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Topology of phosphatidylserine synthase 1 in the endoplasmic reticulum membrane

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Abstract

Phosphatidylserine (PS) synthase 1 (PSS1) of mammalian cells is a multiple membrane-spanning protein of the endoplasmic reticulum (ER) and regulated by inhibition with the product PS. Alanine-scanning mutagenesis of PSS1 has revealed eight amino acid residues as those crucial for its activity and six as those important for its regulation. Furthermore, three missense mutations in the human PSS1 gene, which lead to regulatory dysfunctions of PSS1 and are causative of Lenz–Majewski syndrome, have been identified. In this study, we investigated the membrane topology of PSS1 by means of epitope insertion and immunofluorescence. According to a 10-transmembrane segment model supported by topology analysis of PSS1, all the 8 amino acid residues crucial for the enzyme activity were localized to the luminal side of the lipid bilayer or the lumen of the ER, whereas all the 9 amino acid residues involved in the enzyme regulation were localized to the cytosol or the cytoplasmic side of the lipid bilayer of the ER. This localization of the functional amino acid residues suggests that PSS1 is regulated by inhibition with PS in the cytoplasmic leaflet of the ER membrane and synthesizes PS at the luminal leaflet.

KEYWORDS

biosynthesis, endoplasmic reticulum, membrane topology, metabolic regulation, phosphatidylserine, phosphatidylserine synthase, phospholipids

1 | INTRODUCTION

Phosphatidylserine (PS) is an essential phospholipid for the growth of mammalian cells,[1] comprising 3–15% of the total phospholipids in various mammalian tissues and cultured cells. Although PS in bacteria and yeast is synthesized from cytidine diphosphate (CDP) diacylglycerol and L-serine,[2,3] PS in mammalian cells is synthesized through the exchange of L-serine with the choline moiety of phosphatidylcholine (PC) or the ethanolamine moiety of phosphatidylethanolamine (PE).[4–6] The PS synthesis from PC and PE, respectively, are catalyzed by PS synthase (PSS) 1 and 2.[4–10]

Biosynthesis of PS in cultured mammalian cells, such as CHO-K1 cells, is inhibited on the addition of PS to the culture medium, indicating the presence of negative feedback control for PS biosynthesis.[11] A mutant strain (No. 29) isolated from CHO-K1 cells exhibits exogenous PS-resistant PS biosynthesis[12] and has been shown to harbor a missense mutation resulting in the replacement of Arg-95 of PSS1 with Lys, which causes a regulatory dysfunction of PSS1.[13] Through alanine-scanning mutagenesis of a cDNA clone of Chinese hamster PSS1, we have identified eight amino acid residues as those crucial for

Abbreviations: CDP, cytidine diphosphate; ER, endoplasmic reticulum; HA, hemagglutinin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PSS, PS synthase.

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its activity; namely, their mutation to alanine causes almost complete inactivation of PSS activity, but does not affect protein level of the PSS1.[14] Among these amino acid residues, Asn-209 is probably involved in the recognition and/or binding of L-serine, because, like the wild-type PSS1, the mutant PSS1 with the replacement of Asn-209 with Ala has activities of the choline and ethanolamine base exchange to synthesize PC and PE, in spite of defective in PS synthesis by the serine base exchange.[14] In addition, we have identified six amino acid residues as those important for its regulation; their mutation to alanine increases activity of PSS1 and attenuates inhibition of PSS1 by PS.[14] Furthermore, three missense mutations in the human *PTDSS1* gene encoding PSS1 have been identified as causative mutations of Lenz–Majewski syndrome and shown to lead to exogenous PS-resistant PS biosynthesis.[15] These functional amino acid residues involved in the activity and regulation of PSS1, except for Leu-265 mutated in individuals with Lenz–Majewski syndrome, are found not only in PSS1 of various species, but also in PSS2 enzymes (Figure S1).

PSS1 is a multiple membrane-spanning protein[7] localized to the endoplasmic reticulum (ER) and a subcompartment of the ER, mitochondria-associated membranes.[16,17] However, the membrane topology of PSS1 has not been elucidated. Transmembrane prediction-programs HMMTOP,[18,19] TMHMM,[20,21] and SOSUI[22] predict nine-transmembrane segment models for PSS1. On the other hand, the MEMSAT2 program[23] predicts a 10-transmembrane segment model. As shown in Figure 1a, in the 10-transmembrane segment model of PSS1, all of the 8 amino acid residues crucial for its enzyme activity are localized to the luminal side of the lipid bilayer or the lumen of the ER, while all of the

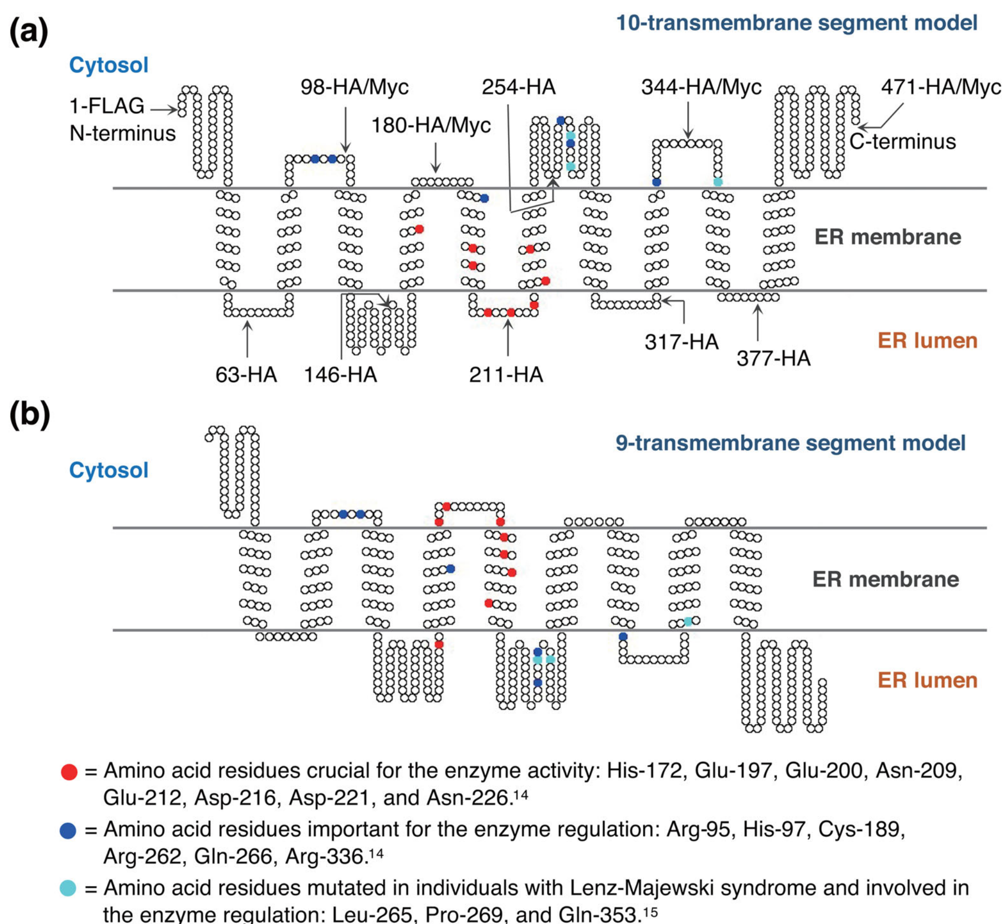

FIGURE 1 Membrane topology models of Chinese hamster phosphatidylserine synthase 1 (PSS1). (a) The 10-transmembrane segment model predicted by the MEMSAT2 transmembrane-prediction program of a public server (http://www.sacs.ucsf.edu/cgi-bin/memsat.py). Internal hemagglutinin (HA)- and Myc-epitope tags of the recombinant PSS1 proteins used in this study were inserted after amino acids corresponding to the native protein's amino acids 63, 98, 146, 180, 211, 254, 317, 344, and 377 (63-HA, 98-HA/Myc, 146-HA, 180-HA/Myc, 211-HA, 254-HA, 317-HA, 344-HA/Myc, and 377-HA). The arrows point to where the epitope tags indicated were inserted. (b) The nine-transmembrane segment model predicted by the SOSUI transmembrane-prediction program of a public server (https://harrier.nagahama-i-bio.ac.jp/sosui/mobile/)

9 amino acid residues involved in the enzyme regulation are localized to the cytosol or the cytoplasmic side of the lipid bilayer of the ER. In contrast, in the nine-transmembrane segment model (Figure 1b), the amino acid residues important for the enzyme activity and regulation are randomly distributed to the lipid bilayer of and near the ER membrane. In this study, we investigated the membrane topology of PSS1 and our results favored the 10-transmembrane segment model rather than the 9-transmembrane one, providing the basis for further structure–function studies on PSS1.

2 | RESULTS

To investigate the membrane topology of PSS1, we employed the approach of epitope insertion and immunofluorescence.[24–28] To visualize cytosolic epitopes selectively or both cytosolic and ER luminal epitopes by immunofluorescence microscopy, HeLa cells producing recombinant proteins with epitope insertion(s) were permeabilized in one of the following manners: (a) digitonin-treated selective permeabilization of the plasma membrane or (b) Triton X-100-treated complete permeabilization of membranes including the ER and plasma ones. To determine whether the digitonin-treated selective permeabilization was adequately performed, HeLa cells producing a fusion protein, ER-EGFP,[29] localized to the lumen of the ER, or an ER-membrane fusion protein, EGFP-Cyt b_5,[29] exposing an EGFP-tag to the cytosol were permeabilized by digitonin treatment and then subjected to immunofluorescence with an anti-EGFP antibody. As shown in Figure 2a, the cytosolic EGFP tag of EGFP-Cyt b_5, but not the ER luminal EGFP tag of ER-EGFP, was visible in the digitonin-treated cells as immunofluorescence, although the fluorescence of EGFP itself was equally visible, indicating normal production (Figure 2a). This control experiment indicated that the selective permeabilization of the plasma membrane by digitonin was adequately performed for analysis of the membrane topology of PSS1.

To determine whether the 9- or 10-transmembrane segment model of PSS1 (Figure 1) was more reliable, we first investigated the localization of the N- and C-termini of PSS1 by means of epitope insertion and immunofluorescence. We constructed cDNA clones encoding recombinant Chinese hamster PSS1 with a FLAG-epitope tag after the first methionine (1-FLAG) or a hemagglutinin (HA)-epitope tag at the C-terminus (471-HA). After introduction of these cDNA clones into HeLa cells, the cells expressing the recombinant proteins were subjected to the immunofluorescence assay described above. As

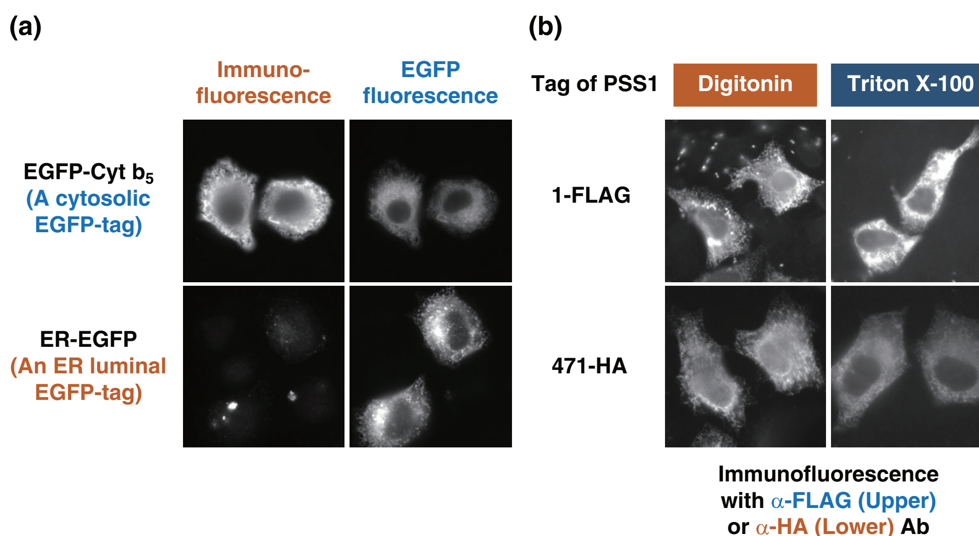

FIGURE 2 The N- and C-termini of recombinant phosphatidylserine synthase 1 (PSS1) proteins are oriented toward the cytosol. (a) Evaluation of digitonin-treated selective-permeabilization of the plasma membrane. HeLa cells were transiently transfected with the plasmid pcDNAZeo/EGFP-Cyt-b_5[29] and pcDNAZeo/endoplasmic reticulum (ER)-EGFP[29] that encode EGFP-Cyt b_5 and ER-EGFP, respectively, fixed with paraformaldehyde, and then treated with digitonin for selective permeabilization of the plasma membrane. The digitonin-treated cells were observed by immunofluorescence with an anti-EGFP antibody (Immunofluorescence). In addition, fluorescence of EGFP in the cells was observed directly with a fluorescence microscope (EGFP fluorescence). (b) HeLa cells were transiently transfected with a cDNA clone encoding the PSS1 protein carrying an epitope tag indicated, fixed with paraformaldehyde, treated with digitonin for selective permeabilization of the plasma membrane or Triton X-100 for complete permeabilization, as indicated, and then subjected to immunofluorescence with an anti-FLAG (α-FLAG) or an anti-hemagglutinin (α-HA) antibody (Ab)

shown in Figure 2b, the 1-FLAG tag was visible in digitonin-treated selective-permeabilized cells, as well as in Triton X-100-treated complete-permeabilized cells as immunofluorescence with an anti-FLAG antibody, indicating that the N-terminus of the recombinant PSS1 protein is oriented toward the cytosol. Similarly, the 471-HA tag was visible in both digitonin- and Triton X-100-treated cells as immunofluorescence with an anti-HA antibody (Figure 2b), indicating that the C-terminus of the recombinant PSS1 protein is also oriented toward the cytosol. These orientations of the N- and C-termini were consistent with the 10-transmembrane segment model of PSS1. Furthermore, because a recombinant PSS1 protein with a FLAG-epitope tag after the first methionine and an HA-epitope tag at the C-terminus was previously shown to be functional,[30] the cytoplasmic orientations of the N- and C-termini of the recombinant PSS1 proteins seemed to reflect the topology of the native PSS1 protein.

For further evaluation of the reliability of the 10-transmembrane segment model, we constructed additional nine cDNA clones encoding recombinant Chinese hamster PSS1 proteins that had the 1-FLAG tag and one of nine internal HA-epitope tags inserted into the loop regions of the 10-transmembrane segment model (Figure 1a). These internal HA-epitope tags were designated as 63-, 98-,146-, 180-, 211-, 254-, 317-, 344-, and 377-HA, respectively (Figure 1a). HeLa cells were transiently transfected with these cDNA clones and then subjected to the immunofluorescence assay described above. As shown in Figure 3a, the 63-, 146-, 211-, 317-, and 377-HA tags were clearly visible in the Triton X-100-treated complete permeabilized cells, but not in the digitonin-treated selective permeabilized cells,

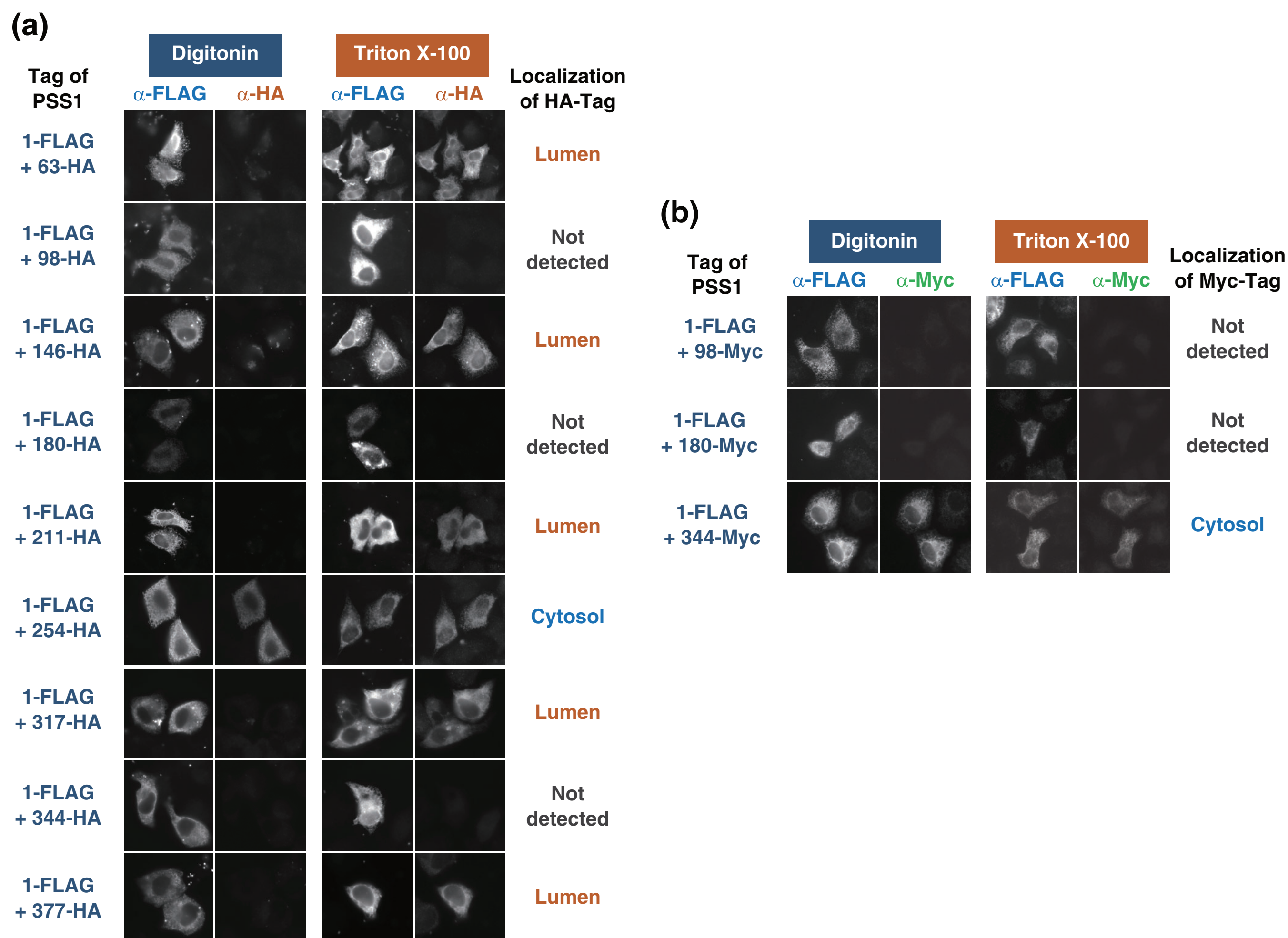

FIGURE 3 Orientations of epitope tags inserted into the loop region of the 10-transmembrane segment model of phosphatidylserine synthase 1 (PSS1). HeLa cells were transiently transfected with a cDNA clone encoding the PSS1 protein with the epitope tag indicated, fixed with paraformaldehyde, treated with digitonin for selective permeabilization of the plasma membrane or Triton X-100 for complete permeabilization, as indicated, and then subjected to immunofluorescence with anti-FLAG (α-FLAG), anti-hemagglutinin (α-HA), or anti-Myc (α-Myc) antibodies indicated

although the 1-FLAG tag of the same protein constructs was visible in both digitonin- and Triton X-100-treated cells. These results indicated that the 63-, 146-, 211-, 317-, and 377-HA tags reside in the lumen of the ER, consistent with the 10-transmembrane segment model. Furthermore, the 254-HA tag, as well as the 1-FLAG tag of the same protein construct, was clearly visible in both digitonin- and Triton X-100-treated cells as immunofluorescence (Figure 3a), indicating the cytoplasmic orientation of the 254-HA tag. This was also consistent with the 10-transmembrane segment model.

Unexpectedly, the 98-, 180-, and 344-HA tags were not visible in either digitonin- or Triton X-100-treated cells as immunofluorescence (Figure 3a), then we tried a different epitope tag, Myc. We constructed cDNA clones encoding recombinant PSS1 proteins with the 1-FLAG tag and an internal Myc-epitope tag (98-, 180-, or 344-myc tag) that substituted for the 98-, 180-, or 344-HA tag, and introduced these cDNA clones into HeLa cells. Like the 98- and 180-HA tags, the 98- and 180-Myc tags were not visible in either digitonin- or Triton X-100-treated cells as immunofluorescence with an anti-Myc antibody; however, the 344-Myc tag was clearly visible in both digitonin- and Triton X-100-treated cells as immunofluorescence (Figure 3b), indicating the cytoplasmic orientation of the 344-Myc tag. This was also consistent with the 10-transmembrane segment model.

Although the 98-, 180-, and 344-HA tags and the 98- and 180-Myc tags were not visible in either digitonin- or Triton X-100-treated cells as immunofluorescence, the recombinant proteins carrying these tags appeared to be significantly produced, because the 1-FLAG tag of the same protein constructs was visible in both digitonin- and Triton X-100-treated cells as immunofluorescence (Figure 3a,b). To confirm this and examine whether the full-length proteins with the 98-, 180-, or 344-HA tag were produced, we constructed cDNA clones encoding recombinant PSS1 proteins with triple tags, the 1-FLAG tag, a C-terminal Myc-epitope tag (471-Myc) (Figure 1a), and the 98-, 180-, or 344-HA tag, and transfected these cDNA clones into HeLa cells. Again the internal HA tags of these protein constructs were not visible, but both the 1-FLAG and 471-Myc tags of the same protein constructs were visible in Triton X-100-treated cells as immunofluorescence (Figure S3), indicating that the full-length proteins with the 98-, 180-, or 344-HA tag were produced. We speculated that the 98-, 180-, and 344-HA tags and the 98- and 180-myc tags could not be recognized by the corresponding antibodies for some reason, such as masking by protein(s) and/or lipid(s) that interact(s) with PSS1. Alternatively, the structure of these epitope tags might change to a form that is not recognized by the corresponding antibodies.

3 | DISCUSSION

All orientations relative to the ER membrane of the FLAG-, HA-, and Myc-epitope tags added to the PSS1 protein (Figures 2 and 3), except for those of undetected tags, were consistent with the 10-transmembrane segment model (Figure 1a), while the 9-transmennbrane segment model (Figure 1b) was inconsistent with the orientations of the 211-, 254-, 317-, 377-, and 471-HA tags and the 344-Myc tag. Thus, the 10-transmembrane segment model of PSS1 was more reliable than the 9-transmembrane one.

According to the 10-transmembrane segment model (Figure 1a), all of the eight amino acid residues crucial for the enzyme activity[14] including the Asn-209 involved probably in the recognition and/or binding of L-serine[14] are localized to the luminal side of the lipid bilayer or the lumen of the ER, whereas all of the nine amino acid residues involved in the enzyme regulation[14,15] are localized to the cytosol or the cytoplasmic side of the lipid bilayer of the ER. This localization of functional amino acid residues suggests that PSS1 senses the level of PS in the cytoplasmic leaflet of the ER membrane for its own regulation and synthesizes PS at the opposite luminal leaflet. In general, the synthesis of phospholipids including PC, PE, phosphatidylinositol, and PS in the ER is thought to occur at the cytoplasmic leaflet of the lipid bilayer, due to utilization of water-soluble precursors produced in the cytosol, such as CDP-choline, CDP-ethanolamine, *myo*-inositol, and L-serine. Given that PS is synthesized at the luminal leaflet of the ER membrane, transport of L-serine to the lumen or the luminal leaflet must be required. Such transport might be catalyzed by Serinc proteins,[31] because (a) Serinc proteins are ER membrane proteins that have 11-transmembrane segments, like amino acid transporters[31]; (b) a Serinc protein of yeast forms a protein complex with key enzymes for biosynthesis of L-serine[31]; and (c) overproduction of mammalian Serinc1, Serinc2, and Serinc5 in COS cells results in enhancement of the enzyme activity of PSS(s).[31]

The distributions of PS in the lipid bilayer of the ER membrane are probably important for the roles of PS in cell biology and physiology and must depend on the site of synthesis and the transbilayer flip-flop of PS, at least in part. It was recently suggested that PS is more abundant in the cytoplasmic leaflet than in the luminal leaflet of the ER membrane in mammalian cells,[32] and that this imbalance in the PS distribution is attenuated by stimulation of a phospholipid scramblase, TMEM16K, by Ca^{2+} [32] and enhanced by depletion of other scramblases, TMEM41B and VMP1.[33] It is therefore likely that these scramblases and PSSs cooperatively maintain a normal

level and distribution of PS in the lipid bilayer of the ER membrane. In such a cooperative function, the topological positions of catalytic and regulatory sites of PSS in the ER membrane may be key factors, and thus future structural investigations of PSS1 at a high resolution would facilitate the elucidation of the specific roles of PS in cell biology and physiology as well as the catalytic and regulatory mechanisms of PSS1.

4 | MATERIALS AND METHODS

4.1 | Cell culture and DNA transfection

HeLa cells were cultured in DMEM medium (GIBCO) supplemented with 10% fetal bovine serum under 5% CO_2–95% air at 37°C. Transfection of HeLa cells with cDNA clones was performed using Lipofectamine LTX reagent and PLUS reagent (Invitrogen), according to the manufacturer's instructions.

4.2 | Plasmids

DNA fragments encoding Chinese hamster PSS1 with a FLAG-epitope tag inserted after the first methionine (1-FLAG) and an internal HA-epitope tag inserted after amino acid corresponding to the native protein's amino acid 63, 98, 146, 180, 211, 254, 317, 344, or 377 were generated by PCR overlap extension recombination, using two pairs of primer (universal SP6 primer and respective reverse primer [Table S1], and universal T7 primer and respective forward primer [Table S1]), and a template plasmid, pSV/F-*pssA* encoding Chinese hamster PSS1 with the 1-FLAG tag.[34] The resulting DNA fragments were digested with SalI and NotI, and then ligated with the pSVOK*neo* vector[35] digested with SalI and NotI, to yield expression plasmids of PSS1 with the 1-FLAG tag and the internal HA-epitope tag at various sites.

For construction of a plasmid encoding PSS1 with a C-terminal HA-epitope tag, a DNA fragment was generated by PCR using an N-terminus primer (Table S1) and a 471HA-reverse primer (Table S1), and a template plasmid, pcDPSSA.[7] The resulting DNA fragment was digested with SalI and NotI, and then ligated with the pSVOK*neo* vector[35] digested with SalI and NotI.

The expression plasmids of PSS1 with the 1-FLAG tag and an internal Myc-epitope tag were constructed by the same procedures as those for the construction of the expression plasmids of PSS1 with the 1-FLAG tag and the internal HA-epitope tag, using PCR primers shown in Table S1.

For construction of plasmids encoding PSS1 with the 1-FLAG tag, the internal HA-epitope tag at various sites, and a C-terminal Myc-epitope tag, DNA fragments were generated by PCR using universal SP6 primer and a 471myc-reverse primer (Table S1), and template plasmids encoding Chinese hamster PSS1 with the1-FLAG tag and the internal HA tag at various sites. The resulting DNA fragments were digested with SalI and NotI, and then ligated with the pSVOK*neo* vector[35] digested with SalI and NotI.

The expression plasmid pcDNAZeo/ER-EGFP[29] and pcDNAZeo/EGFP-Cyt-b5,[29] which, respectively, encode fusion proteins, ER-GFP comprising of amino acid 1–414 of human Hsp47, EGFP, and a KDEL retention signal, and mouse cytochrome b_5 tagged with N-terminal EGFP are gifts from Dr Yukio Fujiki (Kyushu University).

4.3 | Topology analysis

HeLa cells transfected 24 hr prior to analysis with the plasmids were fixed with 3.7% paraformaldehyde in PBS. Fixed cells were treated with 50 μg/ml digitonin in PBS or 0.2% (wt/vol) Triton X-100 in PBS for 5 min at room temperature, followed by washing with PBS three times. Cells were then blocked with 1% fetal bovine serum in PBS for 10 min and immunostained with the indicated antibodies diluted to 1:1,000 in PBS with 1% fetal bovine serum. Mouse antibodies against the FLAG epitope (M2; Sigma-Aldrich) and the c-Myc epitope (9E10; Invitrogen), and rabbit antibodies against the FLAG epitope (anti-DYKDDDDK antibody; Cell signaling technology), the HA epitope (MBL) and EGFP (Invitrogen) were used as primary antibodies. Alexa Fluor 568-conjugated goat anti-mouse IgG and goat anti-rabbit IgG, and Alexa Fluor 488-conjugated goat anti-mouse IgG and goat anti-rabbit antibodies (Invitrogen) were used as secondary antibodies. Images were acquired using a fluorescent microscope (Leica DMRB).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Non Miyata: Investigation; analysis; funding acquisition; writing-review and editing. **Osamu Kuge**:

Conceptualization; investigation; funding acquisition; writing-original draft; writing-review and editing.

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