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Phosphatidylserine-binding protein lactadherin inhibits protein translocation across the ER membrane

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ABSTRACT

Secretory and membrane proteins are translocated across and inserted into the endoplasmic reticulum membrane via translocon channels. To investigate the effect of the negatively-charged phospholipid phosphatidylserine on the translocation of nascent polypeptide chains through the translocon, we used the phosphatidylserine-binding protein lactadherin C2-domain. Lactadherin inhibited targeting of nascent chain to the translocon by signal sequence and the initiation of translocation. Moreover, lactadherin inhibited the movement of the translocating polypeptide chain regardless of the presence or absence of positively-charged residues. Phosphatidylserine might be critically involved in translocon function, but it is not a major determinant for translocation arrest of positively-charged residues.

1. Introduction

Secretory and membrane proteins are translocated across and inserted into the membrane of the endoplasmic reticulum (ER) via protein conducting channel, so-called translocon [1]. Nascent chains with signal sequences elongating from ribosomes are targeted to the translocon together with the synthesizing ribosomes. As soon as the signal sequence emerges from the ribosome, it is recognized by the signal recognition particle and the ribosome-nascent chain complex (RNC) is targeted to the translocon [2]. This is the default pathway of organella targeting of hydrophobic transmembrane (TM) segments, which occurs in the absence of a regulatory sequence on the upstream polypeptide chain [3,4]. The signal sequence is then released from the particle by its receptor and transferred to the translocon [5]. The hydrophobic segment penetrates the translocon to initiate translocation. In eukaryotes, the main part of the translocon comprises a Sec61 complex [5]. X-ray structures of the archaeal and bacterial SecY complexes, homologs of the Sec61 complex, suggest that the translocation pore is surrounded by 10 TM helices of the SecY molecule [6], providing an aqueous environment through which a variety of hydrophilic polypeptides can be translocated. The pore also appears to open laterally to allow TM segments of the translocating polypeptide chain to exit into the membrane lipid environment [5].

Signal sequence functions are mainly determined by their hydrophobic segments and their orientations are modulated by the flanking positively-charged residues [7–9]. Insertion of the hydrophobic segment of the signal sequence into the translocon is a common process for initiation of the translocation. The positive charges are retained on the cytoplasmic side of the membrane and the less positive side is eventually positioned in the lumen. The type I signal-anchor (SA-I) causes translocation of the upstream portion, forming the N(lumen)–C(cytosol) orientation, while the type II signal-anchor (SA-II) and the signal peptide cause translocation of the downstream portion, forming the N(cytosol)–C(lumen) orientation. The signal sequences not only trigger membrane targeting, but also provide a significant motive force for translocation [10]. In the case of SA-I, N-terminal translocation is initiated during the partitioning of the hydrophobic segment into the lipid environment [11]. The motive force depends on the amino acid sequence of the TM segment.

After the initiation stage, translocation of an elongating polypeptide chain can be pushed by protein synthesis at the ribosome. Movement of the polypeptide chain through the translocon is restricted by the hydrophobic sequence and positive charge. TM sequences of the polypeptide chain stop the movement and insert into the lipid bilayer. Hydrophobicity is the primary determinant of stop-translocation [12,13]. Simple hydrophobic partitioning between the aqueous and hydrophobic environment causes membrane insertion [12–14]. In some cases, a marginally hydrophobic sequence stops translocation depending on the downstream positive charges [15]. Such cooperation between the hydrophobic

Abbreviations: CHX, cycloheximide; ER, endoplasmic reticulum; LA, C2 domain of bovine lactadherin; mLA, binding deficient mutant of LA; PS, phosphatidylserine; RM, rough microsomal membrane; RNC, ribosome nascent chain complex; SA-I, type I signal-anchor; SA-II, type II signal-anchor; SAv, streptavidin; SBP-tag, streptavidin-binding peptide tag; TM, transmembrane.

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segment and positive charges occurs even when they are separated by more than 60 amino acid residues [15,16]. Moreover, the positive charges arrest translocation even in the absence of a hydrophobic segment, indicating that the positive charges are translocation regulators independent of the hydrophobic sequences [17]. The fact that translocation arrest can be relaxed under high salt conditions [17] suggests that translocation arrest by positively-charged residues is primarily due to an electrostatic interaction with membranous charged factors.

The ER membrane contains phosphatidylserine (PS) or phosphatidylinositol as negatively-charged phospholipids. Here, we examined the effect of the C2 domain of bovine lactadherin (LA), which specifically binds to PS [18], and found that LA inhibited membrane targeting, translocation initiation by SA-I, and movement of the hydrophilic polypeptide chain. These findings suggest that although the negative charge of PS is not a major determinant of protein translocation arrest by positively-charged residues, PS is critically involved in efficient ER translocon channel function.

2. Materials and methods

2.1. Materials

Biotin and cycloheximide (CHX) were purchased from Sigma-Aldrich. Streptavidin (SAv) was purchased from Wako Pure Chemicals. Plasmids harboring model proteins were previously described [19,20]. The plasmid LactC2-GFP [18] was obtained through a non-profit organization, Addgene.

2.2. Construction of expression plasmids for wild-type and mutant LA

The DNA fragment encoding LA was amplified by PCR and inserted into the XhoI-SacI site of pET-28b. The construct encodes the LA fused to an N-terminal hexa-histidine tag and thrombin cleavage site. Binding-deficient mutant (W26A, W33A, F34A; mLA) [18] was prepared by the quick-change method.

2.3. Purification of wild-type and PS-binding mutant of LA

Expression of LA in *Escherichia coli* strain BL21(DE3) was induced with IPTG for 3 h at 25 °C. Cells were disrupted by sonication in buffer A (150 mM NaCl and 20 mM Tris, pH 7.5). The supernatant was applied to a HisTrap FF (GE Healthcare) and eluted with 500 mM imidazole. The fusion protein was subjected to thrombin cleavage at 25 °C for 2 h to remove the hexa-histidine tag and further purified by gel filtration. The fractions including LA protein were concentrated and the buffer was exchanged with 10 mM Hepes–KOH [pH 7.4]. The molecular weight was estimated to be 22 kDa based on dynamic light-scattering, indicating that LA is the monomer in the solution. The binding-deficient mutant of LA (mLA) was similarly expressed and purified.

2.4. In vitro transcription and translation

In vitro protein synthesis and translocation experiments were performed essentially as previously described [21,22]. Template plasmids for transcription were linearized with AflII. The mRNA for SytII-model proteins did not contain an in-frame termination codon and ended at Arg^{200}. When translated in vitro, the nascent chain formed the ribosome nascent chain complex (RNC). [^{35}S]-Labeled proteins were detected with a bioimage analyzer (Fuji, BAS1800). Translocation percent was calculated using the following formula: [diglycosylated form] × 100/([monoglycosylated form] + [diglycosylated form]).

For the translocation chase experiments, translation reactions were performed in the presence of SAv (0.5 mg/ml) for 60 min and chain elongation was terminated with 2 mM CHX. LA (0.2 μg/μl in final concentration) was then added and the solution was incubated for 10 min at 30 °C. After incubation, biotin was added at a concentration of 2 mM to induce translocation of the N-terminal portion of the model proteins and aliquots were sampled at the indicated times.

For the targeting experiment, the translocation reactions were performed at 30 °C for 40 min in the presence of RM and terminated with 2 mM CHX. Then, a 40-fold volume of high salt buffer (500 mM potassium acetate and 10 mM Hepes–KOH [pH 7.4]) was added and ultracentrifuged at 100,000*g* for 10 min. The supernatant was precipitated by 15% trichloroacetic acid. Proteins were analyzed by SDS–PAGE. Samples were dissolved in sample buffer containing RNaseA (1 mg/ml) to remove the tRNA moiety, and incubated for 10 min at 37 °C and for 5 min at 95 °C before SDS–PAGE.

2.5. Binding analysis of LA to RM

One microgram of LA or mLA and one microgram of the bovine serum albumin was incubated in the presence or absence of RM (5.5 μg protein) for 40 min at 30 °C. Then, a 40-fold volume of buffer comprising 110 mM potassium acetate, 0.8 mM magnesium acetate, and 10 mM Hepes–KOH [pH7.4] was added and the mixture was ultracentrifuged at 100,000*g* and 4 °C for 10 min. The supernatant was precipitated with trichloroacetic acid and analyzed by SDS–PAGE. The gel was stained with Coomassie blue R250.

3. Results

3.1. LA inhibited the translocation of the N-terminal domain by SA-I

The integration of SA-I into the membrane can be dissected into three stages: targeting of the nascent chain together with the ribosome to the translocon, initiation of translocation of the N-terminal domain, and movement of the polypeptide chain through the translocon (Fig. 1A). We examined the effect of LA on the integration of model proteins into the RM. To this end, LA and mLA were expressed in *E. coli* and purified to homogeneity (Fig. 1B, lanes 1 and 2). We confirmed the ability of the LA to bind to RM. The purified LA and mLA were incubated in the presence or absence of RM and separated into supernatant and precipitate fractions by ultracentrifugation. The supernatants were analyzed by SDS–PAGE and Coomassie blue staining. Although some portions were lost, significant amounts of LA and mLA were recovered in the supernatant in the absence of RM (Fig. 1B, lanes 4 and 7). The loss of LA was most likely due to nonspecific adsorption to the tube and/or aggregation. When RM was included, no LA was recovered in the supernatant (Fig. 1B, lane 5). In the case of mLA, the same amount of mLA was recovered in the supernatant as in the absence of mLA. These results indicated that the purified LA bound to the RM, but mLA did not.

We then examined the effect of LA on translocation of the N-terminal domain by SA-I using the SBP-SytII models (Fig. 1C). The models comprised the N-terminal 200 residues of synaptotagmin II and the streptavidin-binding peptide tag (SBP-tag). In addition, a 38-residue spacer and the 4-residue lysine cluster were included as indicated (Fig. 1C). The models were expressed in the cell-free translation system supplemented with RM. The translocation behavior of the model proteins was monitored based on the glycosylation status. In the cases of SBP-SytII and SBP-0K-38-SytII, the majority of the products were 4-kDa larger than the product

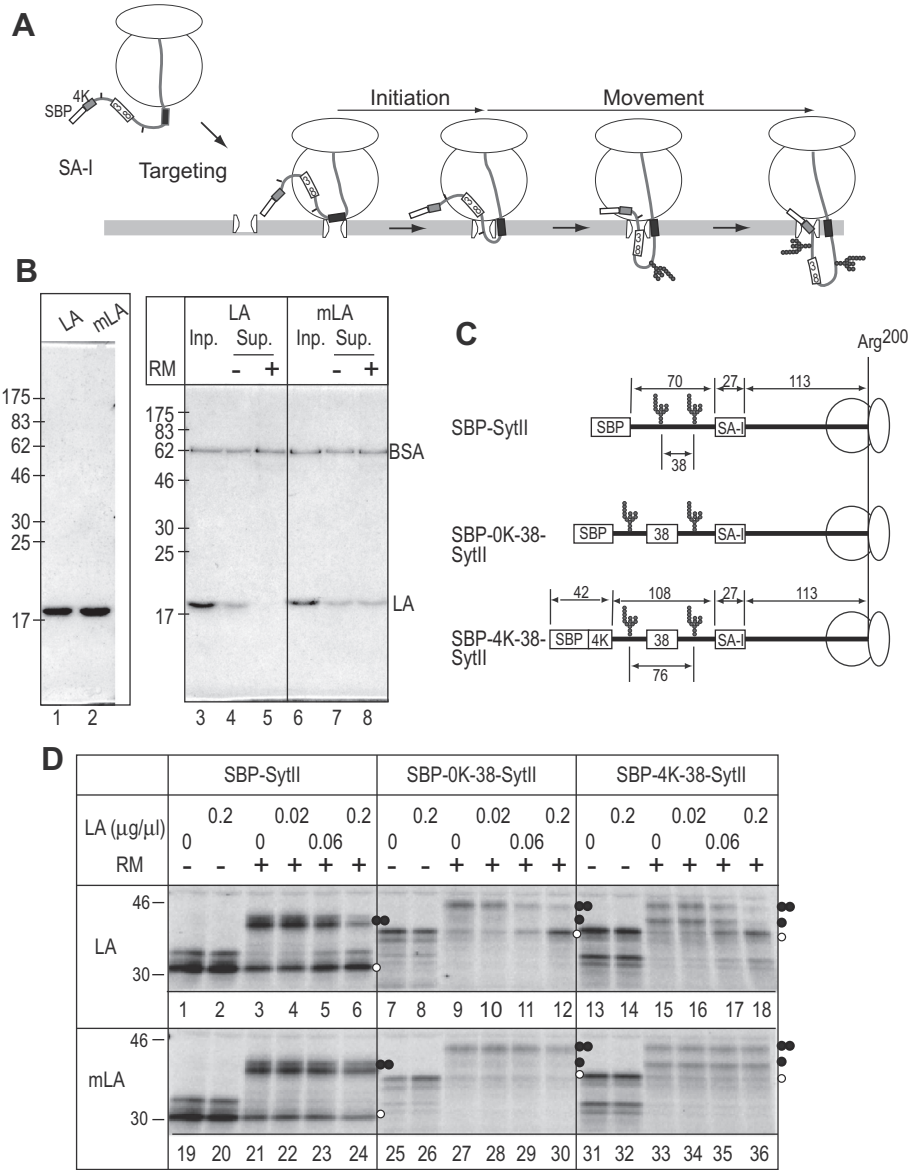

Fig. 1. LA inhibits translocation of the N-terminal domain by SA-I. (A) Stages of translocation of the N-terminal domain by SA-I (black rectangle). First, the nascent chain is targeted to the ER translocon with a ribosome. Second, SA-I initiates translocation of the upstream portion, forming the TM orientation. Third, the N-terminal portion moves through the translocon. Potential glycosylation sites are glycosylated in the lumen (forks). In the presence of a 38-residue spacer, the second glycosylation site can be glycosylated, even when the N-terminal portion is still on the cytoplasmic side of the membrane. (B) Binding of the purified LA and mLA to the RM. One microgram of the purified proteins was subjected to SDS–PAGE and Coomassie blue staining (left panel). LA or mLA and bovine serum albumin were incubated in the presence or absence of RM, and separated to precipitate and supernatant. The supernatants were analyzed by SDS–PAGE and subsequent Coomassie blue staining. Input protein (Inp), proteins in the supernatant fractions obtained in the presence (+) and absence (−) of RM were analyzed (right panel). (C) Model proteins included a backbone of SytII, an N-terminal SBP-tag, and two potential glycosylation sites (forks). Where indicated, a 38-residue spacer between the glycosylation sites and 4 K-cluster after the SBP-tag were included. The residue number of each portion is indicated. The mRNA was truncated at Arg^{200} to make RNC. (D) Effects of LA and mLA on translocation. Model proteins were translated in the absence (−) and presence (+) of RM supplemented with the indicated amounts of LA or mLA. Products were subjected to SDS–PAGE and image analysis. Nonglycosylated (open circles), monoglycosylated (single dots), and diglycosylated (double dots) forms are denoted.

synthesized in the absence of RM, indicating diglycosylation of the product (Fig. 1D, lanes 3 and 9). When LA was included in the translation reactions, the diglycosylated forms were substantially reduced (Fig. 1D, lane 4–6 and 10–12). In the case of SBP-4K-38-SytII, the products were monoglycosylated or diglycosylated and became 2-kDa or 4-kDa larger (Fig. 1D, lane 15), indicating that the 4K-cluster partially arrested the movement of the N-terminal domain and suppressed glycosylation of the upstream site, as indicated in Fig. 1A. In the presence of LA, the level of glycosylated forms decreased (Fig. 1E, lane 16–18). In contrast to the drastic inhibition by LA, no significant inhibition was observed with mLA. These results indicate that LA inhibited the overall translocation of the N-terminal domain.

3.2. LA inhibited targeting to the RM

To examine the effect of LA on the targeting process, RM in the translation mixture was extracted with high salt buffer and sedimented by ultracentrifugation (Fig. 2). As previously shown, when the precursor polypeptide of SytII-200 is targeted to RM, it is resistant to high salt extraction [23]. Diglycosylated products synthesized in the absence of LA were recovered in the membrane precipitate (Fig. 2B, lanes 3, 13, and 23). It is reasonable because the diglycosylated forms span the membrane. On the other hand, a substantial fraction of the nonglycosylated form synthesized in the presence of LA was recovered in the supernatant fraction (Fig. 2B, lanes 7, 17, and 27), indicating that the nonglycosylated

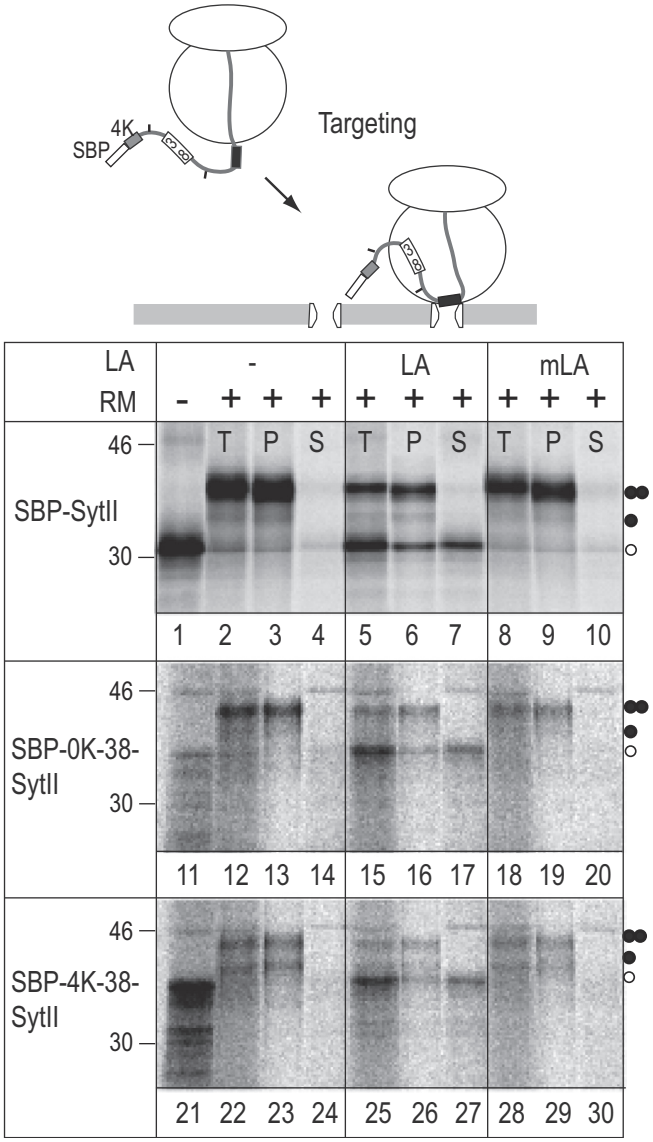

Fig. 2. LA inhibits SA-I targeting to the translocon. Model proteins were translated in the presence of RM and LA for 40 min to form the RNC. Translation was terminated by CHX and the reaction mix was supplemented with a high salt buffer. After the mixture was ultracentrifuged, the supernatant (S) and precipitates (P) were subjected to SDS–PAGE and subsequent image analysis. Total translation products (T) were also analyzed.

form of the model protein was not targeted to the RM and that LA inhibits the targeting step.

3.3. LA inhibited translocation initiation

To examine the effect of LA on translocation initiation after the targeting step, we used the SBP-tag strategy (Fig. 3A). Because the N-terminal SBP-tag is tightly bound to SAv, the SBP-tag on the nascent polypeptide chain is trapped by the SAv on the cytoplasmic side of the RM. Even under this condition, the SA-I is correctly targeted to the translocon [10]. Biotin causes the release of the SBP-tag from the SAv and thus induces movement of the N-terminal region. Because the mRNAs encoding the models were truncated at Arg^{200} of SytII, the C-terminus of the nascent chain is retained in the ribosome as a peptidyl tRNA and the N-terminal region is maintained in the translocation-competent state.

Neither potential site of SBP-SytII was glycosylated in the presence of SAv, but both were glycosylated as soon as the N-domain

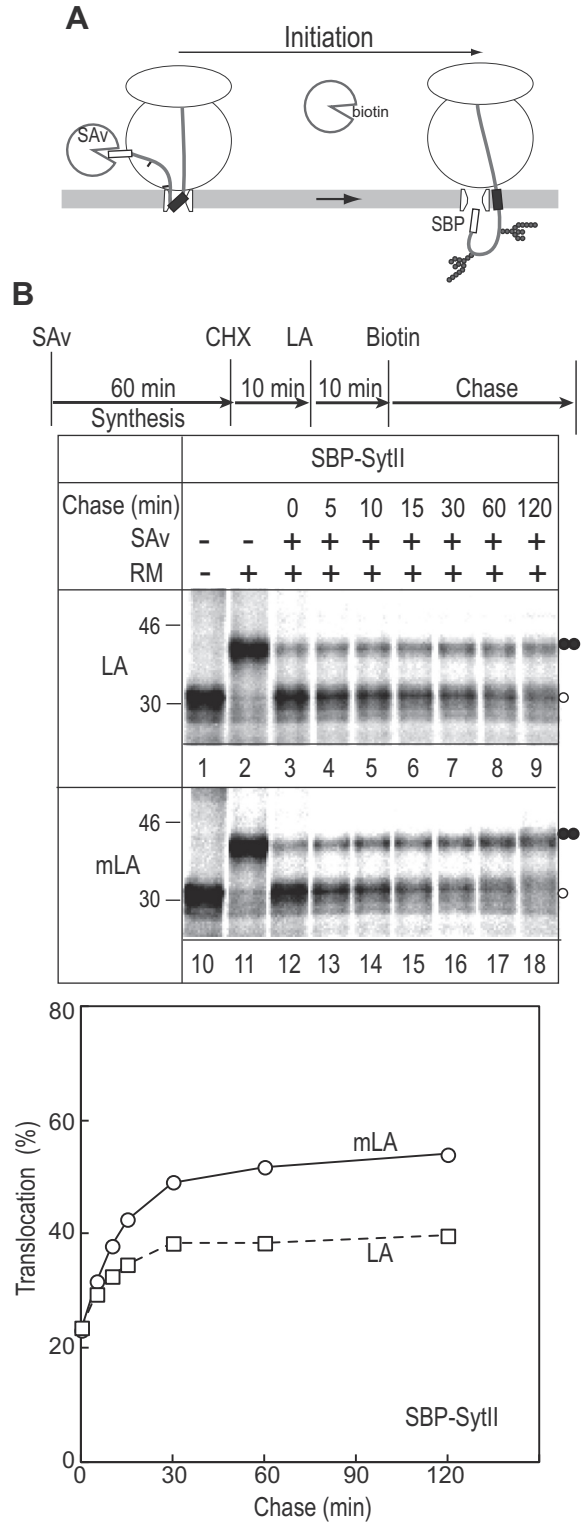

Fig. 3. LA inhibits the translocation initiation by SA-I. (A) SAv binds to the N-terminal SBP-tag and arrests the translocation. Even under these conditions, RNC can bind to the membrane. The addition of biotin releases SAv from the SBP-tag and translocation resumes. For the protein with no spacer, translocation and its initiation were well coupled. SA-I (filled rectangles) and sugar chains (forks) are denoted. (B) Effect of LA on the translocation initiation. Model proteins were translated in the presence of RM and SAv for 60 min, elongation was inhibited with CHX, and then LA was added. Translocation was resumed by the addition of biotin, and aliquots were sampled at the indicated time. Percentages of diglycosylated forms were calculated.

was translocated into the luminal side by biotin. In the context of this model protein, the translocation and glycosylation of the N-terminal domain were tightly coupled with the translocation initiation. Thus, the glycosylation can be used as an indicator of translocation initiation [11]. When translated in the absence of SAv, SBP-SytII was almost completely diglycosylated. In the presence of SAv, however, translocation was arrested and it was mainly nonglycosylated. When mLA was loaded to the RM after the translation reaction, the nonglycosylated form was converted to the diglycosylated form during the biotin chase (Fig. 3B, lower panel, lanes 13–18). In contrast, in the presence of LA, conversion to the diglycosylated form was largely suppressed (Fig. 3B, upper panel, lanes 4–9), indicating that LA inhibited the translocation initiation even after the targeting was completed.

3.4. LA inhibited movement of the polypeptide chain through translocon

We next examined the effect of LA on movement of the hydrophilic segment after the initiation step. To uncouple the movement step from the initiation step, a 38-residue spacer was incorporated between the glycosylation sites (Fig. 4A). In the presence of the spacer, the second site can be glycosylated even when the N-terminal SBP-tag is trapped on the cytoplasmic side. To examine the

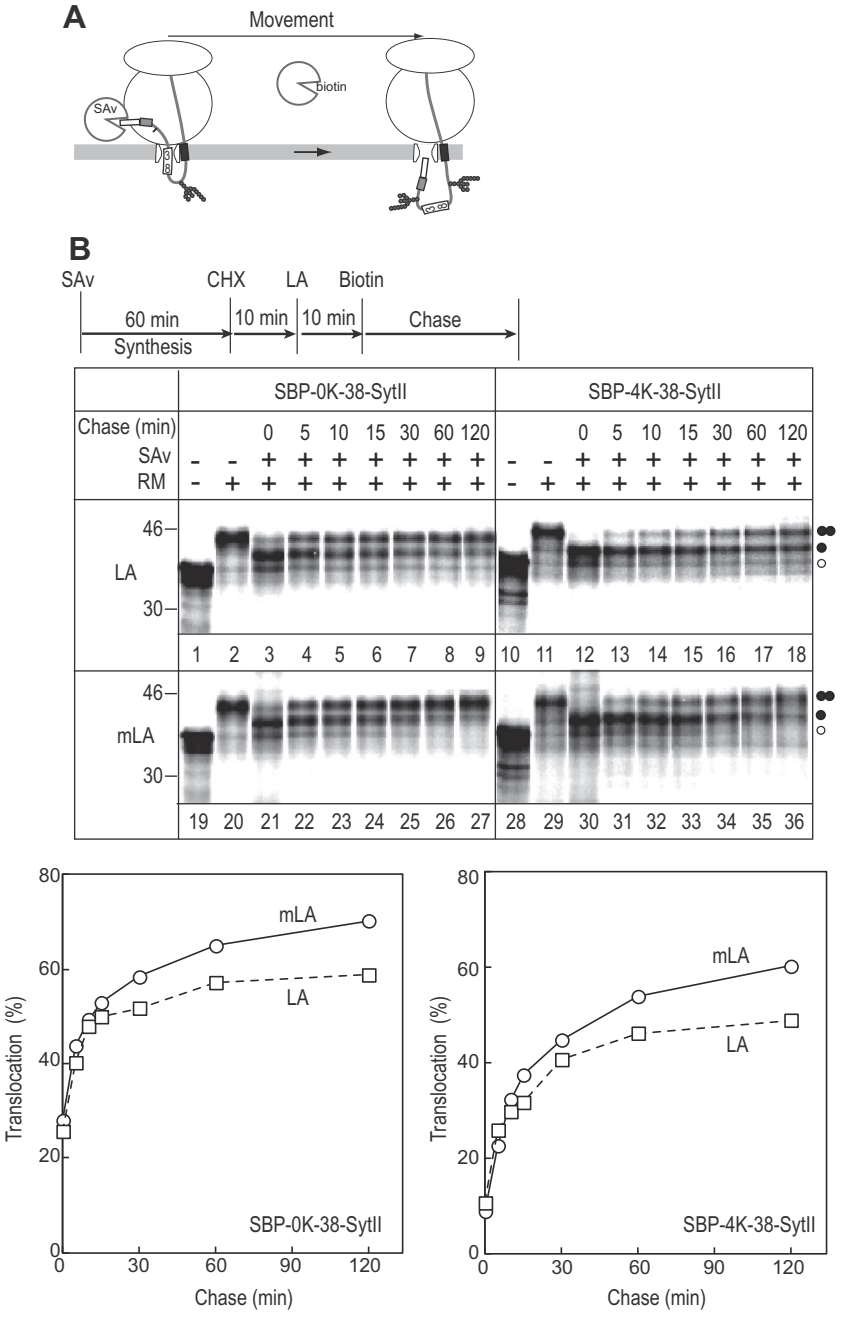

Fig. 4. LA inhibits the movement of hydrophilic polypeptides through the translocon. (A) Even when SAv binds to the N-terminal SBP-tag and arrests translocation of the N-terminus, the 38-residue spacer allows the hydrophilic segment to span the membrane and thus the second glycosylation site to be glycosylated in the ER lumen. Biotin releases SAv from the SBP-tag and resumes movement of the polypeptide through translocon. SA-I (filled rectangles) and sugar chains (forks) are denoted. Using this system, LA can be added after the initiation stage. (B) Effect of LA on the movement. Model proteins were translated in the presence of RM and SAv for 60 min, and LA was added. Translocation was resumed by the addition of biotin and aliquots were sampled at the indicated time. Percentages of diglycosylated forms were calculated.

movement efficiency of positive charges, the 4K-cluster was included in the N-terminal portion (Fig. 1A). When translated in the absence of SAv, the model proteins were almost completely diglycosylated by RM. When synthesized in the presence of SAv, the model proteins were monoglycosylated (Fig. 4B, lanes 3 and 12), indicating that translocation movement was arrested while the chains were spanning the membrane. LA was loaded into the RM in this situation. In the absence of LA, the translocation is resumed by biotin and the monoglycosylated form was converted to the diglycosylated form. When mLA was loaded instead of LA, the monoglycosylated form was converted to the diglycosylated form during the biotin chase (Fig. 4B, lanes 22–27). The conversion was inhibited by LA (Fig. 4B, lanes 4–9), but not by mLA. Movement of the N-terminal portion through the translocon was inhibited by LA, even after translocation had been initiated.

Positively-charged residues on the nascent chain electrostatically interact with membrane lipids or translocon components and suppress polypeptide chain movement [17,20]. If PS functions as an interaction partner, LA is expected to promote the translocation of the positive-charge cluster. When LA was included in the movement chase reaction of the SBP-4K-38-SytII model, translocation was not accelerated, but rather inhibited (Fig. 4B, lanes 13–18). This finding suggests that the negatively-charged head group of PS is not a determinant of translocation-suppression by the positive charges and that PS has a role in polypeptide movement of hydrophilic residues regardless of the presence of positively-charged residues.

4. Discussion

The findings of the present study demonstrated that the PS-binding protein LA inhibited multiple stages of SA-I mediated translocation; targeting, translocation initiation, and translocation movement. PS is likely involved in the general action of the translocon.

What is the primary action of LA on translocon function? First, it is possible that when LA binds PS molecules in the lipid bilayer, PS cannot diffuse into the close proximity of the translocon by steric hindrance, resulting in the sequestration of PS from the channel. PS specifically binds the translocon and actively regulates the protein translocation functions. In fact, the regulation of membrane protein function by membrane lipids has been reported, e.g., one sphingomyelin species specifically binds to the TM domain of p24 on a COPI vesicle and modulates the oligomeric state of the protein and protein transport in cells. Membrane lipids can act as cofactors to regulate the function of TM proteins [24]. We previously demonstrated that cholesterol has pleiotropic effects on translocon function [20]; cholesterol inhibits the translocation of positive charges through the translocon. Cholesterol also enhances the translocation of marginally hydrophobic segments. Membrane lipids likely influence translocon function. PS might be required to maintain proper conformational transitions or translocon dynamics, such as accepting the incoming SRP-RNC complex, signal recognition, gating of the lateral exit site for the membrane lipid, and open-close motion of the plug for hydrophilic polypeptide movement. Second, translocon function might be modulated by generic bilayer properties of the PS-containing ER membrane other than electrical surface charges. Third, with regard to the targeting stage, the LA bound to the RM surface might simply interfere with the access of the SRP-RNC complex to the translocon. Despite this possibility, LA inhibited translocation initiation and movement after the targeting was completed, indicating that the action of LA is not bulk coverage of the membrane vesicle.

With regard to the translocation suppression by positive charges, we initially expected that sequestration of PS by LA would suppress the action of positively-charged residues and enhance their movement. The movement of positively-charged residues, however, was inhibited by LA. PS might be a key factor to maintain efficient translocation of the hydrophilic segment. It is likely that the major interaction partner of the positive charges is not the negative charges of PS. It remains possible, however, that although LA enhances the positive-charge movement, the inhibition effect of LA on the translocon dominates over the enhancing effect.

Negatively-charged phospholipids other than PS are reported to be involved in various stages of protein translocation. Phosphatidylethanolamine is a determinant of the membrane topology of multispanning membrane proteins, such as lactose permease in *E. coli* [25]. Also, negatively-charged phospholipids are essential for TM helix formation of synthetic prePhoE signal peptide analogs and interact with N-terminal positively-charged residues [26]. The LA and SBP-tag system used in this study could become powerful tools for investigating the effects of membrane lipids on translocon function.

Acknowledgments

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References

[1] E. Hartmann, T. Sommer, S. Prehn, D. Gorlich, S. Jentsch, T.A. Rapoport, Evolutionary conservation of components of the protein translocation complex, Nature 367 (1994) 654–657.
[2] A.E. Johnson, M.A. van Waes, The translocon: a dynamic gateway at the ER membrane, Annu. Rev. Cell Dev. Biol. 15 (1999) 799–842.
[3] E. Miyazaki, M. Sakaguchi, S. Wakabayashi, M. Shigekawa, K. Mihara, NHE6 protein possesses a signal peptide destined for endoplasmic reticulum membrane and localizes in secretory organelles of the cell, J. Biol. Chem. 276 (2001) 49221–49227.
[4] S. Iwashita, M. Tsuchida, M. Tsukuda, Y. Yamashita, Y. Emi, Y. Kida, M. Komori, Y. Kashiwayama, T. Imanaka, M. Sakaguchi, Multiple organelle-targeting signals in the N-terminal portion of peroxisomal membrane protein PMP70, J. Biochem. 147 (2010) 581–590.
[5] T.A. Rapoport, Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes, Nature 450 (2007) 663–669.
[6] T. Tsukazaki, H. Mori, S. Fukai, R. Ishitani, T. Mori, N. Dohmae, A. Perederina, Y. Sugita, D.G. Vassylyev, K. Ito, O. Nureki, Conformational transition of Sec machinery inferred from bacterial SecYE structures, Nature 455 (2008) 988–991.
[7] M. Sakaguchi, Eukaryotic protein secretion, Curr. Opin. Biotechnol. 8 (1997) 595–601.
[8] V. Goder, M. Spiess, Topogenesis of membrane proteins: determinants and dynamics, FEBS Lett. 504 (2001) 87–93.
[9] Y. Kida, F. Morimoto, K. Mihara, M. Sakaguchi, Function of positive charges following signal-anchor sequences during translocation of the N-terminal domain, J. Biol. Chem. 281 (2006) 1152–1158.
[10] Y. Kida, F. Morimoto, M. Sakaguchi, Signal anchor sequence provides motive force for polypeptide chain translocation through the endoplasmic reticulum membrane, J. Biol. Chem. 284 (2009) 2861–2866.
[11] Y. Kida, C. Kume, M. Hirano, M. Sakaguchi, Environmental transition of signal-anchor sequences during membrane insertion via the endoplasmic reticulum translocon, Mol. Biol. Cell 21 (2010) 418–429.
[12] T. Kuroiwa, M. Sakaguchi, K. Mihara, T. Omura, Structural requirements for interruption of protein translocation across rough endoplasmic reticulum membrane, J. Biochem. 108 (1990) 829–834.
[13] T. Kuroiwa, M. Sakaguchi, K. Mihara, T. Omura, Systematic analysis of stop-transfer sequence for microsomal membrane, J. Biol. Chem. 266 (1991) 9251–9255.
[14] T. Hessa, H. Kim, K. Bihlmaier, C. Lundin, J. Boekel, H. Andersson, I. Nilsson, S.H. White, G. von Heijne, Recognition of transmembrane helices by the endoplasmic reticulum translocon, Nature 433 (2005) 377–381.
[15] H. Fujita, Y. Kida, M. Hagiwara, F. Morimoto, M. Sakaguchi, Positive charges of translocating polypeptide chain retrieve an upstream marginal hydrophobic segment from the endoplasmic reticulum lumen to the translocon, Mol. Biol. Cell 21 (2010) 2045–2056.

[16] M. Yamagishi, H. Fujita, F. Morimoto, Y. Kida, M. Sakaguchi, A sugar chain at a specific position in the nascent polypeptide chain induces forward movement during translocation through the translocon, J. Biochem. 149 (2011) 591–600.

[17] H. Fujita, M. Yamagishi, Y. Kida, M. Sakaguchi, Positive charges on the translocating polypeptide chain arrest movement through the translocon, J. Cell Sci. 124 (2011) 4184–4193.

[18] T. Yeung, G.E. Gilbert, J. Shi, J. Silvius, A. Kapus, S. Grinstein, Membrane phosphatidylserine regulates surface charge and protein localization, Science 319 (2008) 210–213.

[19] Y. Kida, F. Morimoto, M. Sakaguchi, Two translocating hydrophilic segments of a nascent chain span the ER membrane during multispanning protein topogenesis, J. Cell Biol. 179 (2007) 1441–1452.

[20] H. Yamamoto, H. Fujita, Y. Kida, M. Sakaguchi, Pleiotropic effects of membrane cholesterol upon translocation of protein across the endoplasmic reticulum membrane, Biochemistry 51 (2012) 3596–3605.

[21] M. Sakaguchi, N. Hachiya, K. Mihara, T. Omura, Mitochondrial porin can be translocated across both endoplasmic reticulum and mitochondrial membranes, J. Biochem. 112 (1992) 243–248.

[22] M. Takahara, H. Sakaue, Y. Onishi, M. Yamagishi, Y. Kida, M. Sakaguchi, Tail-extension following the termination codon is critical for release of the nascent chain from membrane-bound ribosomes in a reticulocyte lysate cell-free system, Biochem. Biophys. Res. Commun. 430 (2013) 567–572.

[23] Y. Kida, M. Sakaguchi, M. Fukuda, K. Mikoshiba, K. Mihara, Membrane topogenesis of a type I signal-anchor protein, mouse synaptotagmin II, on the endoplasmic reticulum, J. Cell Biol. 150 (2000) 719–730.

[24] F.X. Contreras, A.M. Ernst, P. Haberkant, P. Bjorkholm, E. Lindahl, B. Gonen, C. Tischer, A. Elofsson, G. von Heijne, C. Thiele, R. Pepperkok, F. Wieland, B. Brugger, Molecular recognition of a single sphingolipid species by a protein's transmembrane domain, Nature 481 (2012) 525–529.

[25] W. Zhang, H.A. Campbell, S.C. King, W. Dowhan, Phospholipids as determinants of membrane protein topology. Phosphatidylethanolamine is required for the proper topological organization of the gamma-aminobutyric acid permease (GabP) of *Escherichia coli*, J. Biol. Chem. 280 (2005) 26032–26038.

[26] R.C. Keller, J.A. Killian, B. de Kruijff, Anionic phospholipids are essential for alpha-helix formation of the signal peptide of prePhoE upon interaction with phospholipid vesicles, Biochemistry 31 (1992) 1672–1677.