peptide ions were observed in the fragmentation of the sulfated peptides (C and E). The arrow indicates a position of the unsulfated R6-N2 peptide in a TOF mass spectrum (D). The (3)s in the de-sulfated peptide (**) ions indicate triply charged ions (C-E).

2005). Therefore, TPST-1 exhibited tyrosylprotein sulfotransferase activity towards tyrosine residues of ROL-6, *in vitro*.

Tyrosine residues are important for ROL-6 cuticle localization

Three tyrosine residues of ROL-6 are sulfated by TPST-1, *in vitro*; thus, we examined the significance of ROL-6 sulfation, *in vivo*. We made transgenic worms expressing ROL-6::GFP to visualize ROL-6 protein localization. ROL-6::GFP was expressed through the whole larval and adult stages and localized in stripes at the cuticle (Fig. 2A and data not shown). Since ROL-6::GFP rescued the Rol phenotype of *rol-6 (e187)* loss-of-function (*lf*) mutant (data not shown), ROL-6::GFP may function properly in the cuticle. Following this, we made a mutant series of ROL-6::GFP, in which tyrosine (Y) residues were replaced with phenylalanine (F) to minimize structural changes (Blott et al., 2003). Unexpectedly, mutant ROL-6::GFP proteins with a single phenylalanine substitution exhibited normal cuticle localization patterns (Figs. 2A-2D). However, mutant ROL-6::GFP proteins with either 2 or 3 phenylalanine substitutions failed to localize to the cuticle (Figs. 2E-2H). These results suggest that sulfation of more than two tyrosine sites is needed for the proper cuticle localization of ROL-6.

Next, we also confirmed the cuticle localization of ROL-6::GFP series in *rol-6 lf* and *null* mutants to exclude the effect of endogenous ROL-6. We observed similar results in ROL-6::GFP expression in wild-type and two mutant backgrounds (Table 1). Therefore, multiple tyrosine sites are needed for the proper cuticle localization and function of ROL-6.

TPST-1 localizes to the *trans*-Golgi network

TPST localizes and functions at the TGN in human (Lee and Huttner, 1983), and the TGN is a terminal of secretory vesicles containing secretory and membrane proteins. Since the subcellular localization of TPST-1 in *C. elegans* has not yet been confirmed, we made a transgenic worm expressing TPST-1::GFP.

Table 1. Tyrosine O-sulfation sites in ROL-6 are important for function

Transgene product[a] (ROL-6::GFP)	GFP expression in cuticle furrows		
	wild type	*rol-6* (*e187*)[b]	*rol-6* (*e187n1270*)[c]
WT	+++++	+++++	++++
Y74F	+++++	+++++	++++
Y77F	++++++	++++++	+++++
Y326F	+++++	++++	+++
Y74F; Y77F	-	-	-
Y77F; Y326F	-	+	-
Y74F; Y326F	+	+	+
Y74F; Y77F; Y326F	-	-	-

Numbers of '+' represent relative fluorescence intensities of ROL-6::GFP proteins in the cuticle. GFP signals as much as background indicated as '-'. We observed 20 worms for each strain and took the pictures of worms with the same exposure time (880 ms). Intensities of GFP fluorescence in the prepared images were measured using the AxioVision Rel. 4.5 software (data not shown). Fluorescence densities were determined in equivalent circle areas around the pharynx (diameter 12.4 μm). Background signals as determined in wild-type worm controls were subtracted before analysis. [a]Tyrosine (Y) residues at Y74, Y77 and Y326 in ROL-6 are replaced by phenylalanine (F) respectively. [b]This mutant is a recessive rolling allele. [c]This mutant is a null allele of *rol-6*.

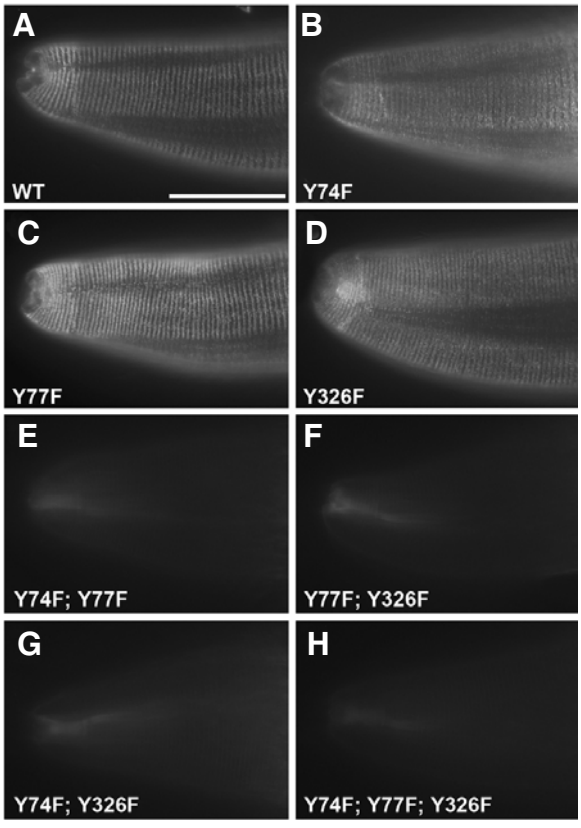

Fig. 2. Tyrosine residues are important for the cuticle localization of ROL-6. Pictures of wild-type ROL-6::GFP (A) and mutant ROL-6::GFPs (B-H) are shown. Y74F (B), Y77F (C), and Y326F (D) indicate that tyrosine residues at Y74, Y77, and Y326 in ROL-6 are replaced by phenylalanine, respectively. Multiple mutant forms are indicated by Y74F; Y77F (E), Y77F; Y236F (F), Y74F; Y236F (G), and Y74F; Y77F; Y236F (H). Cuticle localization of three single tyrosine substituted ROL-6::GFP proteins are similar to wild-type ROL-6::GFP, but multiple sites mutant ROL-6::GFP proteins are barely detectable at the cuticle. Scale bar, 50 μm.

TPST-1::GFP was expressed in the cytoplasm around the nuclei of the hypodermal cells (Fig. 3C). We tried to express some organelle markers with TPST-1::GFP, but failed to elucidate the exact subcellular localization of TPST-1::GFP in the worm. Alternatively, we made another TPST-1::GFP construct using a pEGFP vector, and expressed it in HeLa cells. TPST-1::GFP localization perfectly overlapped with TGN38, which is a TGN marker (Figs. 3D-3F), but partially overlapped with Golgi-58K, which is a Golgi marker (Figs. 3G-3I). Therefore, worm TPST-1 localizes to the TGN, indicating that protein sulfation occurs at the TGN in *C. elegans*.

The cuticle localization of ROL-6 needs TPST-1

Since multiple TPST-1 target sites of ROL-6 are needed for its proper cuticle localization, we focused on the effects of *tpst-1* RNA*i* on ROL-6 localization. ROL-6::GFP is expressed in parallel lines, and most ROL-6::GFP proteins were located at a region distal to the alae (Fig. 4A). However, similar to the localization patterns of the multiple tyrosine mutant forms of ROL-6::GFP (Figs. 2E-2F), GFP signals at the cuticle were hard to detect in *tpst-1* RNA*i*-treated worms (Fig. 4B). These results suggest that TPST-1 is critical for the cuticle localization of ROL-6.

Finally, we assayed the roles of other collagen modifying enzymes in ROL-6 localization. *C. elegans* BLI-3 is a mammalian homolog of Duox, which catalyzes the formation of di-tyrosine bonds among collagens (Edens et al., 2001). ROL-6::GFP was blurred and diffuse in the *bli-3* mutant (Figs. 4E and 4F). DPY-11 is a membrane-associated thioredoxin-like protein expressed in the cytoplasm that affects body shape and ray morphology (Ko and Chow, 2002). The usual stripes of ROL-6::GFP proteins were sharpened and their orientations were disturbed in the *dpy-11* mutant. The number of stripes was reduced in the region proximal to the alae (Figs. 4G and 4H). DPY-18 is the alpha subunit of propyl-4-hydoxylase, which is a procollagen modifying enzyme (Friedman et al., 2000). The ROL-6::GFP localization defect in the *dpy-18* mutant was not severe, but its stripe pattern was discontinuous in the middle of the region distal to the alae (Figs. 4I and 4J). These collagen modifying enzymes seem to function in ROL-6 arrangement at the cuticle without affecting ROL-6 secretion. In contrast, TPST-1 affects ROL-6 transport from the TGN to cuticle.

In summary, from the molting defect and Rol suppression phenotypes of the *tpst-1* RNA*i*, we hypothesize that TPST-1 sulfates collagens and regulates cuticle formation. We selected ROL-6 as a putative target for TPST-1, and verified that sulfation occurred at three tyrosine residues of ROL-6. Next, to

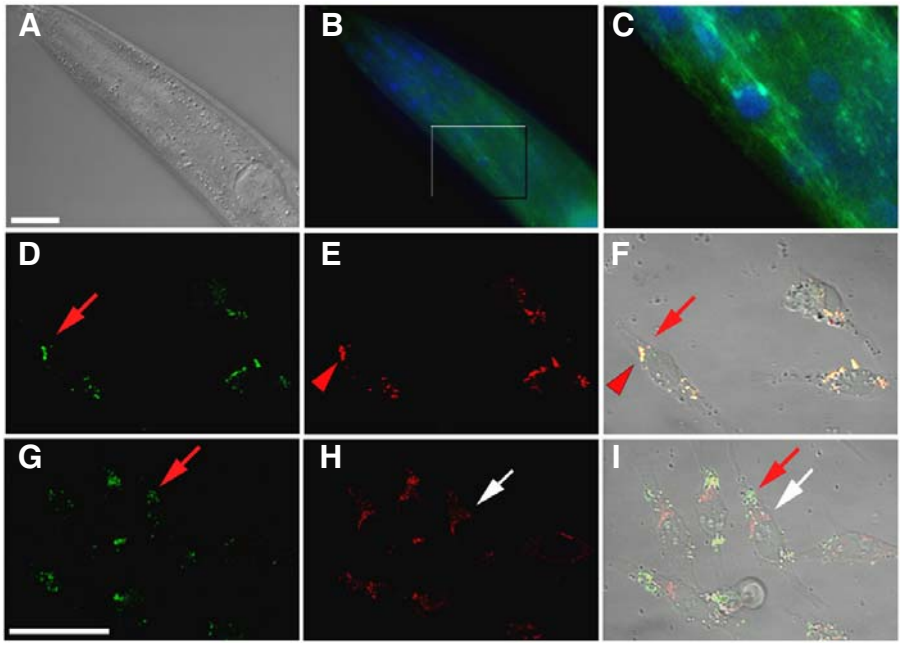

Fig. 3. TPST-1::GFP is localized at the TGN. TPST-1::GFP proteins were expressed in both worms (A-C) and HeLa cells (D-I). TPST-1::GFP is expressed in the cytoplasm of the hypodermal cells (B and C). (A) DIC image, (B, D) merge of fluorescence images (Blue color indicates nuclei stained by Hoechst 33342 and green color is TPST-1::GFP). (C) A large view of a rectangular region of (B). (D, G) TPST-1::GFP in HeLa cells. (E) TGN38 as a TGN marker and (H) Golgi-58K as a Golgi marker. (F) Merged image of (D, E). (I) Merged image of (G, H). TPST-1::GFP proteins (red arrow) are completely co-localized with TGN38 (red arrow head), but partially colocalized with Golgi-58K (white arrow). Scale bars, 30 μm.

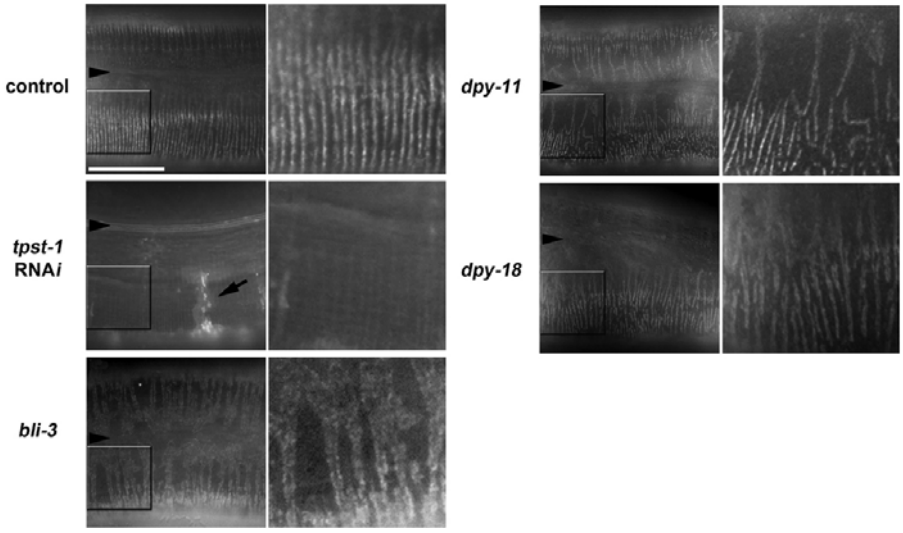

Fig. 4. ROL-6::GFP localization after TPST-1 knock down and in mutants of collagen modifying enzymes. ROL-6::GFP is expressed at the cuticle in a striped pattern and strong in the region distal to the alae (A, B). TPST-1 knock down by *tpst-1* RNA*i* resulted in the elimination of ROL-6::GFP expression at the cuticle (C, D). ROL-6::GFP patterns in *bli-3* (E, F), *dpy-11* (G, H), and *dpy-18* (I, J) mutants. The right-hand panels are amplified versions of rectangular regions in left-hand panels. Arrowheads indicate the alae, and the arrow indicates non-specific fluorescence from cuticle clots (C). Scale bar, 50 μm.

elucidate the biological significance of ROL-6 sulfation, we visualized the localization of various forms of the ROL-6 protein using GFP fusion constructs, including tyrosine site mutants, and verified that the tyrosine sites of ROL-6 are critical for its proper localization at the cuticle. We also observed that TPST-1 was localized to the TGN, and that *tpst-1* RNA*i* resulted in the mislocalization of ROL-6::GFP.

Sulfation of tyrosine residues occurs almost exclusively on secreted and transmembrane spanning proteins (Baeuerle and Huttner, 1985). A functional genomics study in a *Drosophila* cell culture system concluded that *tpst-1* knockdown causes a protein secretion defect without affecting the morphology of the Golgi (Bard et al., 2006). The results presented here indicate that TPST-1 likely participates in the regulation of protein secretion. However, the exact function of protein sulfation still remains to be uncovered. Since the sulfation of many proproteins occurs in the TGN and cleavage processing occurs in secretory granules (Beinfeld, 2003; Bundgaard et al., 1995), the sulfation of pro-ROL-6 is likely to promote its maturation through the next cleavage process and to affect the stability of the protein. There are several possible mechanisms of TPST-1 function in the secretory pathway. One is that tyrosine sulfation may provide recognition sites for proteases. For example, the cleavage of Secretogranin II takes advantage of its tyrosine sulfation in the TGN (Muller et al., 1997). Another possibility is that tyrosine sulfation functions in stabilizing the structure of the collagen. For example, the efficacy of gastrin-6 is increased by tyrosine sulfation, which also protects the protein from elimination (Palnaes Hansen et al., 2000). These two hypothetical mechanisms are potentially complementary because proprotein cleavage may promote protein stability. Indeed, putative sulfation target sites exist near conserved cleavage sites in many collagens, including DPY-7, DPY-10, COL-1, COL-2, COL-8, COL-12, COL-40, SQT-1, and ROL-6 (Kramer and Johnson,

1993). However, our data do not fully support this model, as we found that multiple tyrosine sites were needed for ROL-6 function and localization. Thus, future experiments will focus on elucidating the role of procollagen processing enzymes and the fate of unsulfated collagens in the secretory pathway.

In conclusion, *C. elegans* TPST-1 can sulfate tyrosine residues of ROL-6 protein and regulate ROL-6 collagen secretion. This is the first report showing that tyrosine sulfation by TPST-1 regulates collagen secretion.

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