이학석사학위논문

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Investigation on the Role of Sec62 in Membrane Protein Biogenesis

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Investigation on the Role of Sec62 in Membrane Protein Biogenesis

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ABSTRACT

Membrane and secretory proteins are synthesized by ribosomes in cytoplasm and targeted to the ER membrane via two pathways; co-translational translocation pathway or post-translational translocation pathway.

Membrane proteins are targeted by either the co- or the post-translational translocation pathway and are inserted into the ER membrane by the Sec61 protein conducting channel, which provide a polar passage for polypeptides. During the translocation of polypeptides, Sec61 recognizes a putative transmembrane (TM) segment of about ~20 hydrophobic amino acids and inserts it to the ER membrane through a lateral gate formed by the second and seventh TM segments of the Sec61 channel. Particular for the post-translational translocation pathway is the Sec63/62 protein complex associated with the Sec61 channel. Here, Sec62 acts as a receptor for incoming polypeptides and is found in close proximity to the lateral gate of Sec61. Recently, Reithinger et al (2013) unraveled a novel function of Sec62 in orienting single spanning membrane proteins with a marginally hydrophobic TM segment. However, the function of Sec62 in integration of multi-spanning membrane proteins remained elusive.

To investigate the function of Sec62 in integration of multi-spanning membrane proteins, mutations were introduced into several domains of Sec62. When a set of model membrane proteins was expressed in Sec62 mutant strains, the translocation of marginally hydrophobic TM segment of multi spanning membrane proteins into the ER membrane was specifically decreased in Sec62 N-terminal mutant strains. In addition, the interaction between Sec63 and Sec62 was lost in these mutants. Also, reduction of C-terminal translocation of multi spanning membrane proteins was found in Sec62 C-terminal mutant strains as well. Taken together, it is concluded that N-terminus of Sec62 functions by associating Sec62 to the Sec62/63 complex, where Sec62 mediates C-terminal translocation of multi

spanning membrane proteins and C-terminus of Sec62 also involves in the process in the ER of *Saccharomyces cerevisiae*.

KEY WORDS

: Yeast, *saccharomyces cerevisiae*, Sec61, Sec62, translocation, membrane protein, Endoplasmic reticulum, membrane topology

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Transmembrane, TM; Endoglucosidase H, Endo H; ER, endoplasmic reticulum; Wild type, WT; Hydrophobic segment, H-segment;

I. INTRODUCTION

In eukaryotic cells, Endoplasmic reticulum (ER) functions as a sorting center for membrane and secretory proteins mediating transport of membrane proteins to the membranes of subcellular organelles such as ER, Golgi and plasma membrane, or in case of secretory proteins, to the extracellular space (1). In addition, membrane proteins are folded in the ER membrane prior to transport to the destined organelle. Therefore, to understand membrane and secretory protein biogenesis in the cell, it is necessary to answer how membrane and secretory proteins are properly targeted to and correctly folded in the ER membrane.

Protein targeting to the Endoplasmic reticulum

Membrane and secretory proteins are synthesized by ribosomes in cytoplasm and targeted to the ER membrane via two pathways; the co-translational translocation pathway or the post-translational translocation pathway. (2). In the co-translational translocation pathway (Fig. 1. Upper figure), proteins are translated by cytosolic ribosomes. As an N-terminal ER-targeting signal emerges from the translating ribosome, it is recognized by signal recognition particle (SRP) (3-5). SRP recognizes proteins possessing a relatively hydrophobic signal sequence (SS) or a TM segment (6). Upon recognition of SS, SRP arrests translation transiently and targets ribosome and nascent polypeptide chain complex (RNC) to the ER membrane by interaction between SRP and SRP receptor (SR), in the ER membrane (7,8) After RNC complex binds to the main protein conducting channel called Sec61, SRP is released from the RNC complex and translation is resumed (2). The Sec61 complex is a heterotrimeric protein complex consisting of Sec61/Sec61α, Sbh1/Sec61β and Sss1/Sec61γ in yeast/human, respectively (2). Being the main subunit of the translocon, Sec61 has 10 transmembrane (TM) segments and forms a polar pore for passing polypeptide chains (Fig. 2) (9,10).

Sec61 also recognizes hydrophobic TM segments composed of about 20 amino acids in a translating polypeptide and releases it through the lateral gate formed by the second and the seventh TM segments (10-12). During translocation of a polypeptide, oligosaccharyltransferase (OST) complex recognizes a consensus sequence of N-X (any proteins except proline) - S/T and attaches oligosaccharide on the asparagine residue (13). This N-linked glycosylation reaction allows us to assess whether polypeptide chain has entered the ER lumen. By taking advantage of this, glycosylation sites are engineered into proteins to monitor their translocation status throughout this study.

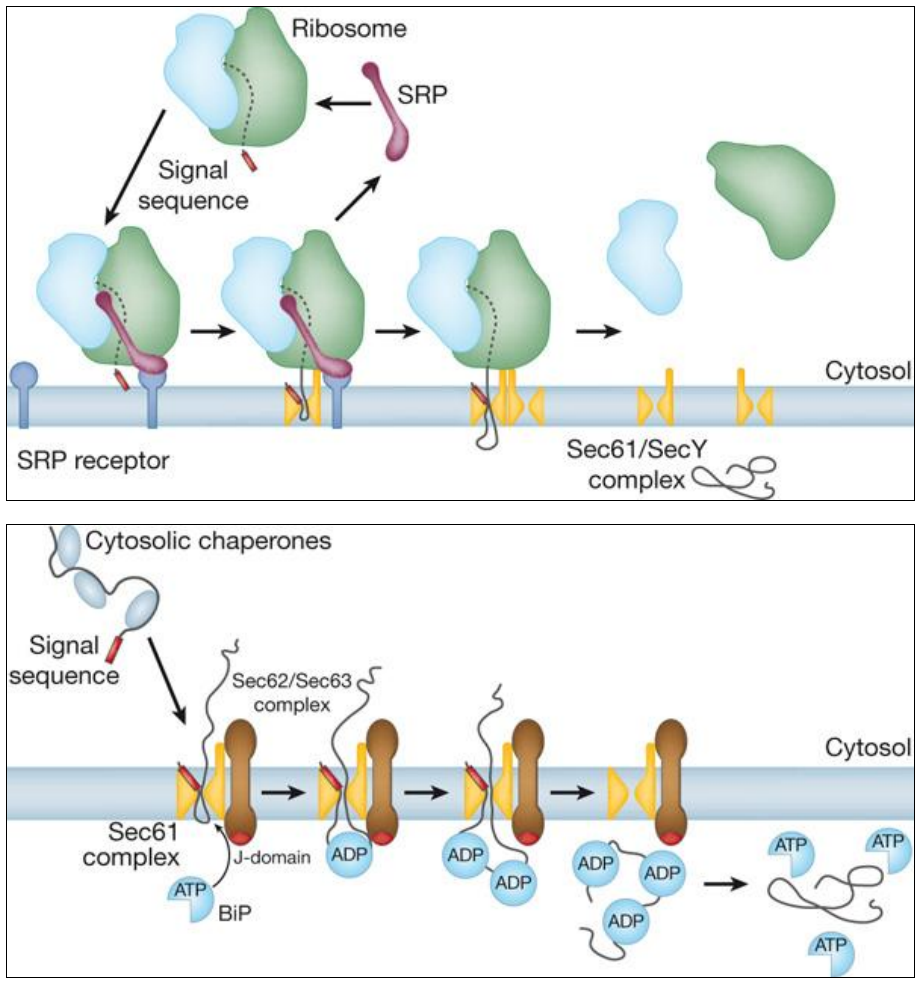

Figure 1. Protein targeting pathways to the ER. The co-translational translocation pathway (upper figure) and the post-translational translocation pathway. (bottom figure). *Nature* 450, 663-669 (29 November 2007) | doi:10.1038/nature06384

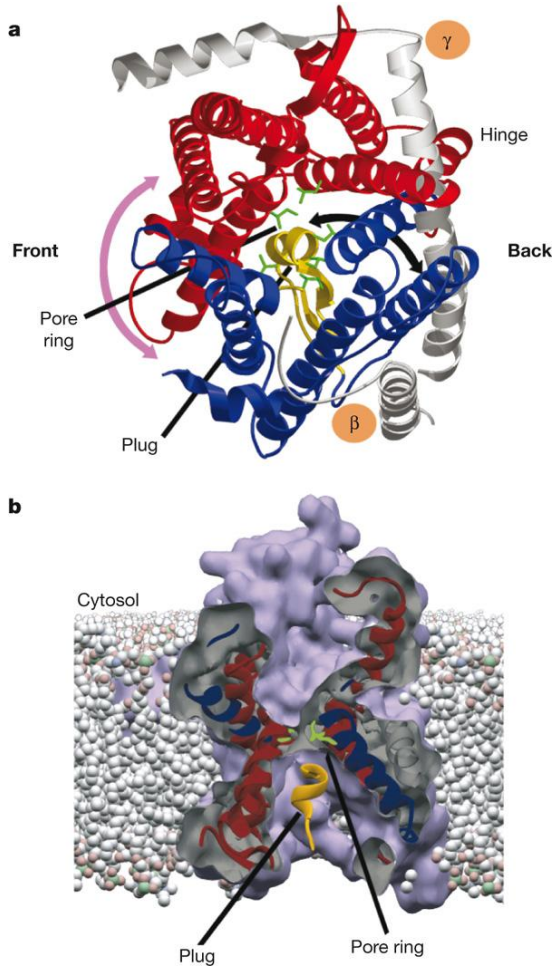

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Figure 2. Sec61 translocation channel. *Nature* 450, 663-669 (29 November 2007) | doi:10.1038/nature06384x. Top figure is Sec61 viewed from the top. The lateral gate opens up in directions indicated with the purpled arrow. Bottom figure shows the cross-sectional view of Sec61.

In the post-translational translocation pathway (Fig. 1. Bottom figure), polypeptides are delivered to the ER membrane after completion of translation (2). Chaperone proteins bind to translated polypeptides to prevent aggregation of hydrophobic patches in the soluble environment of cytoplasm (14). Then, the polypeptides are targeted to the ER membrane by an unknown mechanism. Sec62, a subunit of Sec62/63 complex, might function as a receptor for polypeptides during the process (15,16). Sec61 complex is associated with the Sec63/62 complex (15,17,18). Sec63/62 complex consists of Sec62, 63, 71 and 72, with Sec62 and Sec63 being essential proteins (19,20). When Sec62 is impaired, targeting of secretory proteins with moderately hydrophobic SS are defective (6,21), it is believed that post-translational translocation pathway is utilized by those proteins (6). It has been known that the J-domain in the second loop of Sec63 interacts with Kar2/BiP in yeast/human, respectively, a chaperone protein which works to pull translocating polypeptides into the lumen of the ER in an ATP dependent manner (22-25).

Identification of Sec62

Sec62 was identified from an early study that screened for cells defective in cell surface growth and protein secretion (26). These cells with impaired Sec62 function showed accumulation of secretory proteins in the cytoplasm (21). Subsequent studies found this protein is a double spanning ER membrane protein of 274 amino acids in length and 32-kDa (Fig 3A) (27). Both its N- and C-terminus face the cytoplasmic side (27). A cluster of positively charged residues in the N-terminus of Sec62 was shown to interact with negatively charged amino acids in the C-terminal acidic domain of Sec63, and this interaction was shown to be phosphorylation dependent and important for cell viability (15,28,29). The C-terminus of Sec62 was reported to function on recognizing SS and binding to the

Sec complex (15). In addition, when the region following second TM segment of Sec62 was deleted, the yeast strain was no longer viable (15). A previous cross-linking study showed that Sec62 is located near the translocating polypeptide and the lateral gate of Sec61 to act on insertion and translocation of polypeptides (11,30).

Recently, Reithinger et al. (2013) investigated the role of Sec62 using model single spanning membrane proteins in *sec62-1*, an N-terminal mutant strain (31). In their study, they showed that the C-terminal translocation of signal anchor proteins was specifically reduced in sec62 mutant strains, whereas proteins with their C-terminus already in the cytoplasm were not affected. It implied that Sec62 is involved in specifically translocating C-terminus of single spanning membrane proteins.

To further investigate in greater depth the role of Sec62 we, 1) produced Sec62 mutants of different domains (N-terminus, C-terminus and the TM domains), and using 2) various model membrane proteins differing in the number of TM segments, flanking charges, and hydrophobicity of TM segments, we tested their translocation of the C-terminus within our various Sec62 mutant strains. We found that in case of Sec62 with mutations residing in the N-terminus, translocation of the C-terminus of a subset of our multi spanning membrane proteins to the ER membrane was significantly reduced. Interestingly, we found that not only mutations in the N-terminus, but also a mutation in the C-terminus of Sec62 showed this phenotype. In sum, this study has demonstrated that Sec62 mediates C-terminal translocation of multi spanning membrane proteins.

II. MATERIALS AND METHOD

II. A. Yeast strains

JRY4 strain (*MATα, sec62Δ::HIS3, ade2, can1, his3, leu2, trp1, ura3*) was constructed by homologous recombination (HR) and plasmid shuffling. SEC62 with 1kb upstream region was amplified with 5'-CGATAAGCTTGATATCGAATTCCTGCAGGGGTCTATCTAGGATAC-3' (RP25) and 5'-CGATAAGCTTGATATC5'-GGCGGCCGCTCTAGAACTAGTGGATCCGAGGTTACAATATAGAAGG-3' (RP26) from W303-1α strain (*MATα, ade2, can1, his3, leu2, trp1, ura3)*. SEC62+1kb was cloned into pRS415 or pRS416 vector by HR (recombination sequences are underlined.). The pRS416 carrying SEC62+1kb was transformed into W303-1α. The genomic SEC62 gene of transformant was substituted with HIS selection marker amplified with primers 5'-GGGAGAAGAGTGGGCTTTTATAATTGCAGTTGAATGCAGTCACAGGAAA CAGCTATGACC-3' (RP27) and 5'-GAAGGTTTATACAGTAGAGCTATACAGGATAATGGAAGTGTTGTAAAACG ACGGCCAGT-3' (RP28) from pCgH vector by HR (32). Where indicated, the transformants were transformed once again with pRS415 vector carrying one of the mutant versions of sec62 and grown on FOA plate 3 times at 30 degree.

JRY6 strain (*MATα, SEC63::HA-KanM, sec62Δ::HIS3, ade2, can1, his3, leu2, trp1, ura3)* was constructed by taking the same approach as JRY4 except conjugating genomic SEC63 gene with 3 copies of HA epitope and selection marker for kanamycin cassette. Cassette of HA and kanamycin selection marker was amplified with the primers 5'-CGATACGGATACAGAAGCTGAAGATGATGAATCACCAGAACGGATCCCC GGGTTAATTAA-3' (RP70) and 5'-

CTAAGAGCTAAAATGAAAAACTATACTAATCACTTATATCGAATTCGAGC TCGTTTAAAC-3' (RP71) from pFA6a-3HA-kanMX6 plasmid (33). Amplified PCR product was transformed into W303-1α by HR. All the following procedures were carried out as stated for the construction of JRY4.

II. B. Primer design

Forward and reverse primers contain either 5' or 3' 17-21 nucleotides complementing either N- or C-terminus of the target gene for amplification and 30 nucleotides for HR with proper vector.

II. C. Construction of plasmids

Plasmids were constructed by HR as previously described (29). Amplified genes were transformed into W303-1α with a targeted vector. Site-directed mutagenesis was performed following manufacturer's protocol to introduce mutations at specific sites (Takara, Japan).

II. D. Western blotting

Cells were grown in 5ml medium at 30℃ overnight. Cells were harvested by centrifugation at 3200RPM for 5min and washed once with ddH_2O. Cell pellets were solubilized in 100μl SDS-PAGE sample buffer. Cell debris was spun down at 14000RPM for 5min. The supernatants were incubated at 56℃ for 15min and subjected to SDS-PAGE and western blotting.

II. E. Pulse labeling and autoradiography

JRY4 Sec62 WT and mutant strains expressing model or natural membrane proteins were grown in 10ml medium at 30℃, overnight. Cells were diluted to 0.1 A_{600} and grown until the point where A_{600} reached between 0.2 to 1.0. Then, 1.5

A_{600} unit of each strains was harvested by centrifugation at 3200RPM for 5min and washed twice with 1ml of –Met media. Cells were incubated in 1ml -Met media at 30℃ for 15min to deprive residual methionine. Cells were centrifuged down after the starvation. Cell pellets were resuspended in 150µl -Met media and labeled with 5µl [^{35}S]Met (50 µCi/1.5 A_{600} units of cells) at 30℃ for 5min. Labeling was paused with 750µl Buffer A (20mM Tris-HCl, pH 7.5, 20mM sodium azide) and the cells were pelleted down at 13000RPM for 5min. The supernatants were discarded and the cell pellets were solubilized in 100µl of Lysis buffer (20mM Tris-HCl, pH 7.5, 1% SDS, 1mM DTT, 1mM PMSF, protease inhibitor cocktail). The lysed samples were transferred to ice-cold 1.5ml eppendorf tubes with glass beads and vortexed for 5min. To remove cell debris, samples were spun down and only the supernatant was moved to IP mix (500µl IP buffer, 2 µl anti-HA antibody, 40% slurry Protein G-agarose). Proteins were immunoprecipitated overnight and washed with 1 ml of IP buffer (15mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Triton X-100, 150mM NaCl) twice, Urea buffer (2M Urea, 200mM NaCl, Tris-HCl, pH 7.5, 1% Triton X-100), Con A buffer (500mM NaCl, 10mM Tris-HCl, pH 7.5, 1% Triton X-100) and Buffer C (50mM NaCl, 20mM Tris-HCl, pH 7.5). Washed protein G-agarose beads (Roche Applied Science, Germany) were incubated with SDS sample buffer at 65℃ for 15min and centrifuged at 13000RPM for 5min. The supernatants were subjected to SDS-PAGE and visualized and quantified with Fujifix bio-imaging analyzer IPR BAS-1500 and Multi-gauge, version 3.0 (Fuji, Japan).

For pulse-chase experiment, 5 µl of [^{35}S]Met was added per 1.5 A_{600} unit cell. Labeling was stopped by adding 200mM cold metionine. At each time points, 200 µl labeling reaction was transferred to 750 µl Buffer A solution. The following steps were carried out as described above.

II. F. Co-immunoprecipitation

JRY6 cells expressing FLAG-tagged WT or mutant *sec62* were grown at 30℃ in a selective medium until A_{600} reached 1.0. 10 A_{600} unit cells were harvested by centrifugation at 3000 *g* and washed with dH_2O. Cell pellets were solubilized with 200μl lysis buffer (20mM Tris-HCl, pH 7.5, 10mM EDTA, pH 7.5, 20mM NaCl, 300mM Sorbitol, 1mM PMSF, protease inhibitor cocktail) and vortexed with prewashed glass beads for 10 min at 4℃. The lysates were centrifuged briefly to remove cell debris, and membrane fractions were harvested by centrifugation at 28000 g for 30 min at 4℃. The pellets were solubilized in 200μl of Lysis buffer (20mM Tris-HCl, 1% Triton X-100, 10% glycerol, 100mM NaCl, 1mM PMSF, protease inhibitor cocktail) and incubated on ice for 30 min. 20μl of the lysates were aliquoted to serve as INPUT (In) samples. 150μl of the lysates were mixed with 500μl of IP buffer A (15mM Tris-HCl, 0.2% Trition X-100, 100mM NaCl), 3μl anti-HA antibody, 25μl of protein G-agarose beads, and rotated overnight at 4℃. The agarose beads were washed once with IP buffer B (15mM Tris-HCl, 0.4% Trition X-100, 100mM NaCl), twice with IP buffer A and once with IP buffer C (15mM Tris-HCl, 100mM NaCl). The beads were heated with 50 μl SDS-PAGE sample buffer at 65℃ for 15 min. The beads were removed by centrifugation and the samples in the supernatant fraction were subsequently analysed by SDS-PAGE and Western blotting.

III. RESULTS

III. A. Preparation of Sec62 mutant strains.

To characterize functional domains of Sec62, a systematic mutagenesis was carried out in different parts of Sec62. The patches of positively charged residues are present in both the N- and C- terminus of Sec62. The cluster of positive charges at the N-terminus is required for the interaction with Sec63 while the role of C-terminal charges remained elusive. The C-terminus of Sec62 was proposed to function on binding to SS of secretory proteins and the Sec complex (15). To further characterize the role of these positively charged clusters, 35-35 RQG, 103-105 KKL or 263-265 KKK were substituted by the negatively charged amino acid, DDD. For the TM domains of Sec62, nothing is known apart from the fact that they are required for anchoring into membrane. To explore a novel role of the TM domain, 157-159 VSI and 185-187 FGI residues were each mutated to a sequence of small and non-hydrophobic residues, AAA. Lastly, the region downstream of the second TM, 218-220 FPN, was changed to AAA as the truncation of Sec62 at this point was lethal. (Fig. 3A)

For preparation of the six Sec62 mutant strains stated above, plasmids containing each sec62 mutant gene were transformed into JRY4. JRY4 is a genomic SEC62 deletion strain, carrying a SEC62 WT gene in a URA vector (Fig. 3C. left panel). Transformed cells were grown on 5'-fluoroorotic acid (5'-FOA) plate for 3 days at 23, 30 and 37 degrees Celsius and the functionality of each mutants was assessed (Fig. 3C. right panel). On 5'-FOA plate, yeast cells harboring URA-born plasmid are lethal. Thus, plasmid shuffling (i.e. losing the URA plasmid and taking up a plasmid with a different marker) in the JRY4 strain is required for the survival on FOA plates (34) . Consequently, cells have only one copy of the gene which is either WT or mutant of Sec62.

All mutant strains, except 218AAA, did not show any severe growth defect

compared to SEC62 WT at all tested temperatures (Fig. 3C. left panel). This indicated that residues 218-220 are crucial for Sec62 function. Subsequent single alanine scanning of this region; F218A, P219A and N220A, showed that only P219A exhibit the severe growth defect at 37℃ (Fig. 3C). This identified P219 as a key residue for Sec62 function, although the underlying molecular mechanism of the residue remains largely elusive.

Figure 3

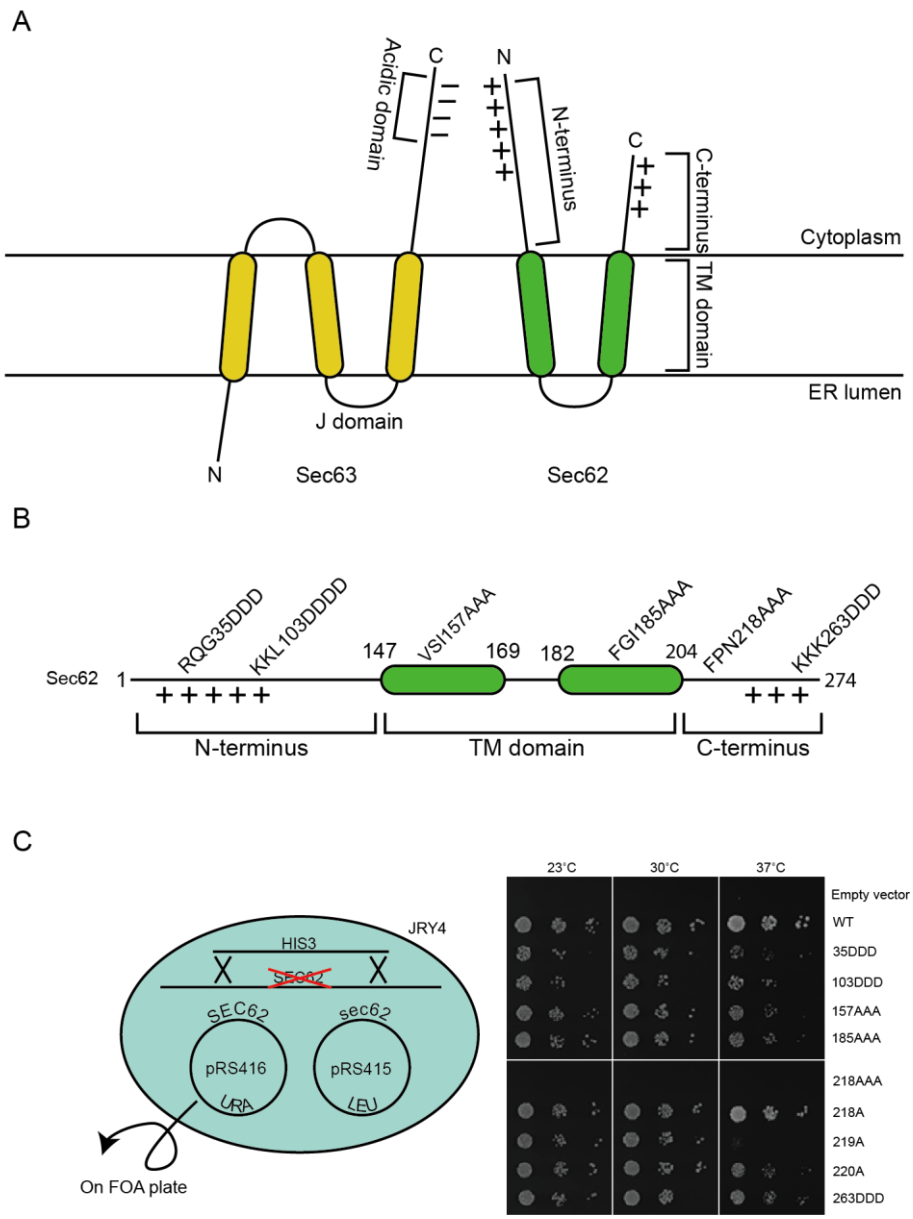

Figure 3. Construction of Sec62 mutant strains. (A) Schematic drawing of Sec63 (left) and Sec62 (right). Yellow and green bars indicate TM segments. '+' and '-' indicate either positively charged or negatively charged amino acids, respectively. N-terminus, C-terminus and TM domains of Sec62 are indicated. J

domain, and loop region between TM2 and TM3 of Sec63 are indicated. Negatively charged amino acids in the acidic domain of the C-terminus of Sec63 interact with positively charged amino acids in the N-terminus of Sec62. **(B)** Locations of each mutation are indicated on Sec62. Green bars indicate TM segments. The start and end of proteins with TM segments are indicated. **(C)** The strategy of construction for Sec62 mutant stains is shown in left. URA-born vector (pRS416) carrying WT Sec62 gene, and LEU-born vector (pRS415) carrying WT Sec62 or mutant Sec62 were transformed into the genomic Sec62 deletion strain. Genomic Sec62 was replaced with HIS3 selection marker by homologous recombination (HR). The transformants were grown on 5'-fluoroorotic acid (5'-FOA) plate to remove the URA-born vector. Growth test of Sec62 mutant strains (right panel). Indicated Sec62 mutant strains were grown on FOA plate for 3 days at 23, 30 or 37℃. 3×10^3 cell was used for first spot and followed by a 10 fold serial dilution.

III. B. Protein translocation assay

To investigate whether the Sec62 mutant strains have defects in translocation of membrane proteins in accordance with the previous studies, a protein translocation assay was carried out. For the protein translocation assay, we used a *E.coli* leader peptidase-based model membrane proteins harboring one, two or three TM segment equipped with multiple glycosylation sites (31,35,36).

For construction of a single spanning model protein, H1, the first and second TM segment of original leader peptidase were substituted by H-segment (hydrophobic segment), a 19 amino acid strech composed of varying number of leucines and alanines for a broad spectrum of hydrophobicity. (Fig. 4A and Table 1). Hydrophobicity (ΔG) represents how much energy is required for a TM segment to insert into a hydrophobic environment. Thus, when a TM segment is hydrophobic, it means that the energy barrier for lipid bilayer insertion is low (hence, a low ΔG value), vice versa. The H-segment acts as both a signal sequence to target polypeptide to the ER membrane, and as a TM segment to anchor the protein in the lipid bilayer. H1 has three glycosylation sites, one in N-terminus and two in C-terminus (Fig. 4A) (36). In the case where the protein is fully translocated into the ER lumen, it gets glycans in all of its glycosylation sites (3G, fully translocated) (Fig. 4B). When H1 is inserted into ER membrane, it can go in two different orientations. Only one N-terminal glycosylation site is modified when the protein is inserted with its N-terminus in the ER lumen (1G, C terminus-in), whereas two C-terminal glycosylation sites are glycosylated if the protein is inserted with its C-terminus in the ER lumen (2G, C terminus-out). When protein remains in the cytoplasm, it is not glycosylated at all (0G, untargeted). Therefore, the glycosylation status of H1 allows not only a direct assessment of targeting efficiency to the ER by comparing the amount of glycosylated (1G, 2G and 3G) and unglycosylated (0G) proteins, but also the orientation H1 proteins in the ER

membrane by comparing the ratio of 1G and 2G forms.

Figure 4

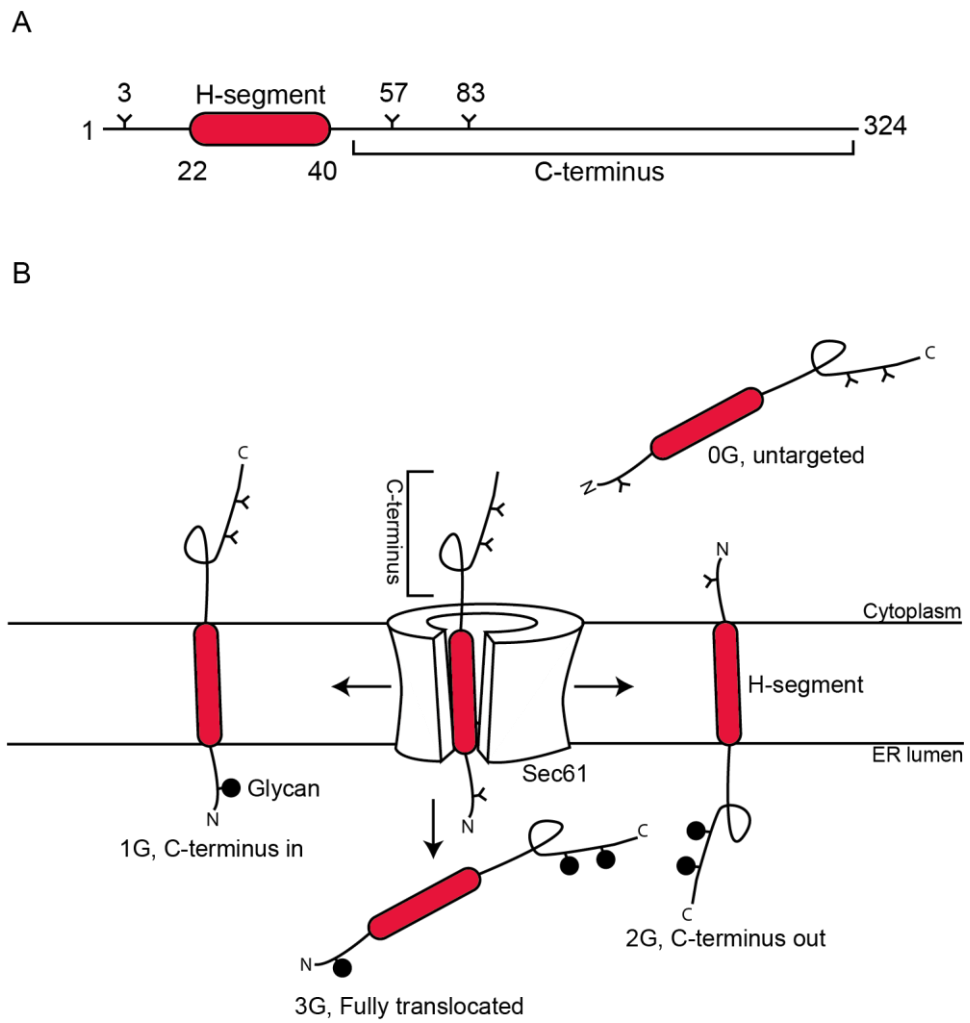

Figure 4. Model single spanning membrane proteins (H1). (A) Schematic drawing of single spanning membrane proteins (H1). Red bar indicates potential test TM segment (H-segment). Sequence and Hydrophobicity (ΔG) of H-segment is indicated in Table 1. Glycosylation sites are indicated in the N-terminus and C-terminus of a protein. Region after H-segment is indicated as 'C-terminus'. Proteins are conjugated with 3 copies of HA epitope in the C-terminus for immunoprecipitation and western blotting. **(B)** Translocation status of H1 proteins. H1 protein is inserted into the ER membrane through the lateral gate of Sec61 or tranlocated into the ER lumen by Sec61. Closed circles indicate glycans. 1G, singly glycosylated; 2G, doubly glycosylated; 3G; triply glycosylated; 0G, non-

glycosylated.

Table 1. Sequence and Hydrophobicity (ΔG) of the test TM segments (H1 ,H2 and H3-segment) in model and natural proteins. ΔG values in kal/mol were predicted by ΔG predictor (http://dgpred.cbr.su.se/) (33). Flanking charged residues of TM segment in model H1, H2 and H3 proteins are indicated. 'EA' indicates the third TM segment of Shr3t (EA), '4A' indicates the third TM segment of Sec63 (4A)

Protein	Name of TM segment	Sequence of Test TM Segment and Flanking Residues	Hydrophobicity (△G)
H1	3L	GGPGAAAALAAAALAAAALAAAAGPGG	-0.006
	4L	GGPGAAAALALAAAAALALAAAAGPGG	-0.490
	5L	GGPGAAAALALAALAALALAAAAGPGG	-0.999
	6L	GGPGAAAALALALALALALAAAAGPGG	-1.540
	7L	GGPGALAALALAALAALALAALAGPGG	-1.76
	10L	GGPGLALALALALALALALALALGPGG	-3.115
H2	P2(0L)	GGPGDKQEGEWPTGLRLSRIGGIGPGG	8.631
	1L	GGPGAAAAAAAAALAAAAAAAAAGPGG	1.060
	K1LG	KKPKAAAAAAAAALAAAAAAAAAGPGG	1.060
	2L	GGPGAAAALAAAAAAAAALAAAAGPGG	0.699
	3L	GGPGAAAALAAAALAAAALAAAAGPGG	-0.006
	5L	GGPGAAAALALAALAALALAAAAGPGG	-0.999
H3	4L	GGPGAAAALALAAAAALALAAAAGPGG	-0.490
	5L	GGPGAAAALALAALAALALAAAAGPGG	-0.999
Shr3t	Original	MFEYCSLGLYVLAICVFLTNVKT	0.179
	EA	MFEYCSLGLYVAAICVFLTNVKT	0.704
Sec63	Original	LLVVCYVALLGLILPYFVSRWWA	-1.606
	4A	LLVVCYVAALGAAAPYFVSRWWA	0.509

III. C. Sec62 N-terminal mutant strains have defects in targeting and translocation of single spanning membrane protein

To screen out Sec62 mutant strains with defects in targeting and/or translocation of single spanning proteins, a set of single spanning membrane proteins of varying hydrophobicity (H1) were expressed in Sec62 mutant strains. In figure 5A, H1-4L and 5L were expressed in Sec62 WT and mutant strains in the presence of radio labeled methionine and immunoprecipitated for SDS-PAGE and autoradiography. To identify non-glycosylated (0G) H1, the proteins were treated with Endoglycosidase H, an enzyme that removes glycans from a peptide. One glycan is about 2kDa, thus it makes proteins to migrate slower in SDS-PAGE, when added. Thus, the bottom bands (Endo H treated) represent non-glycosylated proteins and the other bands above indicate 1G, 2G and 3G form respectively from the bottom. The amount of proteins present in each band were quantified and used to calculate targeting efficiency (Fig 5B) and the relative amount of C_{out} (Fig 5C), respectively.

In figure 5A, glycosylated bands almost disappeared in 35DDD lane of H1-4L proteins. In case of 103DDD with H1-4L, non-targeted form is increased and C-terminal-out form is reduced compared to WT. In other strains, band pattern seems to be similar with WT when screened with H1-4L. Consistent with the screening data with H1-4L, the glycosylated bands were decreased in 35DDD and 103DDD compared to WT in screening H1-5L protein. Again, the rest of mutants did not show much difference compared to WT. The targeting efficiencies of H1-4L and 5L proteins were 63 % and 71 % in WT. (Fig. 5B). But, the targeting efficiency of H1-4L and H1-5L was reduced to 33 % and 50 % in 35DDD strain and to 27 % and 44 % in 103DDD strain, respectively. It indicated that the Sec62 N-terminal mutants, 35DDD and 103DDD, have defects in targeting of single spanning membrane proteins. The rest of the mutant strains displayed similar degrees of targeting efficiency compared to WT., (i.e. These strains are not impaired in targeting of H1-4L/5L proteins.) (Fig 5B). The reduction of the targeting efficiency

in 35DDD and 103DDD was found in H1 proteins harboring 3 to 6 leucines. H1-3L to 6L proteins are said to be marginally hydrophobic compared to less or more hydrophobic H1 variants (Fig. 5B). As hydrophobicity of H-segment was increased in H1-7L/10L, targeting was recovered in the 35DDD and 103DDD. It suggests that Sec62 N-terminal mutant strains have defects in targeting of moderately hydrophobic single spanning membrane protein, but not of strongly hydrophobic proteins.

Next, to check the role of Sec62 in membrane protein topogenesis (i.e. orienting the transmembrane segments in the membrane), the ratio between 1G (C-terminus-in) and 2G (C-terminus-out) form were compared in Sec62 WT and mutant strains. The relative amount of C_{out}, calculated as C_{out} form / C_{out} + C_{in} forms, represents the amount of C-terminal translocated form (2G) out of total membrane inserted H1 proteins. In figure 5A, more H1-4L is originally inserted in C_{out} form than in C_{in} form in WT. In 35DDD and 103DDD strains, the ratio of proteins inserted as C_{out} form was significantly decreased. In other words, C-terminal translocation of proteins into the ER lumen was impaired in Sec62 35DDD and 103DDD. Insertion of H1-5L in WT occurred in two different orientations as well, in C_{in} and C_{out} forms, with both forms present in more or less the equal amount. In 103DDD, the amount of C_{in} was still comparable with that of WT but, C_{out} form was reduced. In 35DDD mutant, C_{out} was completely disappeared whereas there was still a detectable amount of C_{in} form present. Overall, insertion of H1 in C_{out} form was more drastically influenced than in C_{in} form in 35DDD and 103DDD mutants. In figure 5C, the relative amount of C_{out} of H1-4L/5L was decreased in 35DDD and 103DDD, whereas the rest of mutants exhibited no visible defects. In case of H1-6L/7L and 10L, proteins with a strongly hydrophobic TM segment, the relative amount of C_{out} was not significantly changed in 35DDD and 103DDD compared to WT. In sum, the relative amount of C_{out} was specifically decreased in Sec62

35DDD and 103DDD for the proteins carrying a marginally hydrophobic TMs, but not for the proteins with strongly hydrophobic TMs. It demonstrates that the N-terminal domain of Sec62 is involved in inserting single spanning membrane proteins with a moderately hydrophobic TM segment in C_{out} topology. The fact that the relative amount of C_{out} of H1-4L, 5L and 6L in 157AAA, 185AAA and 263DDD mutant strains remained unchanged compared to WT indicate these regions of Sec62 are not involved in translocation of single spanning membrane proteins with marginally hydrophobic TM segment in C_{out} orientation.

Interestingly, even though 157AAA, 185AAA and 263DDD did not show any defects in targeting and translocation of marginally hydrophobic single spanning membrane proteins, when these mutants were screened with a strongly hydrophobic H1-7L/10L proteins, the relative amount of C_{out} was increased to about 55 %. Note that in WT, only about 30% of H1-7L/10L are inserted in C_{out} form. (Fig. 5C). Thus, it shows that the TM domains and the 263-265KKK of Sec62 are involved in translocation of single spanning membrane proteins with a strongly hydrophobic TM segment.

Taking Figure 5 together, in case of N-terminal mutant strains, targeting and C-terminal translocation of marginally hydrophobic single spanning membrane proteins were severely decreased, but not strongly hydrophobic one. And in case of 157AAA, 185AAA (TM domain mutants) and 263DDD, which did not show any targeting defects in single spanning membrane proteins and topogenic defects with marginally hydrophobic proteins, they increase translocation of the C-terminus in strongly hydrophobic single spanning membrane proteins.

Figure 5

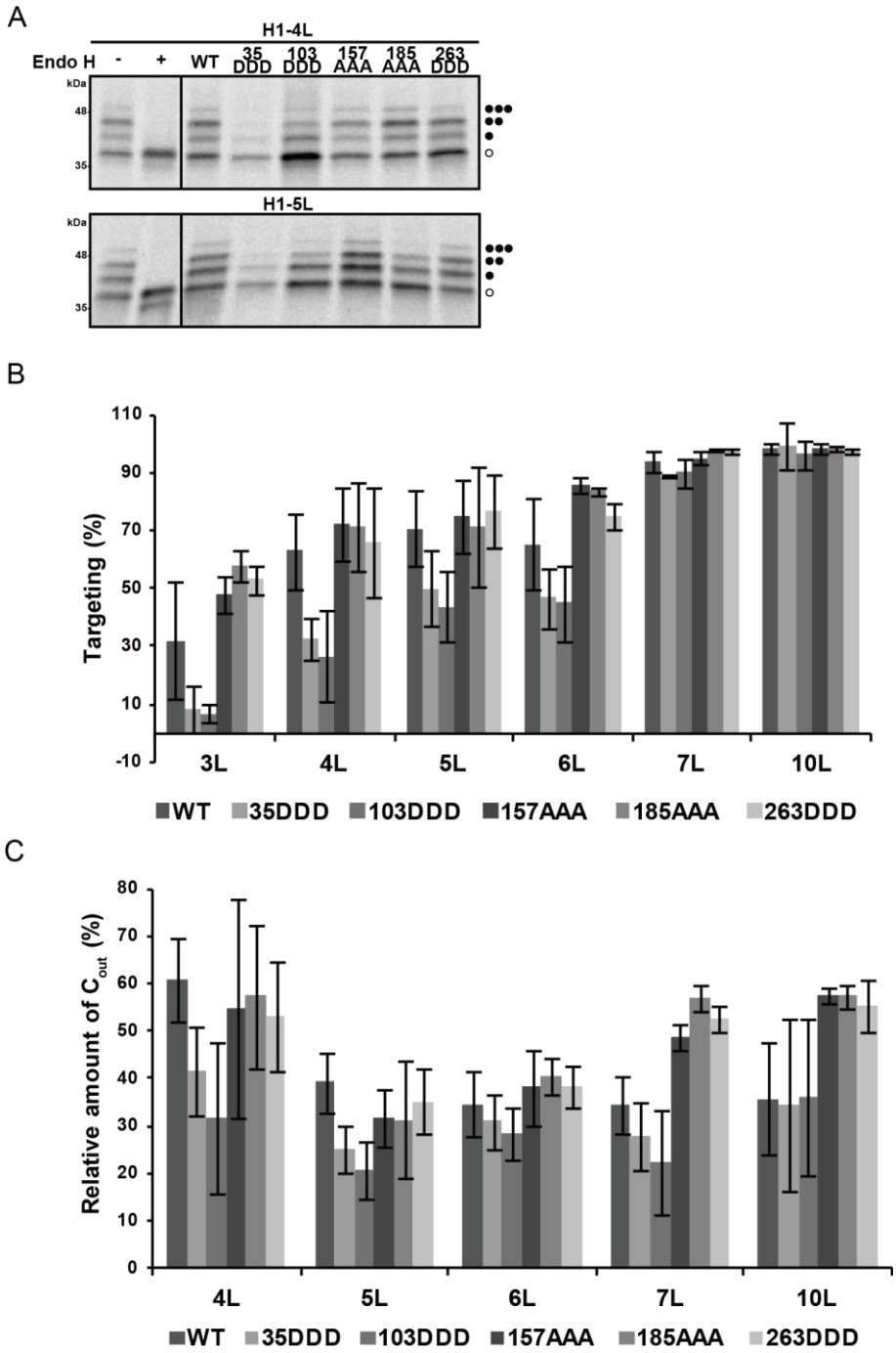
A
H1-4L
Endo H
-
+
WT
35 DDD
103 DDD
157 AAA
185 AAA
263 DDD
kDa
48
35
H1-5L
B
Targeting (%)
110
90
70
50
30
10
-10
3L
4L
5L
6L
7L
10L
WT
35DDD
103DDD
157AAA
185AAA
263DDD
C
Relative amount of C_{out} (%)
80
70
60
50
40
30
20
10
0
4L
5L
6L
7L
10L
WT
35DDD
103DDD
157AAA
185AAA
263DDD

Figure 5. Sec62 N-terminal mutants show reduced targeting and translocation of single spanning membrane proteins. (A) H1-4L/5L proteins were expressed in SEC62 WT or mutant strains. The proteins were radioactively labeled for visualization by autoradiography. H1-4L/5L proteins in SEC62 WT were treated with Endoglycosidase H or mock to identify non-glycosylated bands (0G). Closed circles indicate glycans. Open circle indicates non-glycosylated proteins. **(B)** Targeting efficiency of single spanning proteins (H1). Targeting efficiency was calculated as [(1G+2G+3G) x 100 / Total proteins] based on western blotting or autoradiography data. Averages of at least three independent measurements with standard deviation are shown. **(C)** Orientation of signal anchor proteins. The relative amount of C_{out} of the same set in Fig 5B was calculated as [2G x 100 / (1G+2G)].

With the same approach as in Figure 5, Sec62 218A and 219A mutants that have mutations on region following the second TM segment of Sec62 were examined. H1-4L/5L were expressed in Sec62 218A and 219A mutant strains and visualized by autoradiography (Fig. 6A). Targeting efficiency of H1-4L was decreased from 63% observed in WT to 34 % and 35 % the mutant strains, respectively (Fig. 6B). In case of H1-5L, targeting efficiency was reduced from 71% observed in WT to 61 % and 48 % in 218A and 219A strains. Membrane protein topogenesis was also influenced in the mutant strains. For H1-4L/5L, the relative amount of C_{out} was decreased in Sec62 218A and 219A by a comparable degree observed in Sec62 N-terminal mutant strains as shown in Fig 5C (Fig. 6C).

Taken together, Sec62 N-terminal mutant strains, 35DDD and 103DDD have defects in targeting and translocation of C-terminus in moderately hydrophobic single spanning membrane proteins, but not strongly hydrophobic ones.

157AAA, 185AAA and 263DDD mutant strains did not show any significant difference in targeting and translocation of marginally hydrophobic H1 proteins compared to SEC62 WT. But, in case of strongly hydrophobic H1, the relative amount of C_{out} is increased in these mutants. It indicates that 157AAA, 185AAA and 263DDD mutants may have functions in translocation of single spanning membrane proteins with strongly hydrophobic TM segments.

218A and 219A mutants also showed similar phenotypes with Sec62 N-terminal mutants that showed reduction of targeting and C-terminal translocation of marginally hydrophobic TM segment in single spanning membrane proteins. It indicates that both the N-terminus and C-terminus of Sec62 function on targeting and translocation of single spanning membrane proteins that have moderately hydrophobic TM segments.

Figure 6

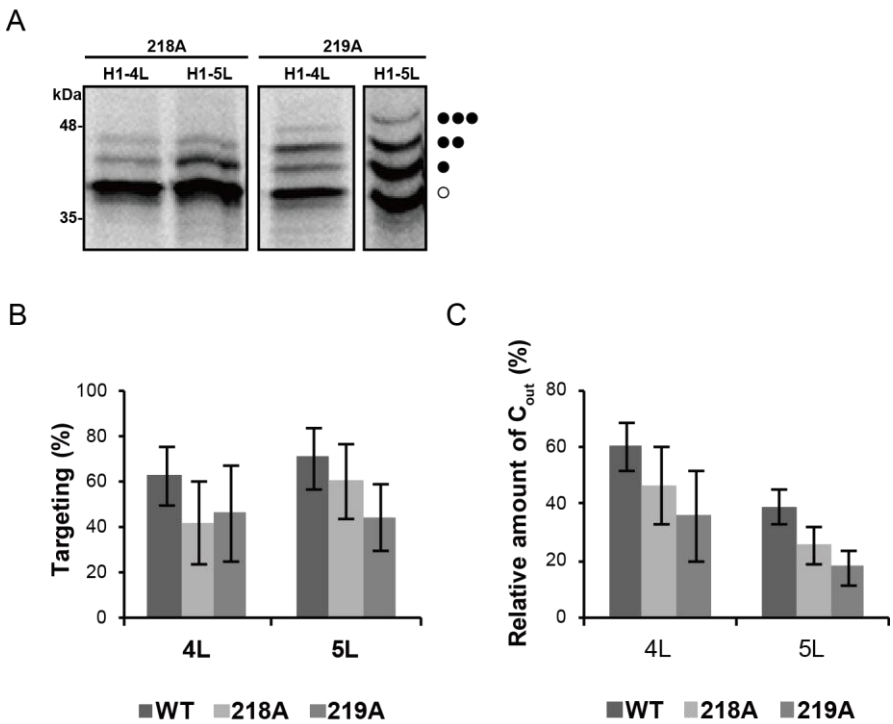

Figure 6. Sec62 218A and 219A mutants have defects in targeting and translocation of single spanning membrane proteins. (A) H1-4L/5L proteins were expressed in *sec62* 218A and 219A strains and analyzed as in Fig 5A. Closed circles indicate glycan. Open circle indicates non-glycosylated protein. **(B)** Targeting efficiency determined by autoradiography or western blotting is calculated as indicated in Fig. 5B. The relative amount of C_{out} is calculated as indicated in Fig. 5C. Averages of three independent measurements with standard deviation are shown.

III. D. Sec62 N- and C-terminal mutant strains show reduction of translocation of C-terminal portions of double spanning membrane proteins.

In consistent with the previous study (29), all N-terminal mutants (35DDD and 103DDD) have defects in translocating the C-terminus of single spanning membrane proteins into the ER lumen (Fig. 5). However, our study showed C-terminal mutants, 218A and 219A mutants, also showed similar defects. We then asked whether C-terminal translocation defect is also seen for proteins with 2 TM proteins. To answer this question, the second TM segment of the original *E.coli* leader peptidase, a double-spanning membrane protein, was varied by changing the number of leucines and alanines to produce what we called H2 proteins (Table 1). H2 proteins have a glycosylation sites on each terminus (Fig 7A). The first, hydrophobic TM segment efficiently targets H2 proteins and when inserted into the ER membrane their N-terminal glycosylation sites become glycosylated (1G). Next, insertion of the second test TM segment (i.e. C-terminal translocation) can be assessed by monitoring the C-terminal glycosylation (2G).

Figure 7

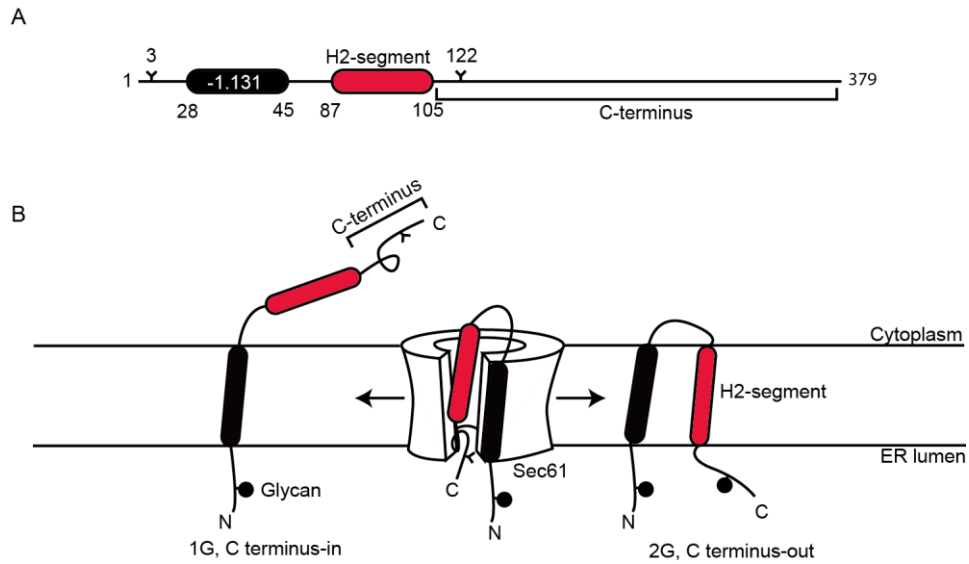

Figure 7. Model double spanning membrane proteins (H2) (A) Schematic drawing of double spanning membrane proteins (H2). H2 protein has two TM segments indicated by black and red bars. Red bar indicates a potential test segment (H2-segment). Sequence and hydrophobicity (ΔG) of H2-segment is indicated in Table 1. H2 protein has two glycosylation sites in its N-terminus and C-terminus. 'C-terminus' refers to the part of protein after H2 segment. ΔG of first TM segment is indicated in black bars. **(B)** Translocation status of H2 proteins. First TM segment is strongly hydrophobic and efficiently inserted into the ER membrane. Second TM segment can be inserted into the ER membrane by the lateral gate of Sec61 or it can remain in cytoplasm. 1G, C-terminus-in indicates that the second TM is not inserted (non-translocated C-terminus). 2G, C-terminus-out indicates translocation of C-terminus to the ER lumen.

The double spanning membrane proteins with H2 segments with composition; P2(0L), 2L, 3L or 5L were expressed in SEC62 WT and mutant strains and the translocation of the C-terminus of H2 proteins was analyzed by autoradiography or western blotting.

In SEC62 WT strain, translocation of H2-2L produces single and doubly glycosylated (1G and 2G) forms. First, absence of 0G form indicates that all protein has been efficiently targeted. Second, from the perspective of C-terminal translocation, the 1G form indicates that C-terminal translocation of H2 proteins has not occurred, whereas 2G form represents efficient C-terminal translocation. The relative amount of C_{out} represents C-terminal translocated (2G) form / total targeted proteins (1G+2G). Thus, in H2-2L, 40% of C-terminal translocation has occurred in WT (Fig 8A). Likewise, H2-3L shows complete targeting, but more C-terminal translocation has occurred (87%), as more hydrophobic segments are likely to embed into the membrane (Fig 8A). Our data show that only in 35DDD and 103DDD mutants, this C-terminal translocated (C_{out}) form is reduced for both H2-2L and 3L. The relative amount of C_{out} in other mutants is comparable to the level seen in WT. The relative amount of C_{out} in H2-2L was reduced to about 15% in sec62 35DDD and 103DDD strains, and further, 15% in 219A. For H2-3L, the relative amount of C_{out} was decreased to 61 % and 63 % in sec62 35DDD and 103DDD mutant strains, respectively; however, no defect in C-terminal translocation was seen in 218A and 219A strains. In WT, the relative amount of C_{out} in H2-P2 and 5L protein was 6 % and 91 %, respectively, and these values were similar in all the mutant strains. It suggested that Sec62 influences only the translocation of C-terminus with marginally hydrophobic TM segments in double spanning proteins.

Next, we investigated the effect of flanking charged residues in translocation of C-terminus. H2-1L and H2-K1LG, which contains a KKPK in N-terminal of

second TMs instead of GGPG, were expressed in SEC62 WT (Fig 8C, Table 1). Translocation of the C-terminus of H2-1L in WT strain is low (18%) (Fig 8D). However, when a positively charged amino acid N-terminally flanks the 2nd TM segment, as shown in Table 1, translocation of the C-terminus increases to 53% (Fig 8D). In Sec62 mutants strains previously shown to decrease C-terminal translocation (35DDD, 35AAA, and 219A), the translocation of H2-1L and H–K1LG is reduced as well. It suggests that effect of flanking charged amino acid on translocation of C-terminus is also dependent on Sec62.

Taken together, it suggests that N- and C-terminus of Sec62 play a role in translocation the carboxy terminal ends of also double spanning membrane proteins. The dependence on Sec62 occurs only when the TM segment is moderately hydrophobic. Further, increase in translocation efficiency by positively charged flanking residues is also dependent on the proper function of Sec62.

Figure 8

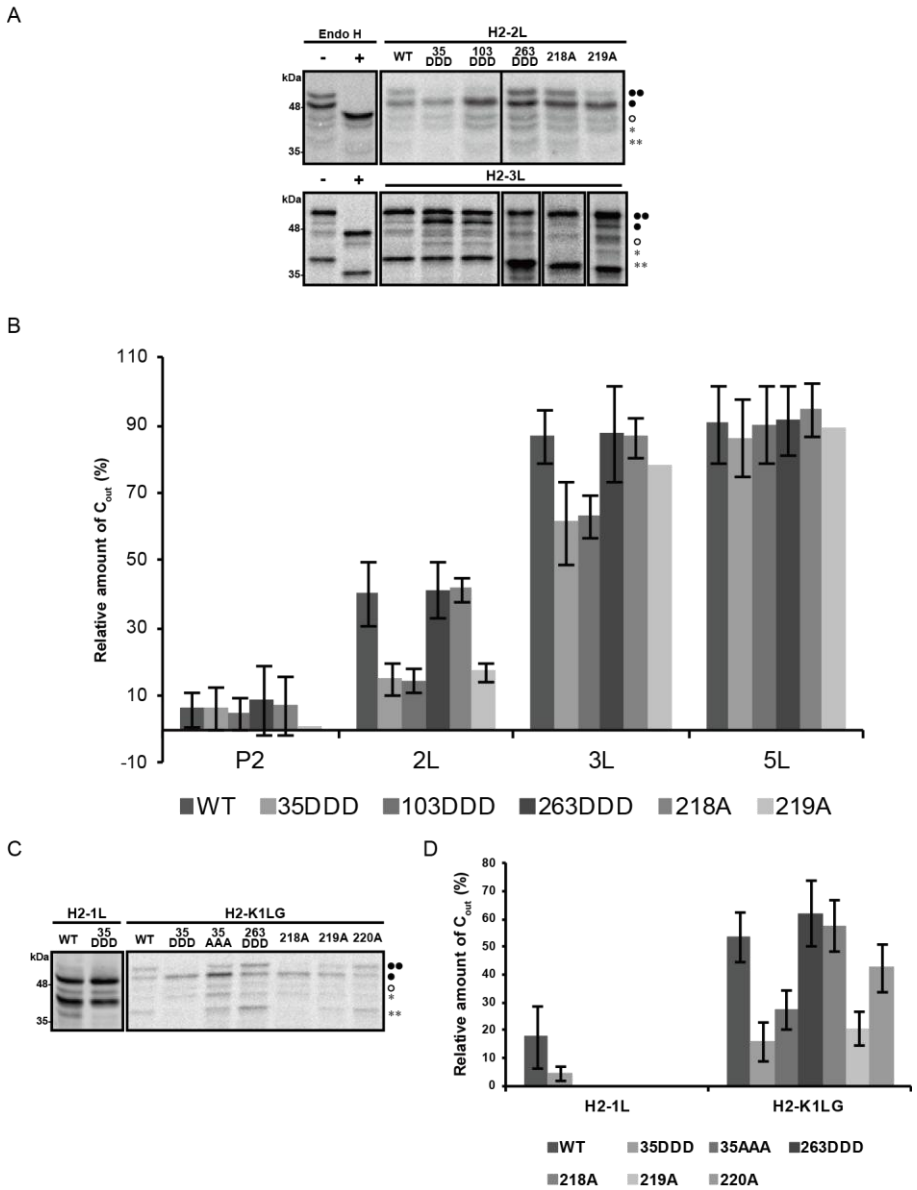

Figure 8. . Translocation of C-terminus of double spanning membrane proteins with a marginally hydrophobic TM segment is impaired in Sec62 N-terminal and C-terminal mutant strains. (A) H2-2L/3L proteins were expressed in SEC62 WT and mutant strains and analyzed as in Fig 5A. H2-2L/3L proteins in SEC62 WT were treated with Endoglycosidase H or mock to identify non-glycosylated band. Closed circles indicate glycan. Open circle indicates non-

glycosylated protein. '*' indicates cleaved products of 1G form and '**' indicates cleaved products of 2G. **(B)** Relative amount of C_{out} determined by Western blotting or autoradiography was calculated as [(2G + 2G cleaved products) x 100 / (Total proteins - 0G proteins)]. Averages of at least three independent measurements with standard deviation are shown except for H2-P2/3L/5L in 219A. H2-P2/3L/5L in 219A is shown with averages of two independent measurement. **(C)** H2-1L/K1LG proteins are expressed in SEC62 WT and mutant strains and analyzed as in Fig 5A. Closed circle indicates a glycan. Open circle indicates non-glycosylated protein. '*' indicates cleaved products of 1G form and '**' indicates cleaved products of 2G. **(D)** Relative amount of C_{out} determined by Western blotting or autoradiography was calculated as in Fig. 8B. Averages of at least three independent measurements with standard deviation are shown.

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III. E. Translocation of C-terminus of multi spanning membrane proteins is decreased in Sec62 N-terminal mutant strains.

Next, we raised question whether Sec62 also functions on translocation of marginally hydrophobic TM segment in multi spanning membrane proteins. To get an answer, triple spanning model proteins derived from *E.coli* leader peptidase were tested in SEC62 WT and mutant strains. *E.coli* leader peptidase-derived Lep4 has 4TM segments (35). With the first, second and the fourth TM segment of Lep4 being very hydrophobic, thus efficiently inserted into membrane. The third TM segment is a test TM segment of a stretch of 19 leucine and alanine to vary hydrophobicity. Sequence and hydrophobicity of H3-segment is shown in Table 1. To exclude effect of the fourth TM segment on insertion of the third TM segment, the fourth TM segment and C-terminal part of the Lep4 protein were truncated. Consequently, this truncated Lep4 (H3) contains three TM segments and three glycosylation sites (Fig. 9A). Triply glycosylated form represents C-terminal translocated proteins while doubly glycosylated form indicates the third TM is inserted (non-translocated C-terminus) (Fig. 9B).

H3 proteins were expressed in SEC62 WT and N-terminal mutant strains, 35DDD and 103DDD, and were subjected to S^{35} pulse labeling and SDS-PAGE. The translocation of C-terminus is assessed by glycosylation status. The relative amount of C_{out} represents a ratio between C-terminal translocated form and 3rd TM inserted form (non-translocated C-terminus). A set of H3 proteins with serially increasing number of leucines from 0 (H3-0L) to 7 (H3-7L) were tested. SEC62 WT and mutant strains, however, did not show significant differences except for H3-4L and 5L (data not shown).

Glycosylation status of H3-4L was examined by Endo H treatment, as doubly and triply glycosylated bands are indicated by closed circles (right). C-terminus-out form is more pronounced than C-terminus-in form (Fig. 9C) while the relative amount of C_{out} is 64 % (Fig. 9D). C-terminus-in form was prominent in 35DDD

and 103DDD. Moreover the relative amount of C_{out} is reduced to 36 % and 39 % in 35DDD and 103DDD, respectively. In case of H3-5L, majority is C-terminus-in form in WT with detectable amount of C-terminus-out form. In mutant strains, however, C-terminus-out form is completely disappeared. Cumulatively, in N-terminal mutant strains, H3-4L/5L containing marginally hydrophobic third TM segment showed reduced C-terminal translocation. Consequently, N-terminus of Sec62 mediates C-terminal translocation of triple spanning membrane proteins.

Figure 9

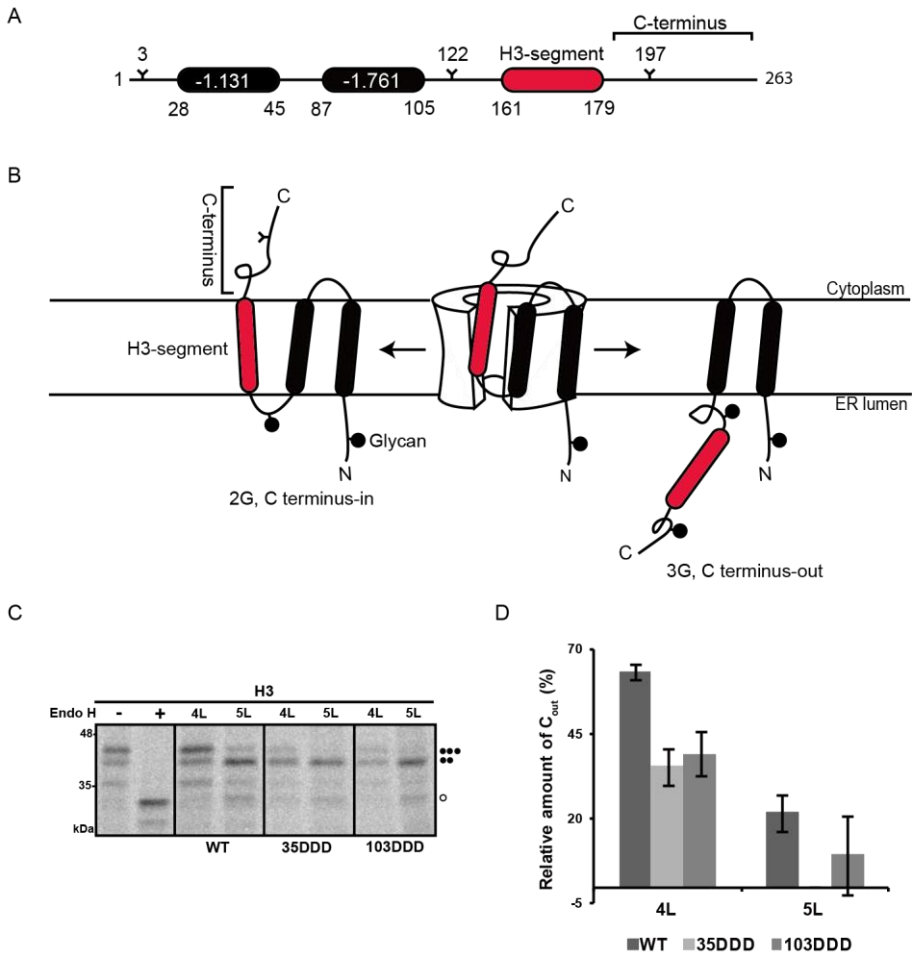

Figure 9. C-terminal translocation of multi spanning membrane proteins is reduced in Sec62 N-terminal mutant strains. (A) Schematic drawing of multi spanning membrane proteins (H3). Hydrophobicity (△G) of the first and second TM segment is indicated in black bars. Red bar is a potential test TM segment. Sequence and hydrophobicity (ΔG) of H3-segment is indicated in Table 1. Glycosylation sites are indicated as 'Y's. **(B)** Translocation status of H3 proteins. First and second TM segments indicated by black bars are strongly hydrophobic and efficiently inserted into the ER membrane. Third TM segment is inserted into the ER membrane by the lateral gate of Sec61 or fully translocated to the ER lumen. 2G, C-terminus-in indicates that third TM segment is inserted. 3G, C-terminus-out indicates the full translocation of the C-terminus of H3. **(C)** H3-4L/5L proteins were expressed in SEC62 WT and N-terminal mutant strains, 35DDD and

103DDD and analyzed as in Fig 5A. H3-4L/5L proteins in SEC62 WT were treated with Endoglucosidase H or mock to identify non-glycosylated band. Closed circles indicate glycans. Open circle indicates non-glycosylated protein. **(D)** Relative amount of C_{out} determined by autoradiography was calculated as [(3G x 100) / (2G+3G)]. Averages of at least three independent measurements with standard deviation are shown.

III. F. Natural multi spanning membrane proteins

The above data was corroborated using different yeast polytopic membrane proteins, Shr3 and Sec63. Shr3 is an ER membrane protein known to function on the biogenesis of amino acid permease (37). It has four TM segments and experimental data suggest both N- and C-termini face the cytoplasm (37). The fourth TM segment of Shr3 was deleted to a total of 140 residues so that the N-terminus of Shr3t is in the cytoplasm, and the C-terminus is in ER lumen (Fig. 10A + B). Further, glycosylation sites and HA tag were introduced in the C-terminus. We engineered different versions of truncated Shr3 with varying hydrophobicity of the third TM segment, our test segment. Doubly glycosylated proteins indicate translocation of the Shr3 C-terminus, while non-glycosylated proteins indicate insertion of the 3rd TM segment (thus Shr3 C-terminus is not translocated).

The C-terminal translocation of Shr3t in SEC62 WT is about 80 %. This is not affected by 35DDD mutation (date not shown). To check whether the effect of Sec62 is indeed dependent on hydrophobicity, hydrophobicity of our test segment was lowered (L103A mutation) (Table 1). As electrostatic interaction between charged amino acids in neighboring TM segments was previously shown to stabilize insertion of TM segment as helix-turn-helix motif (38), K81 in TM1 (which may interact with E94 in TM2) was substituted to glutamate (Shr3t (EA)). Shr3t (EA) was examined in SEC62 WT and 35DDD strains (Fig. 10C). In WT, C-terminal translocation is 51 %. This was decreased to 38 % in 35DDD, thus the relative ratio of 3G and 0G decreases in 35DDD compared to WT.

Sec63 is a subunit of the post-translational translocation complex and contains 3 TM segments (Fig. 10A and 10B). The acidic domain and the Brl domain in the C-terminus of Sec63 have been shown to interact with Sec62 and Sec61, respectively (39). To exclude the possibility of aberrant Sec61 complex formation, the C-terminal domain of Sec63 was truncated to the L305 residue. Glycosylation sites

were introduced in Sec63 as shown in Fig. 10A. Thus, 1G form represents third TM segment insertion into ER membrane, whilst 4G form indicates the ER translocation of Sec63 C-terminus. Again, to confirm the hydrophobicity dependent sensitivity to Sec62, four hydrophobic amino acids in the third TM segment were changed to alanines to produce Sec63 (4A) (Table 1). When this construct was expressed in SEC62 WT, the relative percentage of 4G form was 58% (Fig 10D). However, in 35DDD, this decreased to 27%, indicating that translocation of C-terminus is reduced in the mutant.

To analyze how early the Sec62 acts to decrease C-terminal translocation, pulse-chase experiment of Shr3t (EA) and Sec63 (4A) translocation was carried out at different time-points (Fig. 10E). After 5 min pulse-labelling (0min chase), we see that the relative amount of C_{out} of Shr3t (EA) and Sec63 (4A) is 68 % and 25% in WT, respectively. However, this percentage is decreased in 35DDD even at 0 min (Figure 10E). Although, the relative amount of C_{out} of Shr3t (EA) and Sec63 (4A) gradually increases over time in SEC62 WT, this increase (up to the level of that seen in WT) is not seen in 35DDD. This means that even upon successful membrane anchorage of the first and second TM, the C-terminus cannot be translocated suggesting Sec62 defect acts in the early stage of translocation.

Altogether, as we showed above, translocation of the C-terminus of Shr3t (EA) and Sec63 (4A) is reduced in 35DDD. Again, this apparent defect is only seen with TM segments of less hydrophobicity. Further, a reduced translocation of C-terminus is not recovered in a time dependent manner. This suggests that Sec62 mediates C-terminal translocation at an early stage of protein insertion.

Figure 10

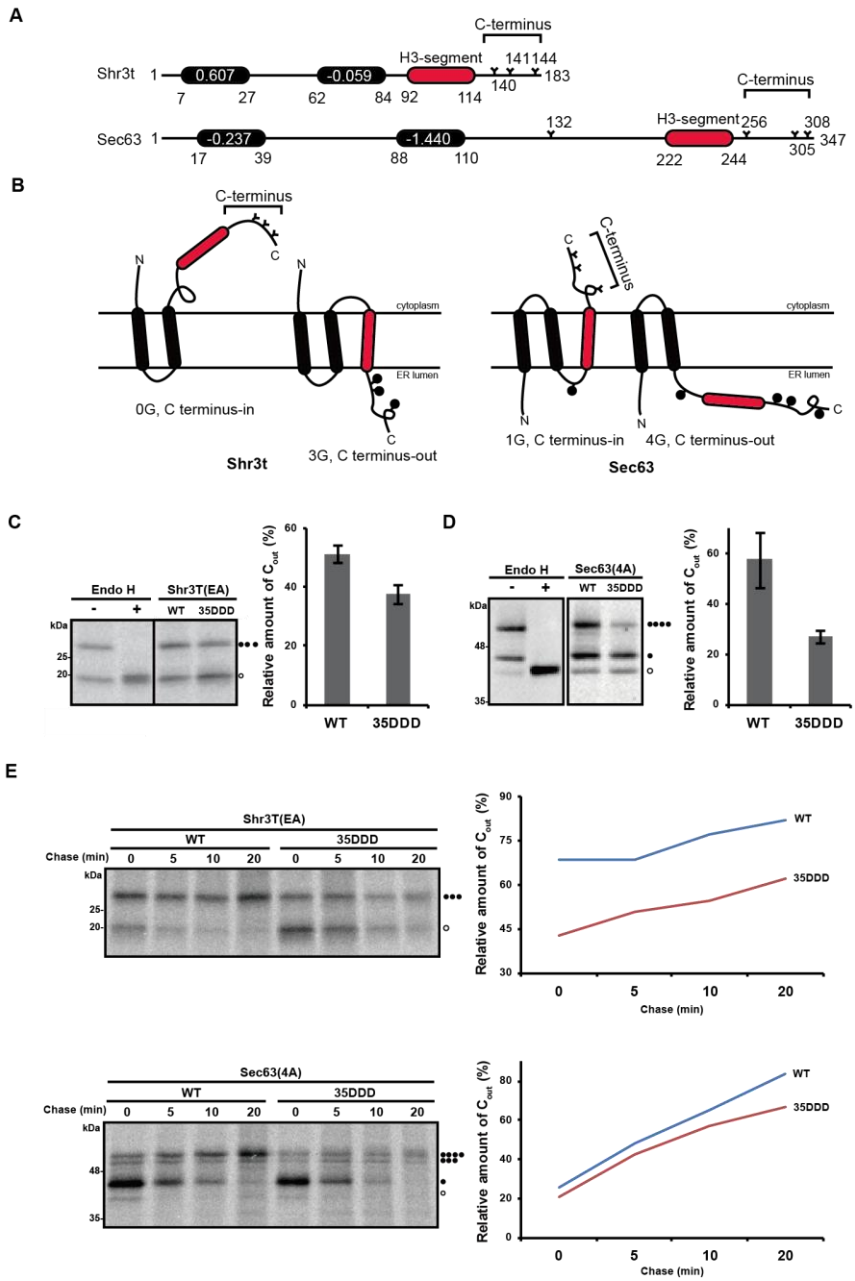

Figure 10. Natural polytopic membrane proteins (A) Schematic drawing of polytopic membrane proteins, Shr3t and Sec63. Shr3t has three TM segments. Predicted ΔG of first and second TM segments are indicated in black bars. ΔG values is predicted by ΔG predictor provided from CBR of Stockholm university

(http://dgpred.cbr.su.se/). 'Y' indicates glycosylation sites. Numbers of glycosylation site are indicated. 'C-terminus' is refers to the part of the protein after the third TM segment. Both Shr3t (EA) and Secc63 (4A) was conjugated with 3 copies of HA epitope in C-terminus. **(B)** Translocation status of Shr3t and Sec63. N-terminus of Shr3t is in cytoplasm. 3G, C-terminus-out indicates translocation of C-terminus. 0G, C-terminus-in indicates insertion of third TM segment (non-translocated C-terminus). N-terminus of Sec63 is in the ER lumen. 4G, C-terminus-out indicates translocation of C-terminus. 1G, C-terminus-in indicates insertion of third TM segment (non-translocated C-terminus). First and second TM segments are inserted through the lateral gate of Sec61. (**C**) Shr3t (EA) was expressed in SEC62 WT and N-terminal mutant strain, 35DDD and followed by S^{35} labeling and autoradiography. Shr3t (EA) in SEC62 WT were treated with Endoglucosidase H or mock to identify non-glycosylated band. Closed circles indicate glycans. Open circle indicates non-glycosylated protein. Relative amount of C_{out} determined by autoradiography was calculated as [(3G x 100) / Total protein]. Averages of at least three independent measurements with standard deviation are shown. **(D)** Sec63 (4A) was expressed in SEC62 WT and N-terminal mutant strains, 35DDD and analyzed by western blotting. Sec63 (4A) in SEC62 WT are treated with Endoglucosidase H or mock to identify non-glycosylated band. Closed circles indicate glycan. Open circle indicates non-glycosylated protein. Relative amount of C_{out} determined by western blotting was calculated as [(4G x 100) / Total protein]. Averages of at least three independent measurements with standard deviation are shown. **(E)** Shr3t (EA) and Sec63 (4A) were expressed in SEC62 WT and 35DDD strain. Cells were labeled with S^{35} for 5 min at 30℃. Cells were harvested at 0, 5, 10 and 20 min time points and subjected to immunopreicipitation with HA antibody to pull down HA tagged Shr3t (EA) and Sec63 (4A). The proteins were subjected to SDS PAGE and autoradiography. The relative amount of C_{out} was calculated as indicated in Fig. 5B for Shr3t (EA). The relative amount of C_{out} was calculated as [(3G+4G) X 100 /(1G+3G+4G)] for Sec63 (4A).

III. G. Protein level of Sec62 is slightly decreased in Sec62 35DDD and 103DDD strains

To see whether the observed defects of Sec62 N-terminal mutants in protein translocation are due to the reduced protein level of Sec62, the stability of Sec62 was checked in WT and Sec62 mutant strains. JRY4 strain carrying either WT Sec62 or each mutant version of Sec62 was analyzed by SDS-PAGE and western blotting. Sec62, Sec61 and Tom70 (translocase of the outer mitochondrial membrane) were immunodecorated with the corresponding antibodies (Fig 11A). Tom70 was included as a loading control. The amount of Sec62 was mildly reduced compared to WT only in Sec62 N-terminal mutant strains.

III. H. N-terminal mutations disrupt the interaction between Sec62 and Sec63

Previous studies showed that Sec62 interacts with Sec63 via its N-terminus and the interaction between the two proteins is crucial for cell viability. To investigate whether the interaction between Sec62 and Sec63 is influenced by the Sec62 mutations, co-immunoprecipitation assay was carried out using JRY6 strain with either WT Sec62 or the mutant versions of Sec62.

JRY6 strain has a genomic SEC63 gene conjugated with a HA tag. JRY6 strain was transformed with a plasmid carrying either the FLAG tagged WT Sec62 or the mutant versions of Sec62. Crude membranes from the transformants were fractionated and solubilized with 1% Triton X-100. Solubilized membranes were immunoprecipitated with Sec63 antibody. Immunoprecipitated samples were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. Sec62 was visualized by using anti-FLAG antiserum (Fig. 11B). Sec62 was not detected in Sec62 35DDD and 103DDD strains. It indicates that Sec62 35DDD and 103DDD could not be pulled down with Sec63. Thus, it suggests that interaction between Sec62 and Sec63 is broken in the N-terminal mutant strains.

In case of Sec62 218A, 219A and 263DDD strains where the mutations were made either at the residues downstream of the second transmembrane segment or at the C-terminus of Sec62, Sec62 was successfully pulled down with Sec63. Immunoprecipitation data from the mutant strains suggest that Sec62 primarily uses its N-terminus for interaction with Sec63 and other domains of Sec62 are dispensable for interaction with Sec63. (Fig. 11C)

Taking Figure 11 together, the mutations at the N-terminus of Sec62 makes the protein slightly unstable and disrupts the interaction between Sec62 and Sec63. The N-terminal mutant versions of Sec62 might be more prone for degradation when it is not bound to Sec63. It can be concluded that the observed impairment on the translocation of C-terminus of membrane proteins in N-terminal mutants are from the loss of Sec62-63 interactions. In case of Sec62 218A-220A mutants, even though Sec62 is attached to Sec63 complex, the translocation of C-terminus of membrane proteins was impaired. It indicates Sec62's 218-220 region is of functional importance when translocating C-terminal parts of membrane proteins.

Figure 11

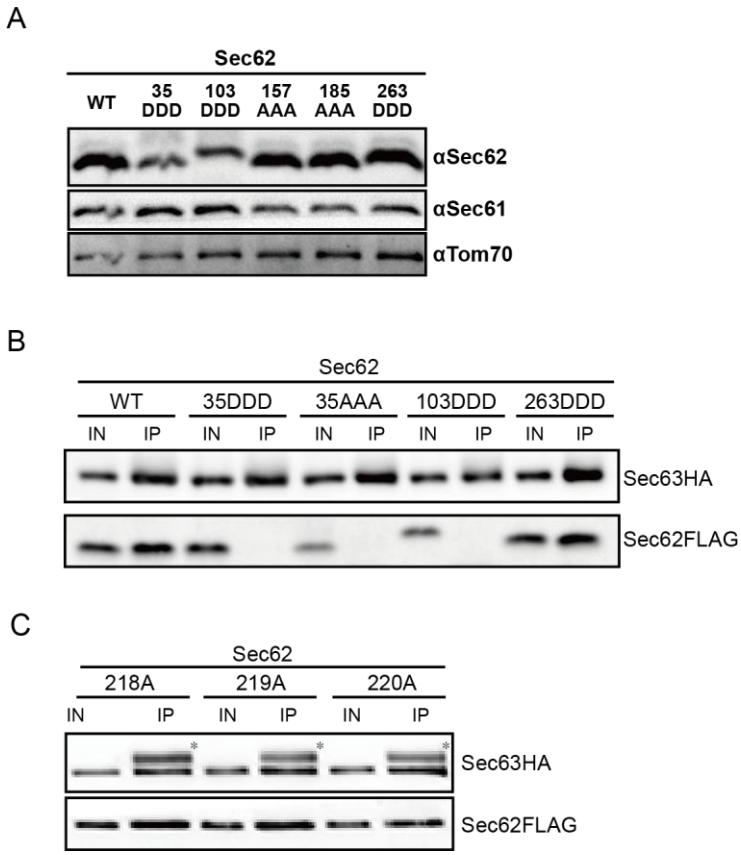

Figure 11. Stability and interaction of Sec62 (A) Stability of Sec62. Crude membrane fractions of JRY4 strain with pRS415 carrying either SEC62 WT or mutant was solubilized with 1 % Trition X-100 and subjected to western blot analysis. Proteins were visualized with corresponding antibodies, Sec62, Sec63, Sec61 or Tom70. **(B)** Co-immunoprecipitation assay to check interaction between Sec63 and Sec62. Crude membrane of SEC62 WT or mutant strains in JRY6 was fractionated. Then, it was solubilized in 1% Triton X-100 and followed by immunoprecipitation with HA antibody to purify HA tagged Sec63. The proteins were analyzed by SDS-PAGE and western blotting. The Proteins are detected either with anti-FLAG or anti-HA antibody **(C)** Interaction of Sec62 with Sec63 is examined in *sec62* 218A, 219A and 220A mutant strains as described in Fig. 11B.

Figure 12

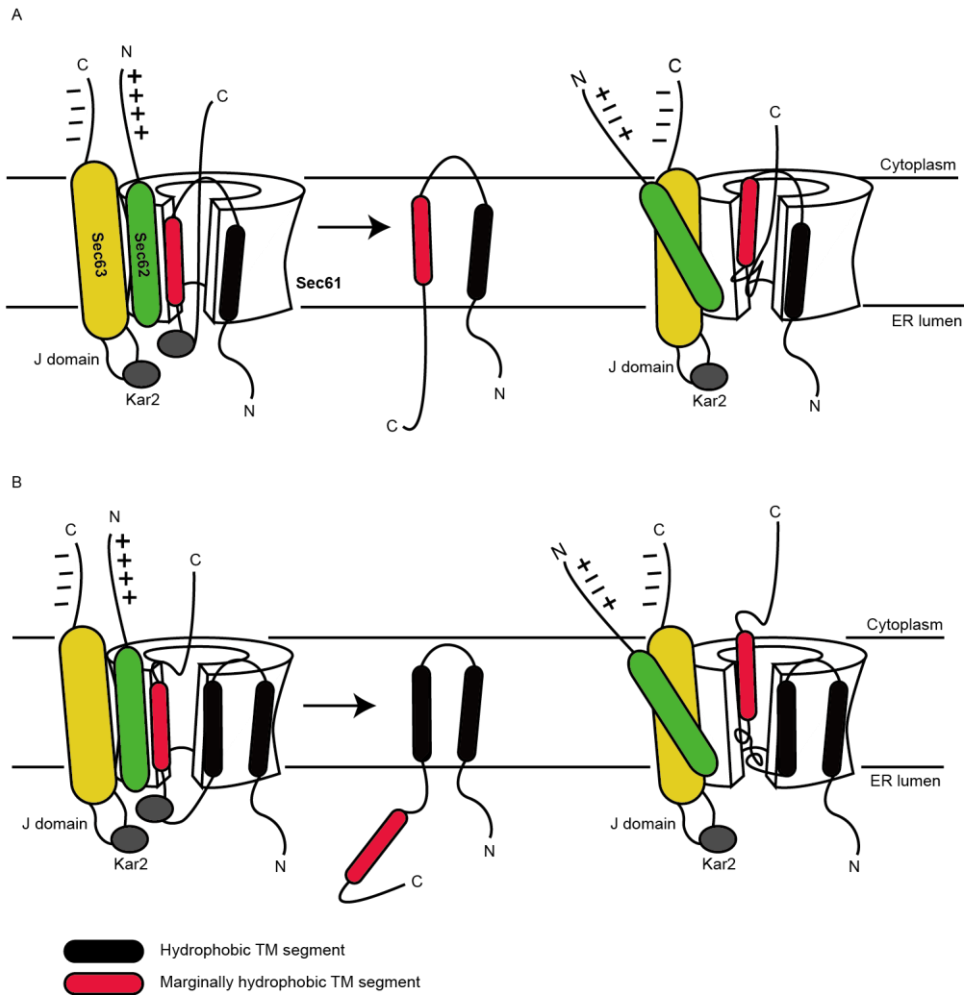

Figure 12. **Model of translocation of C-terminus of membrane proteins by Sec62. (A)** Model for translocation of C-terminus of double spanning membrane proteins. **(B)** Model for translocation of C-terminus of multi spanning membrane proteins.

IV. DISCUSSIONS

Previous studies have shown that the targeting efficiency of secretory proteins with a marginally hydrophobic SS is reduced when the N-terminus of Sec62 is mutated (*sec62-1*). Recently, it was reported that the C-terminal translocation of single spanning membrane proteins with a moderately hydrophobic TM segment is decreased in *sec62-1* strain (29). It implies that the N-terminus of Sec62 is involved in not only secretory protein targeting but also C- terminus translocation of single TM proteins in the ER membrane. But, the function of Sec62 on C-terminus translocation of membrane proteins was only shown with a set of model single spanning proteins. To understand detailed molecular function of Sec62 on translocation of membrane proteins, the role of each domain in Sec62p and its involvement in translocation of multi spanning membrane proteins have been investigated.

The N-terminus of Sec62

Sec62 N-terminal mutant strains, 35DDD and 103DDD were tested by using artificial and natural membrane proteins. The artificial and the selected set of natural membrane proteins feature a broad spectrum of hydrophobicity. In case of multi spanning membrane proteins, the number of transmembrane segments was also varied. The screening of mutant strains with the model proteins showed that the mutant strains have a defect in translocation of C-terminus of single, double and multi spanning membrane proteins. Particularly, reduction of C-terminal translocation was observed only with model proteins containing a marginally hydrophobic TM segment. Therefore, it implies that Sec62 may recognize a marginally hydrophobic TM segment during the process of translocation of membrane proteins. Since, interaction between Sec62 and Sec63 is broken in Sec62 N-terminal mutant strains, Sec62 is no longer tightly associated with

Sec62/63 complex, even though Sec62 was shown to bind Sec61 with weak affinity independent of the N-terminal region (15). Thus, the loss of tight interaction with the translocon results in the impairment on the Sec62's function on facilitating the C-terminal translocation of proteins. Therefore, Sec62 35DDD and 103DDD are impaired in C-terminal translocation of membrane proteins because it is not precisely located at the site of action. As the protein level of Sec62 35DDD and 103DDD seems to be decreased, it is possible that when Sec62 is not associated with Sec63, the protein is degraded easily compare to the ones that form a functional complex.

The C-terminus of Sec62

Although the interaction of Sec62 218A and 219A with Sec63 was intact, these mutants displayed decreased C-terminal translocation of single spanning membrane proteins. It indicates that this region of Sec62 is crucial for proper function of Sec62 regardless of interaction between Sec62 and Sec63. Further investigations are necessary to characterize the function of C-terminus of Sec62 in greater depth. It was suggested that C-terminus of Sec62 recognizes SS of secretory proteins and binds to the Sec complex (15). To reveal how this region of Sec62 influenced on translocation of membrane proteins, stability of Sec62 should be checked to see whether protein levels of Sec62 218A, 219A are changed. In addition, earlier cross-linking studies showed that the C-terminal part of incoming peptides are in close proximity to Sec62 during translocation (Plath K et al , 1998). By employing the same method, it could be tested whether 218-220 region of Sec62 interacts with C-terminus of membrane proteins (11).

The TM domain of Sec62

Sec62 157AAA and 185AAA did not show any difference with WT in targeting

and translocation of marginally hydrophobic single spanning membrane proteins. However, in case of strongly hydrophobic single spanning membrane proteins (H1-7L/10L), translocation of C-terminus to the ER lumen was increased in Sec62 157AAA and 185AAA. It is the opposite result to Sec62 N-terminal mutant strains that showed a reduction of C-terminal translocation of marginally hydrophobic single spanning membrane proteins. Therefore, TM domains of Sec62 may be also involved in translocation of C-terminus of hydrophobic proteins with distinct molecular mechanism that differs from that of the N-terminus or C-terminus domains of Sec62.

Previous study showed that ppαF was simultaneously crosslinked both to the lateral gate of Sec61 and Sec62 (11). Although it is unclear which part of Sec62 was cross-linked to the SS of ppαF, it is likely that the TM domain of Sec62 is the site of crosslinking. Since the SS of ppαF was crosslinked to the lateral gate of Sec61 formed by two transmembrane segments, it is likely that the recognition of SS of ppaF by Sec62 occurs through the TM domain of Sec62 in the ER membrane. The rest part of Sec62 is either located in the cytoplasm or ER lumen making them a less likely candidate for the site of crosslink. Overall, it implies that the TM domain of Sec62 have a function on recognizing TM segments of polypeptides.

Future perspectives

So far, we showed that the C-terminal translocation of proteins is abolished when Sec62 is mutated at its N- or C- termini. It is, however, unclear whether Sec62 directly pulls C-terminus of membrane proteins or whether it is the other proteins of Sec translocon complex pulling the polypeptides. One of the possible candidates is Sec63. It is known to interact with Sec62 and Sec61. Sec63 interacts with Kar2 protein which pulls translocating polypeptides in an ATP dependent manner from the luminal side of the ER. Thus, it is possible to hypothesize that Sec62 recognizes and positions a marginally hydrophobic TM segment to a proper location in the

translocon complex. Kar2 is then recruited by Sec63 providing the pulling force to translocate the C-terminus of membrane proteins. To check this hypothesis, the translocation status of membrane proteins should be tested using Sec63 mutants that cannot interact with Kar2. Alternatively, Kar2 mutants that cannot utilize ATP, thus cannot exert pulling force on translocating polypeptides, can be used.

Even though the presence of positively charged residues upstream of the second TM in H2 protein enhances the C-terminal translocation, the effect of positively charged residues disappeared in Sec62 mutants. So far, the study has mainly focused on investigating the correlation between Sec62 and hydrophobicity of TM segments in protein translocation. The current study neglected many other known factors that influence membrane protein biogenesis. These factors include flanking charged residues of TM segments, the length of the C-terminus of proteins, charged residues within TM segments, and the distribution of specific amino acids of different nature in TM segments. (35,38,40,41). Thus, it would be interesting to investigate how Sec62 cooperates with the factors mentioned above on the translocation and topogenesis of membrane proteins.

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국문초록

막단백질과 분비성 단백질들은 리보솜에 의하여 세포질에서 합성되어 두 가지의 경로를 통하여 소포체의 막으로 이동한다. 두 가지의 경로 중 하나는 동시번역전좌 경로이며, 다른 하나는 번역후전좌 경로이다.

위에서 언급한 동시번역전좌 경로와 번역후전좌 경로를 통하여 이동된 막단백질들은 소포체 막의 단백질 통과를 위한 친수성 통로를 제공하는 Sec61 단백질 전달 통로를 통하여 소포체 막에 삽입된다. 폴레펩타이드가 통과하는 동안, Sec61은 20여 개의 소수성 아미노산으로 구성된 서열을 인식하여, Sec61의 두번째와 일곱번째 막관통분절로 구성된 측면 통로를 통하여 소포체 막에 삽입시킨다. 특별히, 번역후전좌 경로에서는 Sec63/62 단백질 복합체가 Sec61 단백질과 결합되어 있다. 막 내부에 존재하는 Sec63/62의 아단위인 Sec62는 Sec61의 측면 통로에 가까이 위치하며, 폴리펩타이드의 수용체 단백질로서 작동함이 알려져 있다. 최근의 연구가 밝힌 것에 따른면, Sec62는 중간 정도의 소수성을 가진 단일 막관통단백질의 삽입 방향에 관련이 있다. 그러나, Sec62가 다중막관통단백질의 삽입에 어떠한 기능을 하는지는 잘 알려져 있지 않다.

다중막관통단백질의 삽입에 관련한 Sec62의 기능에 대하여 조사하기 위하여, 변이들이 Sec62의 몇몇 도메인에 삽입되었다. 그리고 서로 다른 수의 막관통 분절을 가진 다수의 모델 막단백질이 각각의 Sec62 효모 변이주에서 시험되었다. 그 결과, 중간 정도의 소수성을 나타내는 막관통분절을 가진 다중막관통 단백질에서 카르복시 말단의 전좌가 Sec62의 아미노 말단 변이주에서 특히 감소되었다. 또한, Sec62의 아미노 말단 변이는 Sec62와 Sec63의 상호작용을 파괴하였다. 덧붙여, Sec62의 카르복시 말단 변이주에서도 다중막관통 단백질의 카르복시 말단 전좌의 감소가 관찰

되었다. 결론적으로, 본 연구는 효모 소포체에서, Sec62와 Sec63 간의 결합에 작용하는 Sec62 아미노 말단은 다중막관통 단백질의 전좌를 매개하는 역할을 가지고 있음을 보여주었다. 뿐만 아니라, Sec62의 카르복시 말단도 이 과정에 관여하고 있음을 밝혔다.

주요단어:

효모, *Saccharomyces cerevisiae*, Sec61, Sec62, 전좌, 막단백질, 소포체, 막위상