BBA - General Subjects 1863 (2019) 1371–1380

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

Proper insertion and topogenesis of membrane proteins in the ER depend on Sec63

Sung-jun Jung[1], Yunjae Jung[1], Hyun Kim*

School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul 08826, South Korea

ARTICLE INFO

Keywords:
Endoplasmic reticulum (ER)
Protein translocation
Protein targeting
Sec63
Sec62
Sec61
Signal sequence
Saccharomyces cerevisiae

ABSTRACT

Background: In eukaryotic cells, biogenesis of proteins destined to the secretory pathway begins from the cytosol. Nascent chains are either co-translationally or post-translationally targeted to the endoplasmic reticulum (ER) and translocated across the membrane through the Sec61 complex. For the post-translational translocation, the Sec62/Sec63 complex is additionally required. Sec63, however, is also shown to mediate co-translational translocation of a subset of proteins, the types and characteristics of proteins that Sec63 mediates in translocation still await to be defined.

Methods: To overview the types of proteins that require Sec63 for the ER translocation, we prepared Sec63 mutant lacking the first 39 residues (Sec63_ΔN39) in yeast and assessed initial translocation efficiencies of diverse types of precursors in the *sec63_ΔN39* strain by a 5 min metabolic labeling. By employing Blue-Native gel electrophoresis (BN-PAGE), stability of the SEC complex (Sec61 plus Sec62/Sec63 complexes) isolated from cells carrying the Sec63_ΔN39 mutant was examined.

Results: Among the various translocation precursors tested, we found that proper sorting of single- and double-pass membrane proteins was severely impaired in addition to post-translational translocation precursor in the *sec63_ΔN39* mutant strain. Stability of the SEC complex was compromised upon deletion of the N-terminal 39 residues.

Conclusions: The N-terminus of Sec63 is important for stability of the SEC complex and Sec63 is required for proper sorting of membrane proteins *in vivo*.

General significance: Sec63 is essential on insertion of membrane proteins.

1. Introduction

Approximately 30% of the proteome in a eukaryotic organism are localized in subcellular organelles of the secretory pathway or secreted. They first enter the endoplasmic reticulum (ER) to reach their final destinations. For targeting to the ER membrane, secretory and membrane proteins have a signal sequence (SS) or a hydrophobic transmembrane (TM) domain. These regions are recognized by the signal recognition particle (SRP), associate with the SRP and ribosome, and recruited to the ER membrane (The SRP dependent pathway) [1–3]. For the precursors carrying a moderately hydrophobic SS, they are not recognized by SRP but the mature part of a nascent chain is shielded by cytosolic heat shock proteins and escorted to the ER membrane (The SRP independent pathway) [4–6]. Membrane proteins in yeast are suggested to be co-translationally translocated [5], and proximity-specific ribosome profiling study has shown that membrane proteins as well as a large fraction of secretory proteins are co-translationally translocated [7].

Upon reaching the ER membrane, a nascent chain engages the trimeric Sec61 complex, consisting of Sec61 (Sec61α/SecY), Sbh1(Sec61β/SecG), and Sss1(Sec61γ/SecE) [8,9]. Sec61 has 10 TM domains, forming an aqueous pore that a newly synthesized polypeptide passes through and a lateral gate where a TM domain exits into the lipid bilayer [10–12]. For a subset of proteins carrying the moderately hydrophobic SS, the Sec61 trimer forms a larger protein complex with the Sec62/63 complex, so called the SEC complex [13–16]. The Sec62/63 complex is evolutionarily conserved [17,18], associates with the Sec61 complex for translocation of selective substrates of smaller size (less than 160 amino acids) and carrying a moderately hydrophobic SSs [19–21]. In yeast, the Sec62/63 complex contains additional Sec71 and

Abbreviations: ER, endoplasmic reticulum; SS, signal sequence; TM, transmembrane; CPY, carboxypeptidase Y; Endo H, endoglycosidase H

* Corresponding author at: School of Biological Sciences, Institute of Microbiology, Seoul National University, Building 504-421, Seoul 08826, South Korea.
E-mail address: joy@snu.ac.kr (H. Kim).

[1] These authors contributed equally.

https://doi.org/10.1016/j.bbagen.2019.06.005
Received 7 March 2019; Received in revised form 28 May 2019; Accepted 4 June 2019
Available online 10 June 2019

Table 1
List of yeast strains used in this study.

Strain	Genotype	Source
W303-1α	*MATα, ade2, can1, his3, leu2, trp1, ura3*	Wilkinson et al. [27]
sec65–1	*MATα, ade2, can1, his3, leu2, trp1, ura3, sec65-1*	
JRY4 *SEC62_WT*	W303-1α, *sec62Δ::HIS3*, pRS415 1 kb + *SEC62*	Jung et al. [28]
JRY4 *sec62_35DDD*	W303-1α, *sec62Δ::HIS3*, pRS415 1 kb + *sec62_35DDD*	
JRY4 *sec62_P219A*	W303-1α, *sec62Δ::HIS3*, pRS415 1 kb + *sec62_P219A*	
JRY6 *SEC62_WT-FLAG*	W303-1α, *SEC63::HA-KanM, sec62Δ::HIS3*, pRS415 1 kb + *SEC62-FLAG*	
JRY6 *sec62_35DDD-FLAG*	W303-1α, *SEC63::HA-KanM, sec62Δ::HIS3*, pRS415 1 kb + *sec62_35DDD-FLAG*	
JRY6 *sec62_P219A-FLAG*	W303-1α, *SEC63::HA-KanM, sec62Δ::HIS3*, pRS415 1 kb + *sec62_P219A-FLAG*	
YJY1	W303-1α, *sec63Δ::HIS3*, pRS416 1 kb + *SEC63*	This study
YJY1 *SEC63_WT*	W303-1α, *sec63Δ::HIS3*, pRS415 1 kb + *SEC63*	
YJY1 *sec63_ΔN39*	W303-1α, *sec63Δ::HIS3*, pRS415 1 kb + *sec63_ΔN39*	
YJY1 *sec63_A179T*	W303-1α, *sec63Δ::HIS3*, pRS415 1 kb + *sec63_A179T*	
YJY1 *SEC63_WT-HA*	W303-1α, *sec63Δ::HIS3*, pRS315 1 kb + *SEC63-HA*	
YJY1 *sec63_ΔN39-HA*	W303-1α, *sec63Δ::HIS3*, pRS315 1 kb + *sec63_ΔN39-HA*	
YJY3	W303-1α, *SEC71::HA-G418, sec63Δ::HIS3*, pURASEC63	
YJY3 *SEC63_WT-FLAG*	W303-1α, *SEC71::HA-G418, sec63Δ::HIS3*, pRS415 1 kb + *SEC63-FLAG*	
YJY3 *sec63_ΔN39-FLAG*	W303-1α, *SEC71::HA-G418, sec63Δ::HIS3*, pRS415 1 kb + *sec63_ΔN39-FLAG*	

Sec72 subunits. Although Sec71 and Sec72 are dispensable for cell growth at 30 °C, translocation of a subset of proteins were defective upon their deletion [22–24]. Particularly, proteins containing an internal SS are shown to depend on Sec71 [7,25]. A recent cryo EM structure of the yeast SEC complex has revealed that Sec71 and Sec72 clamps Sec63 soluble domain in the cytosolic side and Sec63 interacts with the Sec61 complex through extensive contacts in its cytosolic, membrane and luminal domains [26,27].

Although it has been known that the Sec62/Sec63 complex functions mainly in post-translational translocation of proteins, recent studies present evidences that its subunits are also involved in co-translational translocation process. Translating ribosomes are found in close proximity to Sec63 [7]. A tetratricopeptide repeat (TPR) domain in Sec72 which is associated with membrane-anchored Sec71 and Sec63 interacts with cytosolic Hsp70 chaperones, Ssa1 and Ssb1 [28]. Ssb1 is associated with the ribosome-nascent chain, acting on co-translational translocation [29]. Hence, the functions of the Sec62/63 complex in co- and post-translational translocation still remain to be clarified.

Previously, it has been shown that deletion of the cytosolic Brl domain of Sec63 disrupted the SEC complex even though individual Sec61 trimer and Sec63/71/72 trimer subcomplexes were still intact [14]. The C-terminal acidic domain of Sec63 binds to the N-terminus of Sec62 having a number of basic residues [30,31]. In the luminal side, Sec63 contains a J-domain in its loop between TM2 and 3 where an ER luminal chaperon Kar2 (Bip in mammalian cells) associates with [32]. This association activates ATP hydrolysis by Kar2, providing driving energy for translocation of a nascent chain into the lumen [32–34].

The Brl domain and the J-domain of Sec63 are required for translocation of both SRP dependent and independent substrates [35] whereas truncation of the acidic domain of Sec63 was shown to selectively inhibit SRP-independent translocation [36]. While functions of these domains in Sec63 are better characterized, a role of the N-terminal region of Sec63 remained poorly defined until recent structure shows that it makes contacts with the Sec61 complex in the cytosol and the membrane [26,27].

To investigate the role of the Sec63 N-terminal region in protein translocation and formation of the SEC complex, we characterized a temperature sensitive Sec63 mutant that the N-terminal 39 residues including TM1 were deleted (Sec63_ΔN39). Assessing by the Blue-Native gel electrophoresis (BN-PAGE) and co-immunoprecipitation experiments, we found that integrity of the SEC complex was disrupted upon the N-terminal deletion of Sec63.

Translocation of precursors containing varying hydrophobicity of SSs that are SRP-dependent or Sec62-dependent was assessed by a metabolic labeling of Sec63_ΔN39 mutant cells. Among the precursors containing the hydrophobic SS, translocation of membrane proteins but not secretory precursors was severely impaired in the Sec63_ΔN39 mutant cell. These results suggest that beyond its role in post-translational translocation, Sec63 is essential for proper insertion of membrane proteins *in vivo*.

2. Materials and methods

2.1. Yeast strains

Yeast strains used in this study is listed in Table 1. The *Saccharomyces cerevisiae* haploid W303-1α was used as a WT strain [37]. *sec65-1* strain is isogenic strain to W303-1α [37]. JRY4 and JRY6 strains expressing each *SEC62* or mutant were constructed in [38]. All the *SEC63* and mutant strains were constructed as previously described [38]. Briefly, for YJY1 strain, W303-1α was transformed with pRS416 1 kb upstream + *SEC63*. Genomic *SEC63* was deleted by homologous recombination (HR) of transformed *HIS3* cassette amplified from pCgH [39]. The resultant cells were transformed with pRS415 encoding *SEC63*, *sec63A179T*, *sec63ΔN39* (First 39 amino acids deleted) or pRS315 encoding *SEC63-HA* or *sec63_ΔN39-HA*. pRS416 1 kb upstream + *SEC63* was removed by FOA selection. YJY3 strain was constructed by one step insertion cassette with *HIS* or G418 marker as previously described [40]. For the YJY3, genomic *SEC63* was deleted as done with YJY1 strain. YJY3 strain was transformed with pRS415 1 kb + *FLAG* tagged *SEC63* or *sec63_ΔN39* and subjected to FOA selection.

2.2. Construction of plasmids

Plasmids carrying CPY variants were cloned in [25]. Signal sequence of CPY WT had two versions that one contains Thr at position 5 and the other contains Ser at the same position. Plasmids encoding other model proteins were constructed in [41,42]. For construction of chimeras with an SS of yeast natural proteins and a CPY mature domain, the N-terminal 40 residue coding region from *IRE1*, *SCJ1*, *LHS1* was amplified and replaced the N-terminal 23 amino acids of *CPY* as previously described [25]. Signal anchored proteins were cloned and mutated as previously described [41]. The 1 kb upstream + *SEC63* was amplified from genomic DNA from W303-1α and cloned into *SmaI* digested pRS415 or pRS315HA vector by homologous recombination (HR). Mutations and FLAG epitope were introduced by a site-directed mutagenesis kit, following the manufacturer's protocol (Toyobo, Japan). Vectors encoding *SEC62* or mutant were constructed as described in [38]. All plasmids were confirmed by DNA sequencing.

2.3. Cell viability test

Each strain was cultured in appropriate selection medium at 30 °C overnight. Cells were diluted to OD_{600} (optical density at 600 nm) 0.1 and incubated at 30 °C until OD_{600} reached logarithmic growth phase. Samples were diluted to OD_{600} 0.1 and 10 μl of serially diluted samples was spotted on -Leu -His synthetic defined medium and incubated at indicated temperatures for 2 days.

2.4. Protein stability test

YJY1 *SEC63-HA* and *sec63_ ΔN39-HA* cells were grown at 30 °C until OD_{600} reached 1.0, and 10 OD_{600} unit cells were harvested by centrifugation at 3,200 rpm and washed with distilled H_2O. Cell pellets were resuspended with 100 μl SDS-PAGE sample buffer (50 mM DTT, 50 mM Tris-HCl pH 7.4, 5% SDS, 5 % glycerol, 50 mM EDTA, 1× protease inhibitor mix, 2 μg/ml PMSF), heated for 15 min at 65 °C, and centrifuged at 15,000 rpm for 5 min. 20 μl of supernatant was loaded on SDS-gel and subjected to Western blotting with anti HA and anti GAPDH antibody.

2.5. Pulse-labeling and immunoprecipitation

Metabolic protein labeling using [S^{35}]-MET in W303-1α, JRY4 *SEC62* and mutant, and YJY1 *SEC63* and mutant strains was carried out as previously described [41]. For *sec63_A179T* strain, cells were starved at 37 °C for 15 min. For *sec65-1* strain, cells were incubated at 37 °C for 30 min before starvation at 37 °C for 15 min. Protein samples were subjected to SDS-PAGE and visualized by autoradiography using Typoon™ FLA7000 (GE healthcare, USA). Quantitative data analysis was done by using Multi-Gauge version 3.0. For Fig. 2B pule-labeling experiments, one of the three sets of CPY(1.8) data included data of CPY WT (S5T).

2.6. Blue native-PAGE analysis

Yeast microsome isolation was adapted from methods previously described in [43,44] with the following modifications. Spheroplasts were resuspended in 10 ml homogenization buffer (containing 200 mM sorbitol and 1× protease inhibitor cocktail) and lysed by 20 strokes of glass homogenizer at 2500 rpm on ice. After sequential centrifugation of homogenized lysate, post-nuclear and mitochondria fraction was layered on glycerol cushion (20 mM Hepes-KOH, pH 7.4, 150 mM KOAc, 5 mM MgOAc, 1 mM DTT and 30% (vol/vol) glycerol) and centrifuged at 100,000 *g* at 4 °C for 1 h. Microsomes were washed with 3 ml of B88 buffer (20 mM Hepes-KOH, pH 6.8, 150 mM KOAc, 5 mM MgOAc, 250 mM sorbitol) and resuspended with small volume of B88 to final concentration of 100 μg/μl. 10 μl of solubilization buffer (10 mM Tris-HCl pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$, 1.5% digitonin, and 10% glycerol) was added to 10 μl of isolated microsomes and incubated on ice for 30 min. The solubilized microsomes were ultracentrifuged at 100,000 *g* for 20 min and 7 μl of supernatant was mixed with the same volume of dilution buffer (10 mM Tris-HCl pH 7.4, 1% digitonin, and 10% glycerol), 5 μl of 4× BN-PAGE sample buffer and 1 μl G-250 additive. Samples were subjected to gel electrophoresis and Western Blotting.

2.7. Co-immunoprecipitation

Co-immunoprecipitation experiments were carried out as previously described [38].

2.8. Carbonate extraction

Cells were grown at 30 °C until OD_{600} reached 1.0, and 10 OD_{600} unit cells were harvested at 3,000 *g* and washed with distilled H_2O. Cell pellets were solubilized with 200 μl lysis buffer (20 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8, 20 mM NaCl, 300 mM sorbitol, 1 mM PMSF and protease inhibitor cocktail), transferred to a 1.5 ml tube and vortexed with glass beads for 10 min at 4 °C. Cell debris was removed by a quick spin-down, washed once more with 200 μl of lysis buffer and the supernatant was combined with the previously collected supernatant. 75 μl of 400 μl collected cell lysate was stored as 'Total fraction'. The remaining samples were mixed with 0.1 M Na_2CO_3 (pH 11.5) and incubated on ice for 30 min. Then, it was centrifuged at 15,000 rpm for 20 min and supernatant was transferred for 'Supernatant fraction'. The pellet was resuspended with 200 μl of 0.1 M Na_2CO_3 (pH 11.5) and centrifuged at 15,000 rpm for 20 min. The supernatant was added to the 'Supernatant fraction' and the pellet was saved for 'Pellet fraction'. For the total fraction and the supernatant fraction, 150 μl and 700 μl of 25% TCA, respectively, were added, and 300 μl of 12.5% TCA was additionally added to the pelle fractions. After centrifugation for 15 min, the TCA precipitates were washed with acetone and 50 μl of sample buffer was added. Samples were heated at 65 °C for 15 min and analyzed by SDS-PAGE and Western blotting.

3. Results

3.1. The N-terminal truncation of Sec63 impairs both SRP-dependent and SRP-independent translocations

Previous biochemical studies have demonstrated how the C-terminal cytosolic and the luminal J-domain of Sec63 interacts with other subunits of the Sec translocon and their roles in the ER protein translocation [14,30,31,34]. While these regions of Sec63 are better characterized, physiological roles of the N-terminal region of Sec63 remain undefined. A recent cryo EM structure shows that the N-terminal TM domains of Sec63 form interaction surface with the Sec61 trimer [26,27].

To investigate the role of the N-terminal region of Sec63, we prepared a construct lacking the first 39 residues and tested whether it could replace Sec63 wildtype (WT) by a plasmid shuffling in yeast (Sec63_ΔN39. Fig. 1A, *left* and B). *sec63_ΔN39* strain was viable at 30 °C although exhibited a growth defect at 37 °C, indicating that truncation of the N-terminus did not interfere with correct targeting of Sec63 at permissive temperature (Fig. 1B).

Next, translocation ability of Sec63_ΔN39 mutant was assessed using carboxypeptidase Y (CPY) and dipeptidyl aminopeptidase (DPAPB, Dap2). CPY showed an SRP-independent, Sec62-dependent translocation to the ER while Dap2 exhibited opposite features for SRP and Sec62 dependency in [45]. CPY and Dap2 were transformed into *SEC63*, *sec63_A179T* and *sec63_ΔN39* strains and initial ER translocation efficiency of these proteins was assessed by a 5 min radiolabeling with [^{35}S]-Met (Fig. 1C). A point mutation of residue 179 in the luminal J-domain of Sec63 was shown to cause general translocation defects due to an impaired interaction with a luminal chaperone, Kar2 [34]. Translocation status of CPY and Dap2 was judged by the N-inked glycosylation that only occurs in the lumen, which results in a size upshift on SDS-gels. In the WT strain, CPY and Dap2 were efficiently glycosylated. Translocation of CPY and Dap2 was defective in both *sec63_A179T* and *sec63_ΔN39* mutant strains (Fig. 1C).

Previously, we found that mutations in the N-terminal basic residues of Sec62 (Sec62_35DDD) disrupted an interaction with Sec63 and impaired translocation of proteins carrying a moderately hydrophobic SS or a TM segment [38]. In the same study replacement of residues FPN218-220 to AAA in the downstream of the Sec62 TM2 resulted in a cell death [38]. It was observed that the C-terminal truncation including 218-220 residues exhibited a lethal phenotype in [31]. These studies indicated a functional importance of the region downstream of the TM2 in Sec62, and we prepared a substitution version, Sec62_P219A mutant. When the growth was assessed, its growth was comparable to *SEC62* and *sec62_35DDD* mutant strains (Fig. 1D). Next,

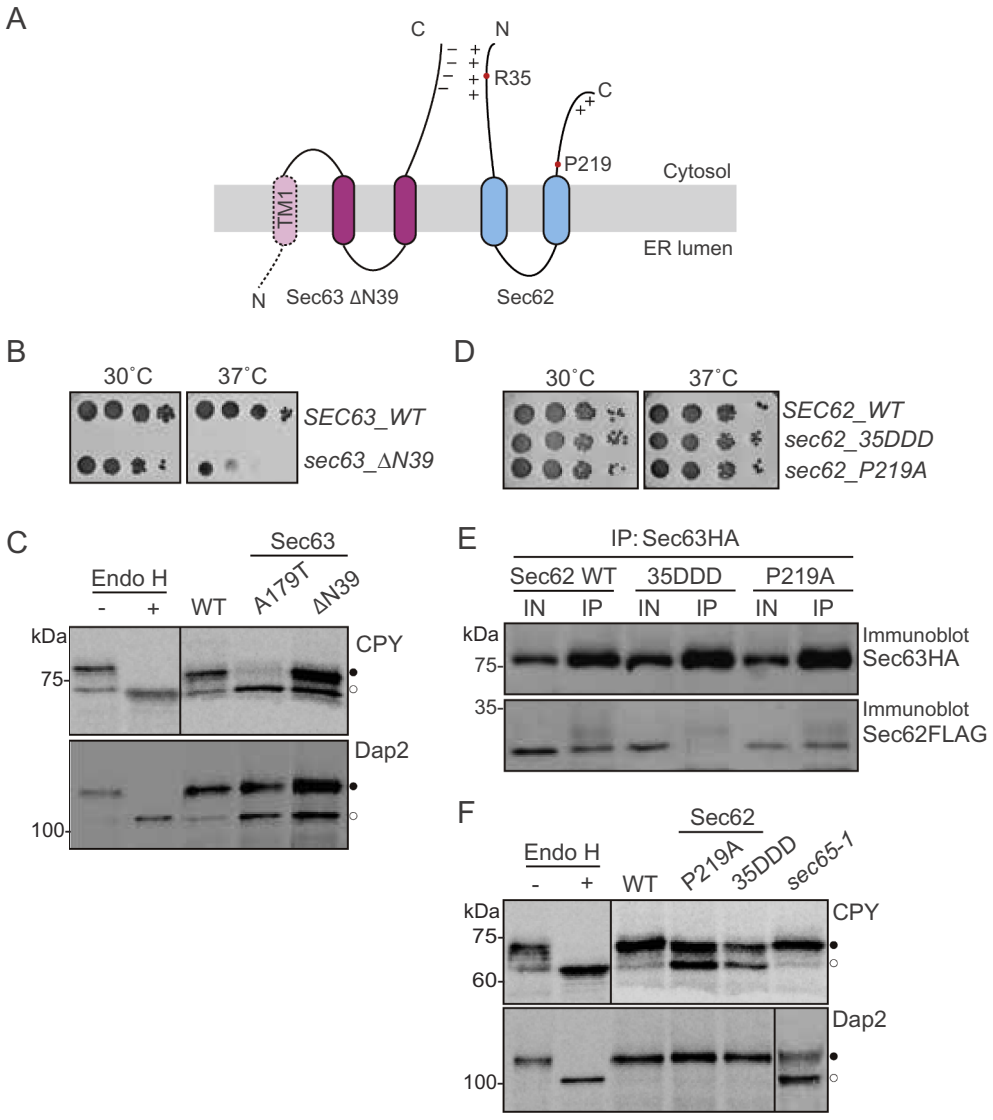

Fig. 1. Translocation of SRP dependent and independent precursors is defective in *sec63_ΔN39* strain. (A) Schematics of Sec63 and Sec62. The N-terminal 39 amino acids including TM1 was truncated in Sec63, indicated as a dotted line. Positions of mutations in Sec62 are indicated. (B) YJY1 strain carrying *SEC63* or *sec63_ΔN39* was grown at indicated temperatures for 2 days. (C) CPY (*top*) and Dap2 (*bottom*) were expressed in indicated YJY1 *SEC63_WT* and *sec63* mutant strains. Proteins were radiolabeled with [^{35}S]-Met for 5 min at 30 °C. For *sec63_A179T* strain, cell culturing and protein labeling conditions were modified as described in the materials and methods. Endoglycosidase H (Endo H) was added in the WT sample. Open and closed circles indicate non-glycosylated and glycosylated bands, respectively. (D) Cell viability of JRY4 strain carrying *SEC62* or mutants was assessed at indicated temperatures as done in Fig. 1B. (E) Crude membranes from JRY6 strains carrying *SEC62-FLAG* or *sec62* mutant-*FLAG* were solubilized with 1% Tx-100 and co-immunoprecipitated by using an HA antibody. Proteins were subjected to SDS-PAGE (12.5%) followed by Western blotting either with FLAG or HA antibodies. (F) Translocation of CPY (*top*) and Dap2 (*bottom*) expressed in JRY4 *SEC62_WT*, *sec62* mutants and *sec65-1* strains were examined as done in Fig. 1C. For *sec65-1* strain, cell culturing and protein labeling conditions were modified as described in the materials and methods.

we assessed interaction of Sec62 P219A with Sec63 (Fig. 1E). Sec63 remained bound to Sec62_P219A in co-IP, indicating that P219A mutation did not disrupt the interaction between Sec62 and Sec63. CPY and Dap2 proteins were expressed in Sec62 and SRP mutant strains and assessed their translocation efficiency (Fig. 1F). *sec65-1* is a temperature sensitive SRP defective strain [37]. CPY was efficiently translocated in *sec65-1* strain, but not in Sec62 mutant strain whereas Dap2 exhibited opposite translocation patterns in these mutant strains as previously shown in [45].

These data show that truncation of the N-terminal 39 residues of Sec63 leads to translocation defects of both CPY and Dap2. It was shown that translocation of Dap2 was unaffected in the Sec63 C-terminal truncation mutant [36]. The C-terminal end is where Sec63 interacts with Sec62. These observations thus suggest that translocation of Dap2 requires Sec63, independent of Sec62.

3.2. Translocation of secretory precursors is mildly or unaffected in *sec63_ΔN39 strain*

Dap2 contains an internal signal sequence (SS) that is hydrophobic and uncleaved by the signal peptidase (signal-anchored sequence). To verify which sequence features in Dap2 make it dependent on Sec63, we engineered CPY in two ways: one increasing the hydrophobicity of a SS and the other lengthening the N-terminus preceding a SS (Figs. 2A and 3A).

First, a set of CPY-based precursors carrying a SS of increasing hydrophobicity (CPY(ΔG_{app})) [25] was transformed into *sec63_ΔN39, sec62_P219A* and *sec65-1* yeast strains and their translocation efficiencies were assessed by a 5 min metabolic labeling of cells (Fig. 2B). *sec62_P219A* and *sec65-1* showed opposite translocation phenotypes that in *sec62_P219A* mutant, translocation of CPY variants having a marginally hydrophobic SS (CPY(1.8) and CPY(0.6)) was defective whereas in *sec65-1* strain, translocation of variants with a more hydrophobic SS (CPY(-0.6) and CPY(−1.8)) was defective. In *sec63_ΔN39* strain, translocation of less hydrophobic variants (CPY(1.8) and CPY (0.6)) was mildly impaired but that of more hydrophobic variants (CPY (−0.6) and CPY(−1.8)) was unaffected (Fig. 2B). Next, we prepared and assessed a set of chimera proteins that contain a hydrophobic cleavable SS from natural proteins fused with a CPY mature reporter domain. Here, translocation of chimera proteins was unaffected by Sec63_ΔN39 mutant, indicating that Sec63 is not required for translocation of proteins with a hydrophobic and cleavable SS (Fig. 2C).

3.3. Translocation of signal anchored proteins is defective in *sec63_ΔN39 strain*

Next, a set of CPY variants carrying the internal SS (signal anchored sequence) of varying hydrophobicity (D26CPY(ΔG_{app})) [25] was

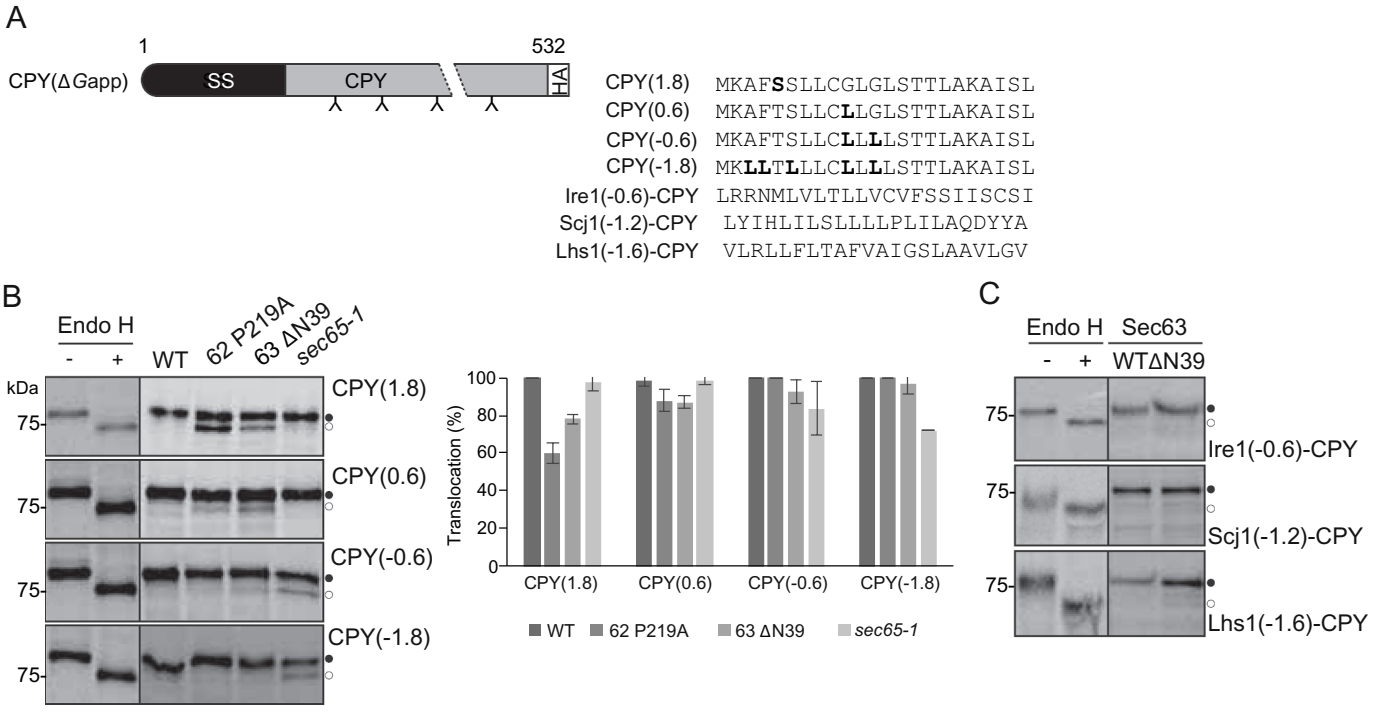

Fig. 2. Translocation of secretory proteins is mildly or unaffected in *sec63_ΔN39* strain.
(A) Schematics of CPY(ΔG_{app}) variants with a signal sequence (SS) colored in black. Sequences of SSs in CPY(ΔG_{app}) variants and chimera proteins are shown with mutated residues in bold and predicted ΔG_{app} values of SSs in parentheses. ΔG_{app} values were predicted by the ΔG predictor (http://dgpred.cbr.su.se/). Three copies of HA epitope were fused at the C-terminus of CPY(ΔG_{app}) variants for immunoprecipitation. 'Y' indicates an N-linked glycosylation site. Indicated CPY(ΔG_{app}) variants were expressed in W303-1α, JRY4 *sec62_P219A*, YJY1 *sec63_ΔN39* and *sec65-1* strains. Proteins were analyzed as done in Fig. 1C. (B) Translocation (%) of CPY(ΔG_{app}) variants was calculated as [glycosylated band ×100/Total products]. Average values of three independent experiments and the standard deviation are shown. (C) Translocation of chimera proteins containing indicated yeast natural protein SS and mature part of CPY was analyzed in YJY1 *SEC63* and *sec63_ΔN39* strains as done in Fig. 2B.

expressed in *sec63_ΔN39*, *sec62_P219A*, and *sec65-1* strains and their translocation efficiencies were assessed.

D26CPY(−2.1) was fully translocated whereas D26CPY(−0.6) was partially translocated in WT strain (Fig. 3B). Less hydrophobic D26CPY (−0.2) variant was not translocated at 5 min in all strains, showing that the internal SS needs to be more hydrophobic than the N-terminal SS for translocation and membrane insertion in the ER (Figs. 2B and 3B). Translocation of D26CPY(−0.6) was defective in both *sec62_P219A* and *sec63_ΔN39* mutant strains whereas translocation of more hydrophobic D26CPY(−2.1) was defective in *sec63_ΔN39* strain but not in *sec62_P219A* strain (Fig. 3B).

To check whether D26CPY(−2.1) was membrane anchored, alkaline carbonate extraction was done (Fig. 3C). CPY and Dap2 were detected mostly in supernatant and pellet fractions, respectively, as expected. CPY(−1.8) was found in the soluble fraction, indicating that the mature domain was released from the membrane after cleavage of the SS. In comparison, the majority of D26CPY(−2.1) was in the pellet fraction, indicating that it became membrane anchored (Fig. 3C). These data suggest that among the SRP-dependent proteins carrying hydrophobic SSs, Sec63_ΔN39 mutant is selectively defective for translocation of signal anchored type proteins.

To confirm that Sec63_ΔN39 mutant impairs translocation of membrane anchored type proteins, we prepared and tested yeast signal anchored proteins of varying TM domain hydrophobicity in *sec63_ΔN39* strain (Fig. 3D and Table 2). Albeit different degrees, translocation of these signal anchored proteins was defective except Pho8(A) (Fig. 3D), suggesting that the N-terminal 39 residues of Sec63 is required for efficient translocation of signal anchored proteins.

3.4. Topogenesis of type II signal anchored proteins is impaired in *sec63_ΔN39 strain*

To determine whether Sec63_ΔN39 mutant impairs other types of single-pass membrane proteins, we tested a set of model proteins that can insert in two orientations.

The *E.coli* leader peptidase based Lep-H1 proteins contain an engineered TM domain of varying hydrophobicity (Fig. 4A and Table 2). Since distribution of flanking charged residues of the TM domain is unbiased, it has potential to be integrated into two different orientations: a type I membrane topology form (N_{out}-C_{in}) and a type II membrane topology form (N_{in}-C_{out}) [42]. Different number of N-linked glycosylation occupancy; one in the N-terminus and two in the C-terminus in Lep-H1 protein allows an easy monitoring of targeting and membrane topology status. An unglycosylated (0 g) form represents a precursor form. Singly glycosylated (1 g) and doubly glycosylated (2 g) forms represent a type I membrane topology (N_{out}-C_{in}) and a type II membrane topology (N_{in}-C_{out}), respectively (Fig. 4A). These model single-pass membrane proteins were expressed in *sec63_ΔN39* strain and their membrane insertion efficiencies were measured by assessing glycosylation status (Fig. 4B).

Membrane insertion of relatively lower hydrophobicity variants (Lep-H1(5 L) and (6 L)) was severely defective in Sec63_ΔN39 mutant, (91% and 80% reduced in translocation compared to WT) (Fig. 4B). Although less severe, membrane insertion of Lep-H1(7 L) was also defective (~30% reduced compared to WT). For high hydrophobic Lep-H1(10*L*), translocation occurred efficiently but doubly glycosylated form was selectively reduced, indicating that membrane insertion as a type II orientation (N_{in}-C_{out}) was selectively reduced (Fig. 4C). In comparison, membrane insertion was defective only for the low hydrophobicity variants (Lep-H1(5 L) and (6 L)) in Sec62_P219A mutant

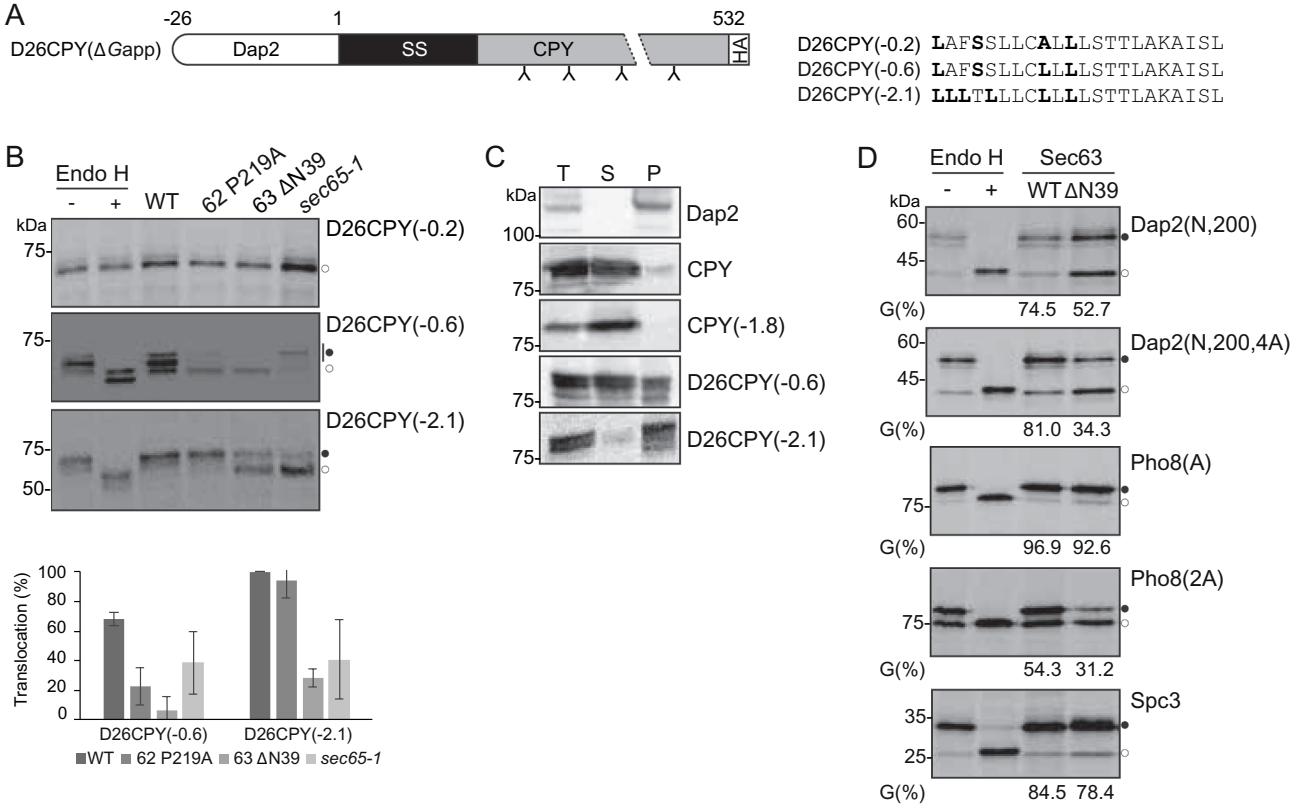

Fig. 3. Translocation of signal anchored proteins is impaired in *sec63_ΔN39* strain. (A) Schematics of D26CPY(ΔG_{app}) variants. The N-terminal 26 residues of Dap2 were fused to the N-terminus of CPY. Sequences and ΔG_{app} values of SSs are indicated in parentheses. Indicated D26CPY(ΔG_{app}) variants were expressed in W303-1α, JRY4 *sec62_P219A*, YJY1 *sec63_ΔN39* and *sec65-1* strains. Proteins were analyzed as done in Fig. 2B. Translocated, SS-cleaved and uncleaved species indicated with black line in D26CPY(−0.6). (B) Translocation (%) of D26CPY(ΔG_{app}) variants was calculated as in Fig. 2B. (C) Crude membranes were extracted from W303-1α strain expressing each indicated protein. Fractionated membranes were incubated with Na_2CO_3. Proteins in Total (T), Supernatant (S), Pellet (P) fractions were subjected to SDS-PAGE and analyzed by Western blotting using an HA antibody. (D) Signal anchored proteins of varying hydrophobicity (Table 2) were analyzed in YJY1 *SEC63* and *sec63_ΔN39* strains as done in Fig. 2B. 'G' indicates glycosylation (%) calculated as [(glycosylated × 100)/Total]. Average values of two independent experiments are shown.

Table 2
Sequences and hydrophobicity of TM domains of signal anchored proteins used in this study.

Proteins		Sequences	ΔG_{APP} (kcal/mol)
Lep	2 L	AAAALAAAAAAAAALAAAA	0.69
	3 L	AAAALAAAALAAAALAAAA	−0.06
	5 L	AAAALALAALAALALAAAA	−1.00
	6 L	AAAALALALALALALAAAA	−1.54
	7 L	ALAALALAALAALALAALA	−1.761
	10 L	LALALALALALALALALAL	−3.155
Dap2(N,200)		KLIRVGIILVLLIWGTVLLL	−2.9
Dap2(N,200,4A)		KLIRVGII**AAAA**IWGTVLLL	−0.8
Pho8(A)		KIIVSTVVCIGL**A**LVLVQLAF	−0.73
Pho8(2A)		KIIVSTVVCIG**AA**LVLVQLAF	−0.11
Spc3		AFSMGIVMVVFIMASSYYQLI	0.337
DT-Spc3		AFSMGIVMVVFIMASSYYQLI	0.337
Sec71		VYTPLIYVFILVVSLVMFASSYR	−1.5

Sequences and ΔG_{APP} (kcal/mol) values of TM domains of Lep proteins and predicted TM domains of yeast signal anchored proteins are shown. TM domains and ΔG_{APP} (kcal/mol) values were predicted by ΔG predictor. Leu residues and mutated amino acids are indicated with bold.

and the degree of defect was milder than in Sec63_ΔN39 mutant (Fig. 4B). Relative amounts of doubly glycosylated form were decreased in Sec62_P219A mutant for less hydrophobic Lep-H1(5 L), indicating that type II membrane orientation (N_{in}-C_{out}) was reduced (Fig. 4C).

To further confirm, we tested a dual topology version of Spc3 (DT-Spc3) which contains a Lysine residue at the N-terminal flanking side of the TM domain [41]. In *sec63_ΔN39* strain, doubly glycosylated band was markedly reduced, indicating that type II, N_{in}-C_{out} orientation was selectively decreased (Fig. 4D). Next, we tested translocation of type I membrane protein, Sec71 in *sec63_ΔN39* strain. Translocation of Sec71 was not affected by Sec63 ΔN39 mutant. These results suggest that Sec63 is required for topogenesis of type II membrane proteins.

3.5. Topogenesis of double pass membrane proteins is impaired in *sec63_ΔN39* strain

Next, we tested whether translocation of membrane proteins carrying more than one TM segment is defective in Sec63_ΔN39 mutant using Lep-derived model proteins with two potential TM segments (Lep-H2) [42]. Lep-H2 proteins contain TM1 of *E.coli* Lep that targets a protein to the ER and an engineered TM segment of varying hydrophobicity (H2) in the downstream (Fig. 4E and Table 2). Two N-linked glycosylation sites are positioned one in the upstream of TM1 and the other in the downstream of TM2, allowing an easy assessment of targeting and membrane insertion. When the first TM targets the protein to the ER, the N-terminal glycosylation site is modified. Subsequent C-terminal translocation and membrane insertion of TM2 lead to modification of the second glycosylation site in the downstream of TM2 (Fig. 4E). It has been previously shown that when TM2 is integrated into the membrane it could be cleaved by the ER signal peptidase, generating a cleaved glycosylated product [42]. Thus, the cleaved product is resulted only when TM2 is membrane inserted. Lep-H2 proteins carrying the H2-segment of varying hydrophobicity were

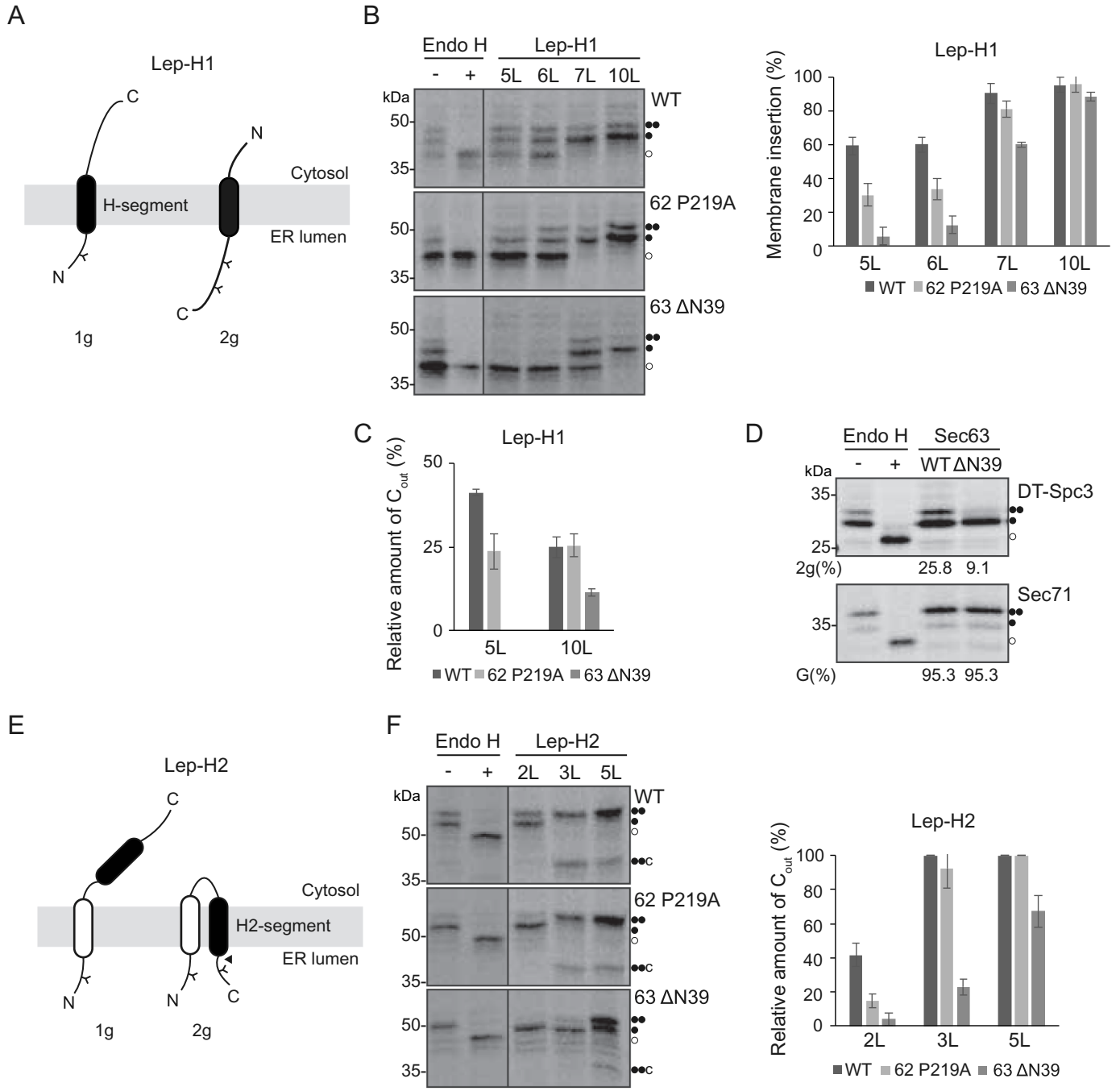

Fig. 4. Topogenesis of membrane proteins is impaired in *sec63_ΔN39* strain. (A) Schematics of an *E.coli* Lep-based H1 (Lep-H1) with a hydrophobic (H) segment composed of varying number of Leucine and Alanine residues shown in black. Three copies of HA epitope were fused at the C-terminus of Lep-H1 variants for immunoprecipitation. (B) Indicated Lep-H1 variants were expressed in W303-1α, JRY4 *sec62_P219A* and YJY1 *sec63_ΔN39* strains. Proteins were analyzed as done in Fig. 2B. Membrane insertion (%) was calculated as [(1 g + 2 g) x 100 / Total proteins]. (C) The relative amount of C_{out} (%) (type II topology) was calculated as [2 g × 100 / (1 g + 2 g)]. Average values of three independent experiments and the standard deviation are shown. (D) Translocation of dual topology variant of Spc3 (DT-Spc3) and type I membrane protein, Sec71 was assessed in YJY1 *SEC63* and *sec63_ΔN39* strains as done in Fig. 2B. DT-Spc3 has three N-linked glycosylation sites, one in the N-terminus and the others in the C-terminus. Sec71 has two N-linked glycosylation sites in the N-terminus. 'G' was calculated as in Fig. 3D. '2 g' was calculated as [(2 g * 100)/(1 g + 2 g)]. Average values of two independent experiments are shown. (E) Schematics of an *E.coli* Lep-based H2 (Lep-H2). The hydrophobic (H2) segment is composed of varying number of Leucine and Alanine residues, shown in black. N-linked glycosylation sites are indicated as "Y". (F) Indicated Lep-H2 variants were expressed in W303-1α, JRY4 *sec62_P219A* and YJY1 *sec63_ΔN39* strains. Proteins were analyzed as done in Fig. 4B. 'Two closed circle with C' indicates a cleaved product derived from doubly glycosylated form. The relative amount of C_{out} (%) was calculated as [(2 g + 2 g cleaved) x 100/(Total - 0 g)]. Average values of three independent experiments and the standard deviation are shown.

expressed in *sec62_P219A* and *sec63_ΔN39* strains and their translocation and membrane insertion efficiencies were assessed (Fig. 4F). All Lep-H2 model proteins were efficiently targeted to the ER as no unglycosylated product was detected. The C-terminal translocation of Lep-H2 with less hydrophobic TM (2 L) was defective in Sec62_P219A mutant whereas the C-terminal translocation of all variants was defective in Sec63_ΔN39 mutant. These data suggest that Sec63 mediates topogenesis of double-pass membrane proteins.

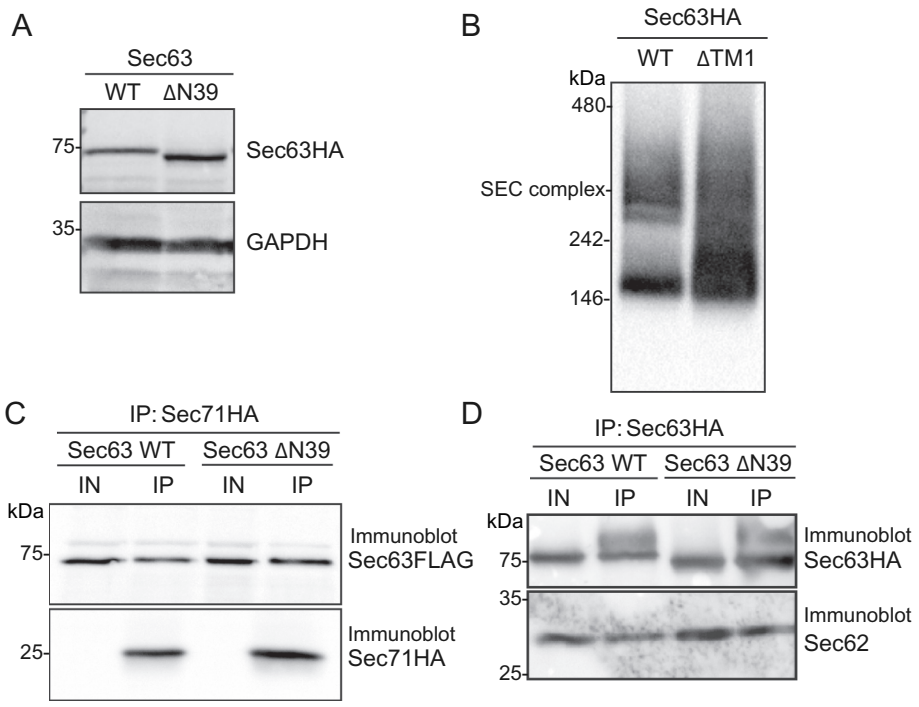

Fig. 5. The SEC complex is destabilized in *sec63_ΔN39 strain*. (A) Whole cell lysates from YJY1 strain carrying *SEC63-HA* or *sec63_ΔN39-HA* strains were analyzed by SDS-PAGE and Western blotting using HA and GAPDH antibodies. (B) Microsomes were isolated from YJY1 strain carrying *SEC63-HA* or *sec63_ΔN39-HA* were analyzed by BN-PAGE and Western blotting using an HA antibody. (C) Crude membranes from YJY3 strain carrying *SEC63-FLAG* or *sec63 ΔN39-FLAG* were solubilized and co-immunoprecipitated by using an HA antibody. Proteins were subjected to SDS-PAGE (12.5%) followed by Western blotting either with HA or FLAG antibodies. (D) Co-immunoprecipitaion experiment was carried out with YJY1 strain carrying *SEC63-HA* or *sec63_ΔN39-HA* as done in Fig. 5C. Proteins were visualized by using HA or Sec62 antibodies.

3.6. The SEC complex is destabilized in sec63_ΔN39 strain

To find out causes of translocation defects in *sec63_ΔN39* strain, first, stability of Sec63 and Sec63_ΔN39 was examined by Western blotting of whole cell lysates obtained from WT and *sec63_ΔN39* strain. Amount of Sec63_ΔN39 was comparable to that of Sec63, indicating that the stability of Sec63 was not compromised in *sec63_ΔN39* strain (Fig. 5A).

Next, to determine whether integrity of the SEC complex was altered upon the N-terminal deletion of Sec63, we carried out the Blue Native (BN)-PAGE and co-immunoprecipitation (IP) experiments.

Microsomes from WT and *sec63_ΔN39* strains were prepared and solubilized with a non-ionic detergent, digitonin, the condition of which has been used to purify the SEC complex [13,14]. Lysates from solubilized microsomes were resolved on BN-PAGE and detected by antibodies directed to an HA-tagged Sec63 (Fig. 5B). In the WT sample, Sec63 was detected in two distinct bands whereas only the lower band was detected in the Sec63_ΔN39 sample. Based on the size and Western blotting profiles shown in [13,14], the large band was estimated to be the SEC complex containing the subunits of the Sec61 and Sec62/Sec63 complexes. These data suggest that the heptameric SEC complex was destabilized in *sec63_ΔN39* mutant strain.

In co-IP experiments, the interaction of Sec63 with Sec71 or Sec62 was intact in *sec63_ΔN39* strain (Fig. 5C and D), indicating that the stability of Sec62/63 complex was stable in *sec63_ΔN39* strain. Considering destabilization of the SEC complex in *sec63_ΔN39* strain, judging by BN-PAGE, the interaction of Sec63 with Sec61 may be compromised.

4. Discussion

How the Sec61 translocon mediates translocation and membrane insertion of diverse types of proteins destined to the secretory pathway remains enigmatic. The sequence characteristics of SS and TM segments, both of which function in initiating translocation *via* the Sec61 channel are highly diverse. The Sec62/Sec63 complex aids the Sec61 complex to properly translocate a subset of precursors. Yet, it is unclear how their association impacts sorting of selective precursors.

To investigate the role of the Sec63 N-terminal domain in sorting of precursor proteins, we prepared and characterized a Sec63_ΔN39 mutant lacking the first 39 residues. Determining complex formation and subunit interactions of the SEC complex by BN-PAGE and co-IP experiments, we found that the SEC complex was destabilized in Sec63_ΔN39 mutant. A recent cryoEM structure shows that Sec63 makes extensive interactions with other subunits of the SEC complex [26,27]. The N-terminus of Sec63 points toward the luminal pore between the loop 5 and 6 of Sec61, and TM1 contacts the Sec61 complex in the membrane. Although point mutations in the N-terminus of Sec63 did not compromise its function [26], it is conceivable that deletion of the N-terminal 39 residues which includes TM1 of Sec63 could have a substantial impact on stability of the SEC complex. Notably, high sequence homology of the N-terminus and TM1 of Sec63 among the species was observed [26].

It was expected that destabilized SEC complex would impair post-translational translocation. However, translocation of CPY, known Sec62-dependent, post-translational translocation substrates was relatively mildly defective in the Sec63_ΔN39 mutant than membrane proteins. These results suggest that Sec63 functions in sorting of hydrophobic membrane proteins beyond its role in post-translational translocation.

Sec71 and Sec72 that tightly associate with Sec63 were originally selected from a yeast genetic screen rescuing the defects of membrane protein translocation [46]. Lately, the EMC has been shown to mediate biogenesis of multi-pass membrane proteins co-translationally [47]. Interestingly, it has been observed that patterns of enriched proteins in ribosome-Sec63 proximity resembles those of ribosome-EMC components proximity, hinting that Sec63 also co-translationally mediates multi-pass membrane proteins [7,47].

A notable feature of the membrane proteins that were defective in translocation in Sec63_ΔN39 mutant is that they contain soluble domains preceding and following the TM sequence, suggesting that membrane proteins with soluble domains residing in both sides of the membrane may need Sec63 for the ER translocation. The cryoEM structure shows that Sec63 is positioned optimally to coordinate chaperones at both ends of the translocation channel [26, 27]. Folding rate of soluble domains in a membrane protein can influence topogenesis of membrane proteins [48]. It is tempting to speculate that Sec63 associates with Sec61 and recruits chaperones at both ends of the channel to transiently stabilize flanking soluble loops for proper positioning of a TM domain during membrane protein topogenesis.

Funding

This work was supported by grants from National Research Foundation of Korea (NRF-2016R1A2B2013459) and Promising-Pioneering Researcher Program through Seoul National University to HK.

Conflict of interests

The authors declare no conflicts of interest with the contents of this article.

Author contributions

SJ, YJ and HK conceived the project, analyzed data and wrote the manuscript. SJ and YJ conducted experiments.

Acknowledgements

Authors thank Tina Junne (University of Basel) for kindly providing a yeast strain carrying the genomically HA tagged SEC61, and the lab members for discussion and critical reading of the manuscript.

References

[1] R.J. Keenan, D.M. Freymann, R.M. Stroud, P. Walter, The signal recognition particle, Annu. Rev. Biochem. 70 (2001) 755–775.
[2] M. Wiedmann, A. Huth, T.A. Rapoport, A signal sequence is required for the functions of the signal recognition particle, Biochem. Biophys. Res. Commun. 134 (1986) 790–796.
[3] T.V. Kurzchalia, M. Wiedmann, A.S. Girshovich, E.S. Bochkareva, H. Bielka, T.A. Rapoport, The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle, Nature 320 (1986) 634–636.
[4] E.A. Costa, K. Subramanian, J. Nunnari, J.S. Weissman, Defining the physiological role of SRP in protein-targeting efficiency and specificity, Science 359 (2018) 689–692.
[5] T. Ast, G. Cohen, M. Schuldiner, A network of cytosolic factors targets SRP-independent proteins to the endoplasmic reticulum, Cell 152 (2013) 1134–1145.
[6] K. Plath, T.A. Rapoport, Spontaneous release of cytosolic proteins from post-translational substrates before their transport into the endoplasmic reticulum, J. Cell Biol. 151 (2000) 167–178.
[7] C.H. Jan, C.C. Williams, J.S. Weissman, Principles of ER cotranslational translocation revealed by proximity-specific ribosome profiling, Science 346 (2014) 1257521.
[8] T.A. Rapoport, Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes, Nature 450 (2007) 663–669.
[9] B. Van den Berg, W.M. Clemons Jr., I. Collinson, Y. Modis, E. Hartmann, S.C. Harrison, T.A. Rapoport, X-ray structure of a protein-conducting channel, Nature 427 (2004) 36–44.
[10] T.A. Rapoport, L. Li, E. Park, Structural and mechanistic insights into protein translocation, Annu. Rev. Cell Dev. Biol. 33 (2017) 369–390.
[11] R.M. Voorhees, R.S. Hegde, Structure of the Sec61 channel opened by a signal sequence, Science 351 (2016) 88–91.
[12] R.M. Voorhees, R.S. Hegde, Toward a structural understanding of co-translational protein translocation, Curr. Opin. Cell Biol. 41 (2016) 91–99.
[13] Y. Harada, H. Li, J.S. Wall, H. Li, W.J. Lennarz, Structural studies and the assembly of the heptameric post-translational translocon complex, J. Biol. Chem. 286 (2011) 2956–2965.
[14] A.J. Jermy, M. Willer, E. Davis, B.M. Wilkinson, C.J. Stirling, The Brl domain in Sec63p is required for assembly of functional endoplasmic reticulum translocons, J. Biol. Chem. 281 (2006) 7899–7906.
[15] S. Panzner, L. Dreier, E. Hartmann, S. Kostka, T.A. Rapoport, Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p, Cell 81 (1995) 561–570.
[16] R.J. Deshaies, S.L. Sanders, D.A. Feldheim, R. Schekman, Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex, Nature 349 (1991) 806–808.
[17] J. Tyedmers, M. Lerner, C. Bies, J. Dudek, M.H. Skowronek, I.G. Haas, N. Heim, W. Nastainczyk, J. Volkmer, R. Zimmermann, Homologs of the yeast Sec complex subunits Sec62p and Sec63p are abundant proteins in dog pancreas microsomes, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 7214–7219.
[18] H.A. Meyer, H. Grau, R. Kraft, S. Kostka, S. Prehn, K.U. Kalies, E. Hartmann, Mammalian Sec61 is associated with Sec62 and Sec63, J. Biol. Chem. 275 (2000) 14550–14557.
[19] B.J. Conti, P.K. Devaraneni, Z. Yang, L.L. David, W.R. Skach, Cotranslational stabilization of Sec62/63 within the ER Sec61 translocon is controlled by distinct substrate-driven translocation events, Mol. Cell 58 (2015) 269–283.
[20] N. Johnson, S. Hassdenteufel, M. Theis, A.W. Paton, J.C. Paton, R. Zimmermann, S. High, The signal sequence influences post-translational ER translocation at distinct stages, PLoS One 8 (2013) e75394.
[21] A.K. Lakkaraju, R. Thankappan, C. Mary, J.L. Garrison, J. Taunton, K. Strub, Efficient secretion of small proteins in mammalian cells relies on Sec62-dependent posttranslational translocation, Mol. Biol. Cell 23 (2012) 2712–2722.
[22] D. Feldheim, R. Schekman, Sec72p contributes to the selective recognition of signal peptides by the secretory polypeptide translocation complex, J. Cell Biol. 126 (1994) 935–943.
[23] T. Kurihara, P. Silver, Suppression of a sec63 mutation identifies a novel component of the yeast endoplasmic reticulum translocation apparatus, Mol. Biol. Cell 4 (1993) 919–930.
[24] D. Feldheim, K. Yoshimura, A. Admon, R. Schekman, Structural and functional characterization of Sec66p, a new subunit of the polypeptide translocation apparatus in the yeast endoplasmic reticulum, Mol. Biol. Cell 4 (1993) 931–939.
[25] C. Yim, S.J. Jung, J.E.H. Kim, Y. Jung, S.D. Jeong, H. Kim, Profiling of signal sequence characteristics and requirement of different translocation components, Biochim. Biophys. Acta Mol. Cell Res. 1865 (2018) 1640–1648.
[26] X. Wu, C. Cabanos, T.A. Rapoport, Structure of the post-translational protein translocation machinery of the ER membrane, Nature 566 (2019) 136–139.
[27] S. Itskanov, E. Park, Structure of the posttranslational Sec protein-translocation channel complex from yeast, Science 363 (2019) 84–87.
[28] A. Tripathi, E.C. Mandon, R. Gilmore, T.A. Rapoport, Two alternative binding mechanisms connect the protein translocation Sec71-Sec72 complex with heat shock proteins, J. Biol. Chem. 292 (2017) 8007–8018.
[29] K. Doring, N. Ahmed, T. Riemer, H.G. Suresh, Y. Vainshtein, M. Habich, J. Riemer, M.P. Mayer, E.P. O'Brien, G. Kramer, B. Bukau, Profiling Ssb-nascent chain interactions reveals principles of Hsp70-assisted folding, Cell 170 (2017) 298–311 (e220).
[30] M. Willer, A.J. Jermy, B.P. Young, C.J. Stirling, Identification of novel protein-protein interactions at the cytosolic surface of the Sec63 complex in the yeast ER membrane, Yeast 20 (2003) 133–148.
[31] S. Wittke, M. Dunnwald, N. Johnsson, Sec62p, a component of the endoplasmic reticulum protein translocation machinery, contains multiple binding sites for the Sec-complex, Mol. Biol. Cell 11 (2000) 3859–3871.
[32] B. Misselwitz, O. Staeck, K.E. Matlack, T.A. Rapoport, Interaction of BiP with the J-domain of the Sec63p component of the endoplasmic reticulum protein translocation complex, J. Biol. Chem. 274 (1999) 20110–20115.
[33] K.E. Matlack, B. Misselwitz, K. Plath, T.A. Rapoport, BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane, Cell 97 (1999) 553–564.
[34] A.K. Corsi, R. Schekman, The lumenal domain of Sec63p stimulates the ATPase activity of BiP and mediates BiP recruitment to the translocon in Saccharomyces cerevisiae, J. Cell Biol. 137 (1997) 1483–1493.
[35] S.K. Lyman, R. Schekman, Interaction between BiP and Sec63p is required for the completion of protein translocation into the ER of Saccharomyces cerevisiae, J. Cell Biol. 131 (1995) 1163–1171.
[36] B.P. Young, R.A. Craven, P.J. Reid, M. Willer, C.J. Stirling, Sec63p and Kar2p are required for the translocation of SRP-dependent precursors into the yeast endoplasmic reticulum in vivo, EMBO J. 20 (2001) 262–271.
[37] B.M. Wilkinson, J.R. Tyson, C.J. Stirling, Ssh1p determines the translocation and dislocation capacities of the yeast endoplasmic reticulum, Dev. Cell 1 (2001) 401–409.
[38] S.J. Jung, J.E. Kim, J.H. Reithinger, H. Kim, The Sec62-Sec63 translocon facilitates translocation of the C-terminus of membrane proteins, J. Cell Sci. 127 (2014) 4270–4278.
[39] K. Kitada, E. Yamaguchi, M. Arisawa, Cloning of the Candida glabrata TRP1 and HIS3 genes, and construction of their disruptant strains by sequential integrative transformation, Gene 165 (1995) 203–206.
[40] M.S. Longtine, A. McKenzie 3rd, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, J.R. Pringle, Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae, Yeast 14 (1998) 953–961.
[41] J.H. Reithinger, J.E. Kim, H. Kim, Sec62 protein mediates membrane insertion and orientation of moderately hydrophobic signal anchor proteins in the endoplasmic reticulum (ER), J. Biol. Chem. 288 (2013) 18058–18067.
[42] C. Lundin, H. Kim, I. Nilsson, S.H. White, G. von Heijne, Molecular code for protein insertion in the endoplasmic reticulum membrane is similar for N(in)-C(out) and N(out)-C(in) transmembrane helices, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 15702–15707.

[43] R.J. Deshaies, R. Schekman, SEC62 encodes a putative membrane protein required for protein translocation into the yeast endoplasmic reticulum, J. Cell Biol. 109 (1989) 2653–2664.
[44] J.A. Rothblatt, D.I. Meyer, Secretion in yeast: reconstitution of the translocation and glycosylation of alpha-factor and invertase in a homologous cell-free system, Cell 44 (1986) 619–628.
[45] D.T. Ng, J.D. Brown, P. Walter, Signal sequences specify the targeting route to the endoplasmic reticulum membrane, J. Cell Biol. 134 (1996) 269–278.
[46] N. Green, H. Fang, P. Walter, Mutants in three novel complementation groups inhibit membrane protein insertion into and soluble protein translocation across the endoplasmic reticulum membrane of Saccharomyces cerevisiae, J. Cell Biol. 116 (1992) 597–604.
[47] M.J. Shurtleff, D.N. Itzhak, J.A. Hussmann, N.T. Schirle Oakdale, E.A. Costa, M. Jonikas, J. Weibezahn, K.D. Popova, C.H. Jan, P. Sinitcyn, S.S. Vembar, H. Hernandez, J. Cox, A.L. Burlingame, J.L. Brodsky, A. Frost, G.H. Borner, J.S. Weissman, The ER membrane protein complex interacts cotranslationally to enable biogenesis of multipass membrane proteins, Elife 7 (2018).
[48] A.J. Denzer, C.E. Nabholz, M. Spiess, Transmembrane orientation of signal-anchor proteins is affected by the folding state but not the size of the N-terminal domain, EMBO J. 14 (1995) 6311–6317.