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이학석사학위논문

Sec62/63 번역후전좌 복합체에서 Sec71의 기능 조사

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이 논문을 이학석사학위논문으로 제출함

2014년 12월

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2014년 12월

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Investigation on the Function of Sec71 Protein in the Sec62/Sec63 Post-translational Translocation Complex

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A Thesis submitted in partial Fulfillment

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Abstract

Membrane and secretory proteins are synthesized by ribosomes in cytoplasm and targeted to the endoplasmic reticulum (ER) membrane via two pathways: co translational translocation pathway and post translational translocation pathway.

In case of post translational translocation pathway it has been reported that Sec61 works with distinct hetero-tetrameric protein complex called Sec62/Sec63 complex. Sec63 complex consists of Sec62, Sec63 essential proteins and Sec71, Sec72 which are dispensable.

Sec62p was previously shown to play a role on the translocation of moderately hydrophobic single and multi spanning membrane proteins.

Since Sec71p and Sec62p belong to the same Sec62/Sec63 complex, we were curious about the role of Sec71 on the translocation of membrane proteins.

To investigate the function of Sec71 in translocation and or insertion of secretory and membrane proteins, a deletion strain lacking *SEC71* was constructed. When a set of model proteins was expressed in *sec71::HIS3* (sec71 deletion strain), the translocation of some presecretory proteins was impaired.

In case of membrane proteins, the effect of *SEC71* deletion was more intense in case of single spanning (Lep-H1 model protein) where sec71 deletion abolished the targeting of moderately hydrophobic proteins and still attenuated the targeting even for the ones having a higher hydrophobicity. The multi spanning membrane proteins (H2 and H3) were affected only with the moderately hydrophobic TMD which insertion was decreased in absence of Sec71p.

Our results on translocation and membrane insertion profiles of single spanning (H1) and multispanning membrane proteins (H2 and H3) in sec71 deletion cell suggest two possibilities: the Sec71 may be responsible for the stability of the Sec62/Sec63 complex or Sec71p may stabilize directly the translocating proteins which are less hydrophobic or large which may require more energy to be translocated across the ER. Sec71p may with some interactions with other translocation components help these proteins to position better for optimal translocation and membrane insertion.

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I. List of abbreviations

Transmembrane domain (TMD)

Endoglycosidase H (EndoH)

Endoplasmic reticulum (ER)

Immunoprecipitation (IP)

Wild type (WT)

Hydrophobic segment (H-segment)

Signal Recognition Particle (SRP)

Dithiothréitol (DTT)

II List of tables

Model proteins	Predicted ΔG_{app}
ppαF	1.706
CPY	1.471
DPAP B	-0.025

Table1. Hydrophobicity (ΔG_{app}) of ER-targeting sequences of presecretoty proteins pp**α**F, CPY and DPAP B.

Protein	Name of TM segment	Sequence of Test TM Segment	Hydrophobicity (ΔG)
H1	4L	**GGPGAAAALALAAAAALALAAAAGPGG**	-0.490
	5L	**GGPGAAAALALAALAALALAAAAGPGG**	-0.999
	6L	**GGPGAAAALALALALALALAAAAGPGG**	-1.540
	7L	**GGPGALAALALAALAALALAALAGPGG**	-1.76
	10L	**GGPGLALALALALALALALALGPGG**	-3.115
H2	3L	**GGPGAAAALAAAALAAAALAAAAGPGG**	-0.006
	5L	**GGPGAAAALALAALAALALAAAAGPGG**	-0.999
H3	5L	**GGPGAAAALALAALAALALAAAAGPGG**	-0.999
	7L	**GGPGALAALALAALAALALAALAGPGG**	-1.76

Table2. Sequence and Hydrophobicity (ΔG) of the test TM segments (H1, H2 and H3-segment) in membrane model proteins

III List of figures

IV Introduction

Trafficking and sorting of proteins through the intra-organellar system have been a focus of interest since Palade (1975) and coworkers formulated the general outline of the secretory pathway in pancreatic exocrine cells. This pathway, common to all eukaryotic cells, defines the transport route by which secretory proteins are delivered from the endoplasmic reticulum (ER) to the plasma membrane via the Golgi apparatus. In yeast, 30% of nascent polypeptides translated from the ribosome are targeted to the endoplasmic reticulum (ER) (Ghaemmaghami et al., 2003), in what is viewed as the initial step of the secretory pathway. It is widely acknowledged that a signal sequence, which is a hydrophobic stretch of amino acids, located within the N-terminal end of these proteins targets them to the ER (Sabatini et al., 1971) (Milstein et al., 1972).

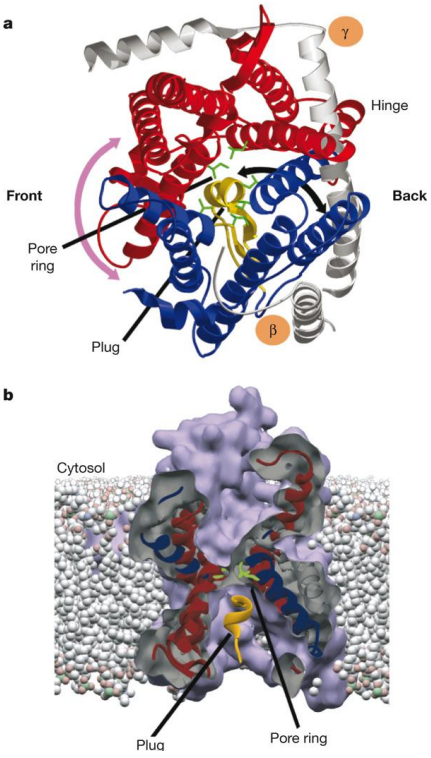


Figure 1.The translocation channel.
A) The crystal structure of the SecY complex from *Methanococcus jannashii* viewed from the cytoplasmic side. The purple double-headed arrow represents how the lateral gate may open. The plug (yellow) is found blocking the aqueous pore and its movement dictates opening and closure of the pore. B) Cross-sectional view of the channel. (Rapoport, 2007)

Secretory proteins enter the ER after or concomitant with their synthesis on cytoplasmic ribosomes in a process known as translocation. In either case, nascent secretory proteins must be targeted to the translocation machinery at the ER membrane and translocate into the ER lumen through the translocation channel called Sec61 in eukaryotic cells or SecY in prokaryotic cells which provides hydrophilic channel across the ER membrane (Osborne et al., 2005; Van den Berg et al., 2004). The protein conducting channel of Sec61p (Figure 1), which comprises of 10 TMDs provides the access for proteins across the ER membrane in the case of secretory protein, and the lateral exit of membrane proteins into the lipid phase (Martoglio et al., 1995). Together with the Sec61p, Ssh1p, Sss1p, Sec63p, Sec62p, Sec71p and Sec72p form the Sec translocon (Figure2), where only Sec61p, Sec63p, Sec62p and Sss1p are essential for viability (Deshaies et al., 1991).

Molecular chaperones in the cytosol and ER lumen assist translocation and facilitate protein folding and assembly in

the lumen. Proteins that achieve their native conformation exit the ER and continue through the secretory pathway. Incompletely folded or unassembled proteins are recognized by a constitutively active quality control pathway in the ER that identifies aberrant proteins and targets them for destruction in the cytosol by the proteasome. This process is known as ER associated degradation (ERAD).

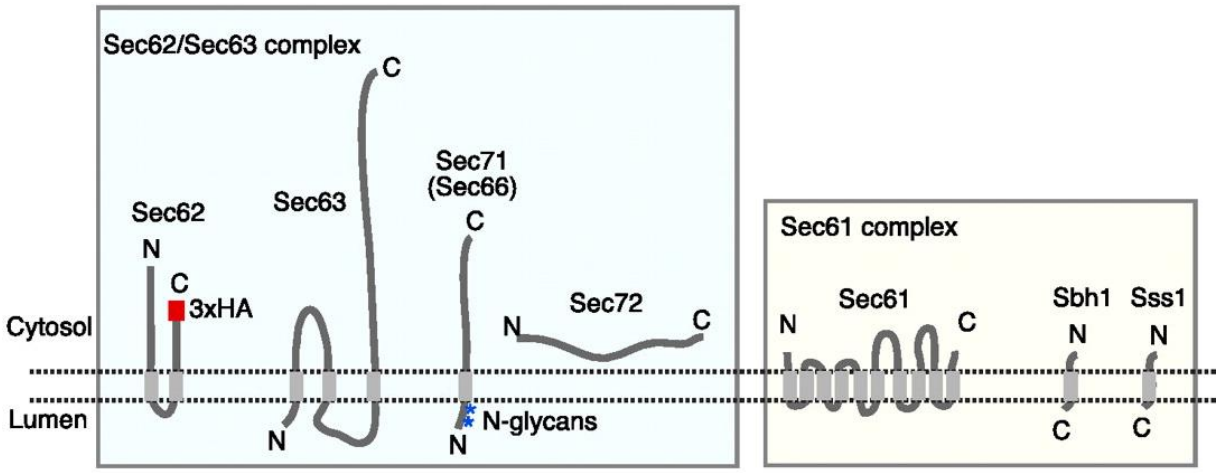


Figure2. Subunit composition of the post-translocon. The two boxes represent the Sec62/Sec63 complex and Sec61 complex. (Harada 2011)

Protein translocation can occur either cotranslationally, during which insertion into the ER lumen or membrane occurs concomitant with protein synthesis, or post-translationally, in which translocation occurs after a polypeptide has been completely synthesized. In either mechanism, the translocation reaction involves: the identification and targeting of proteins to the ER, the association of proteins with the ER translocation machinery, including a pore through which proteins enter the ER, the energy-dependent import of proteins into the ER lumen or membrane, and protein folding and maturation in the ER.

A. Co-translational translocation

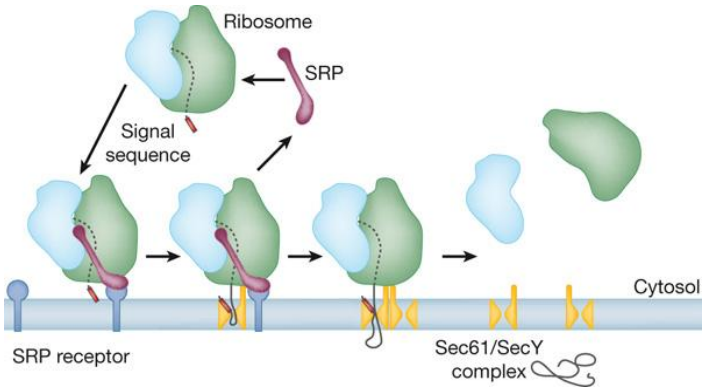
Most proteins that are secretory, membrane-bound, or reside in the endoplasmic reticulum (ER), Golgi or endosomes use the co-translational translocation pathway. This

process begins with the N-terminal signal peptide of the protein being recognized by a signal recognition particle (SRP) while the protein is still being synthesized on the ribosome. This stalls translation temporarily and the ribosome-nascent chain (RNC) SRP complex is targeted to the ER membrane, where binding of SRP to its cognate receptor allows docking of the ribosome to the translocation channel. Once SRP is released from a signal sequence, translation is resumed and polypeptide begins to be translocated through a pore of Sec61. During translocation, if Sec61 recognizes a hydrophobic segment, it is inserted into the ER membrane through the proposal lateral gate (Plath et al., 1998). (Figure3.A)

B. Post-translational translocation

Even though most secretory proteins are co-translationally translocated, some are translated in the cytosol and later transported to the ER/plasma membrane by a post-translational system.
It has been reported that Sec61 works with distinct hetero-tetrameric protein complex called Sec63 complex (Deshaies et al., 1991; Lyman and Schekman, 1997; Panzner et al., 1995; Stirling et al., 1992). It was reported that Sec63 complex consists of Sec62, Sec63 essential proteins and Sec71, Sec72 which are dispensable (Plath and Rapoport, 2000; Tyedmers et al., 2000). In comparison to co-translational translocation, after completion of translation of polypeptides, it is targeted to the ER membrane. During the targeting step, to prevent aggregation of polypeptide, it is protected by chaperon protein such as Hsp70 (Huber et al., 2005). These protected polypeptides are targeted to the ER by an unknown mechanism (Petersen et al., 2011). (Figure3.B) But, previous cross-linking study reported that pre-pro-alpha factor is cross-linked with Sec62, Sec71 and Sec72 in the absence of ATP. On the other hand, in the presence of ATP, pre-pro-alpha factor formed cross-linking product with Sec61 (Lyman and Schekman, 1997) which imply that Sec62, 71 and 72 may function as a receptor in post-translational translocation pathway.

A



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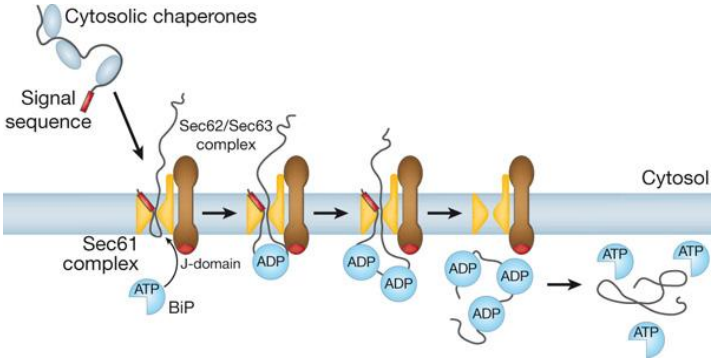

Figure 3. A. Model of co-translational translocation. B.Model of post-translational translocation (Rapoport, 2007)

C. Identification of Sec71 protein

SEC71 encodes the 31.5-kDa transmembrane glycoprotein (p31.5) of the Sec63p-BiP complex. SEC71 is identical to SEC66 (HSS1), which was shown also to encode p31.5. DNA sequence analyses reveal that *sec71-1* cells contain a nonsense mutation that removes approximately two-thirds of the cytoplasmic C-terminal domain of p31.5. The *sec72-1* mutation shifts the reading frame of the gene encoding p23. Unexpectedly, the sec71-1 mutant lacks p31.5 and p23. Neither mutation is lethal, although *sec71-1* cells exhibit a growth defect at 37°C. These results show that p31.5 and p23 are important for the trafficking of a subset of proteins to the ER membrane.

Sec63p, a DnaJ homologue (Sadler et al., 1989), interacts with a 31.5-kDa glycoprotein (p31.5) and a 23-kDa protein (p23) in two distinct complexes containing either Kar2p or Sec62p. The Sec63 complex contains Sec63p, Sec62p, p31.5, and p23 (Deshaies et al., 1991), and the Sec63p-BiP complex contains Sec63p, Kar2p, p31.5, and p23 (Brodsky and Schekman, 1993). Kar2p shares homology with mammalian BiP/GRP78 (Normington et al., 1989; Rose et al., 1989). The gene encoding p31.5, termed *SEC71* or *SEC66* (also *HSS1*), was cloned and sequenced (Feldheim et al., 1993; Kurihara and Silver, 1993). The encoded protein contains a single transmembrane segment and is glycosylated near the N-terminus. Components of the Sec63 complex can be cross-linked to Sec6lp and precipitated as a multi subunit complex (Deshaies et al., 1991).

Although the previous studies concerning Sec71p were not numerous, they were mainly showing the temperature sensitivity due to the deletion of SEC71 gene and the translocation defect of few secretory proteins as consequence of this deletion.
Sec62p was previously shown to play a role on the translocation of moderately hydrophobic single and multi spanning membrane proteins.
Since Sec71p and Sec62p belong to the same Sec62/Sec63 complex, we were curious about the role of Sec71 on the translocation of membrane proteins. The main goal was to determine whether Sec71p affects translocation and insertion of the membrane proteins and if so whether it handles a subset of membrane proteins depending on the hydrophobicity and/or the number of transmembrane domains or all membrane proteins.

V Materials and methods

A. Model proteins

All plasmids were constructed from p424GPDHA (Lundin et al., 2008) by site-directed mutagenesis and homologous recombination (Oldenburg et al., 1997). The sequence of each gene in the plasmid encoding for our tested proteins was confirmed by DNA sequencing then transformed into the strains W303-1α and *sec71::HIS3*. These strains were grown in selective plates (-tryptophan) for 2days at 30°C. The colonies were used for the protein translocation assay which it will be described later.

B. Yeast strains

We tried to reconstitute a strain having a genomic deletion of SEC71 and a plasmid carrying the SEC71 wild type version. This strain was constructed by homologous recombination and plasmid shuffling. SEC71 with 1kb upstream region (1kb+SEC71) was amplified with 5'-ATACCCGGGTAAAGCCTGGGGTGCCTAATGAGTGAGC-3' and 5'-CACTTTATGCTTCCGGCTCGTATG-3' from the genomic DNA of wild type W303-1α strain.

1kb+SEC71 was cloned into the pRS415 plasmid which was *SmaI* linearized then transformed to the Sec71 deletion strain.

For the deletion strain *sec71::HIS3*, genomic SEC71 gene was substituted with HIS selection marker amplified with primers 5'-GGGAGAAGAGTGGGCTTTTATAATTGCAGTTGAATGCAGTCACAGGAAACAGCTATGACC-3' and 5'-GAAGGTTTATACAGTAGAGCTATACAGGATAATGGAAGTGTTGTAAAACGACGGCCAGT-3' from pCgH vector by homologous recombination.

C. Growth assay

Cells were grown in selective media overnight. After measuring OD_{600}, each transformant was subjected to 5-fold serial dilution, and cells were grown in selective plates at different temperatures (23°C, 30°C or 37°C) for 2days.

D. Western blot analysis

Yeast transformants carrying the various protein constructs were grown in 5 ml –Trp or –His –Trp media at 30°C overnight, depending on the strain used, W3031α or *sec71::HIS3*, respectively. Cells were harvested by centrifugation at 3000 *g*, washed with dH_2O, resuspended in 100 µl SDS-PAGE sample buffer, centrifuged down and the supernatant fractions were heated for 15 min at 55°C, then loaded onto 6.5% ,10% or 12.5% SDS gels, depending on the protein size and subsequently subjected to Western blotting using anti HA antiserum. For endoglycosidase H (Endo H) digestion, 15 µl of the whole-cell lysate was mixed with 10.5 µl dH_2O, 4 µl Endo H buffer (800 mM sodium acetate, pH 5.8), and 1.5 µl of Endo H (5 U/ml; Roche) or dH_2O for the mock treatment and incubated at 37°C for 2 h.

E. Pulse chase-labeling and immunoprecipitation

Cells for pulse-chase labeling were either grown at 30°C till an OD_{600} reached between 0.2-0.8 per timepoint, 1.5 OD_{600} units of cells were harvested by centrifugation at 3000 *g*, washed twice with –Met medium without ammonium sulfate, and incubated at 37°C for 15 min. Cells were centrifuged and resuspended in 450 µl of –Met medium without ammonium sulfate and labeled with 15 µl of [^{35}S]-Met (40 µCi/1.5 OD_{600} units of cells) for 5 min at 25°C. Labeling was stopped and chased by addition of 50 µl of 200mM cold Met per 1.5 OD_{600} units for 0, 15, and 60 min. The reaction was stopped by addition of 750 µl of ice-cold stop solution buffer, centrifuged down, and cell pellets were resuspended in 110 µl lysis buffer (20 mM Tris-HCl, pH7.5, 1% SDS, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail (Complete, Roche) and mixed with 100 µl of ice-cold acid-washed glass beads (Sigma). Cell suspensions were vortexed at maximum speed for 3 min. Then, samples were incubated at 65°C for 15 min and centrifuged for 5 min at 20,000 *g*. The supernatant fractions were mixed with 500 µl IP buffer (15 mM Tris-HCl, pH7.5, 0.1% SDS, 1% Triton X-100, and 150 mM NaCl), 1.5 µl mouse anti-HA antibody (Covance, California, USA), and 50 µl of prewashed protein G-agarose beads (Roche; 33% slurry in IP buffer) and rotated at 4°C overnight. The agarose beads were washed three times with IP buffer, once with ConA buffer (500 mM NaCl, 20 mM Tris-HCl, pH7.5, and 1% Triton X-100), and once with buffer C (50 mM NaCl and 10 mM Tris-HCl, pH7.5). Then the beads were incubated with 55 µl of SDS-PAGE sample buffer at 65°C for 15 min, centrifuged down, and the supernatant fractions were loaded onto 6.5% SDS gels. Endo H treatment was carried out as described above. Radiolabelled bands on SDS gels were

quantified using a Fuji FLA-3000 phosphoimager and the Image Reader V1.8J/Image Gauge V 3.45 software.

VI Results

1. Confirmation of the deletion of *SEC71*

Previous studies have shown that Sec71 protein, among the Sec62/Sec63 complex, is a nonessential protein that the deletion of this gene does not affect to the cell viability. However, the absence of Sec71 exhibits growth defect at a non permissive temperature (37°C).

In order to study the role of Sec71p in the translocation of proteins, we first decided to prepare a yeast strain lacking Sec71p to observe the consequence of the absence of this protein from the post translational translocation complex on the translocation of different proteins. For this reason the *SEC71* was substituted with the *HIS3* selection marker by homologous recombination. To verify that the gene was effectively deleted, we first examined the resulting transformants by colony PCR. Each colony was treated with heat to break the membranes then the buffer mix containing two primers which one complements the upstream sequence of SEC71 and the other complements the *HIS3* selection marker region was added. The resulted product was resolved on agarose gel. (Figure4.A)

In Figure4 (A), the first lane was loaded with the size marker. The second lane is the sample from the wild type (WT) yeast strain basically carrying the genomic SEC71 and the third lane is from a putative sec71Δ strain. The expected *HIS* marker size of 1.5KDa was detected only on the third lane, indicating that SEC71 gene was properly replaced with the *HIS* cassette in the genomic DNA. In addition our WT negative control in the second lane did not show any band indicating the primers failed amplification without *HIS* gene incorporation into the chromosome. (Figure4.A)

It is previously shown that sec71 deletion strain is a temperature sensitive strain which does not grow at non permissive temperature. To further confirm that our deletion strain exhibits temperature sensitivity we carried out a growth assay at different temperatures.

Compared to a yeast wild type strain, sec71Δ strain grew at a similar level to the wild type at permissive temperatures (25°C and 30°C). In contrast the effect of the deletion of *SEC71* was shown at higher temperature where the cell growth was prevented comparing to the control

strain (WT) at 37°C. Therefore the temperature sensitivity of *sec71::HIS3* (sec71Δ strain) shown from previous studies is confirmed. (Figure4 (B)).

In addition, *sec71*Δ strain showed a sensitivity in presence of high concentration of DTT. (Figure5). At 4mM of DTT the sec71Δ strain growth is highly slow down and this growth is totally suppressed at 6mM of DTT concentration.

The deletion of *SEC71* was confirmed by both PCR data and growth assay.

2. Recovery of growth defect by Sec71 wild type

To test the growth defects caused by Sec71 deletion, first we carried out a growth recovery assay using the reconstituted isogenic Sec71 wild type.

For that we used the co-transformation method allowing the introduction of a plasmid encoding the wild type copy of Sec71 or an empty vector (as a control) in the background of the Sec71 deletion strain.

Same numbers of cells were grown then the isogenic wild type strain was spotted on the YPD plates. A recovery in growth was observed at a non permissive temperature (37°C) and the cells grew similarly to those from wild type strain. Therefore our isogenic Sec71 wild type was able to recover the growth defect caused by the Sec71 deletion and showed a similar growth rate as the wild type strain.

Next to check the recovery of translocation defect by Sec71deletion, we carried out a translocation assay using a single spanning model protein which we introduced into the isogenic wild type via co-transformation (Figure 4.C). The used model protein H1-5L has one glycosylation site on its N terminus and two glycosylation site on its C terminus as shown in the scheme of the Figure4 (D). When entering the ER a glycan is added to the glycosylation sites in which single glycosylation results in about 2 kDa shift in the protein's molecular weight so that when loaded into the gel it migrates slower than the unglycosylated protein.

If the protein is properly translocated, its transmembrane domain (TMD) should be embedded in the ER membrane with two alternative cases: a singly glycosylated H1-5L (1G) if it inserts with its N-terminus in the lumen or a doubly glycosylated H1-5L (2G) whose C-terminus is in

the lumen. If the protein is not translocated but remains in the cytosol, the H1-5L protein exists as the unglycosylated form (0G). When the protein is completely translocated into the ER lumen, a triply glycosylated form (3G) appears.

The translocation assay was carried out at two different temperatures: a non permissive temperature (37°C) and a permissive temperature (30°C).

As shown in Figure4 (D), at 30°C the H1-5L was mostly targeted to the ER generating predominant 1G form in the wild type strain (W303-1α). However in case of sec71Δ or also the Sec71 isogenic wild type the prominently detected band was the unglycosylated one (0G) meaning that the H1-5L was not efficiently targeted to the ER and was kept in the cytosol. A plasmid carrying WT *SEC71* was not able to rescue the translocation defects, showing similar translocation pattern to that of Δ*sec71*. Even at the permissive temperature the isogenic wild type strain did not recover the defect of translocation induced by the deletion of Sec71p from the Sec62/Sec63 complex. The same result was shown at a higher temperature where the isogenic wild type could not recover the translocation defect of H1-5L.

Although the SEC71 isogenic WT recovered the growth at non permissive temperature it did not recover the translocation defect caused by the deletion of sec71. Therefore we decided to carry out our study using a yeast wild type strain as a control for the next experiments.

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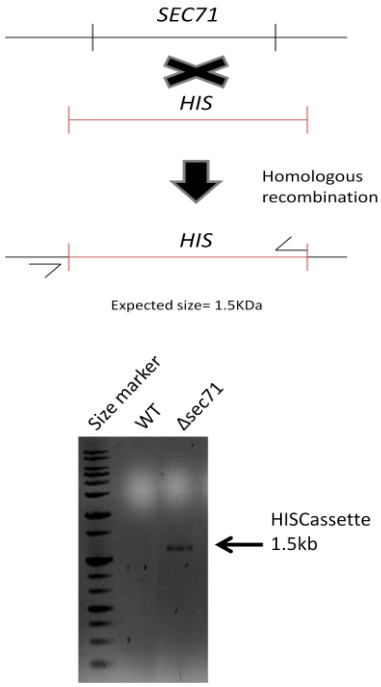

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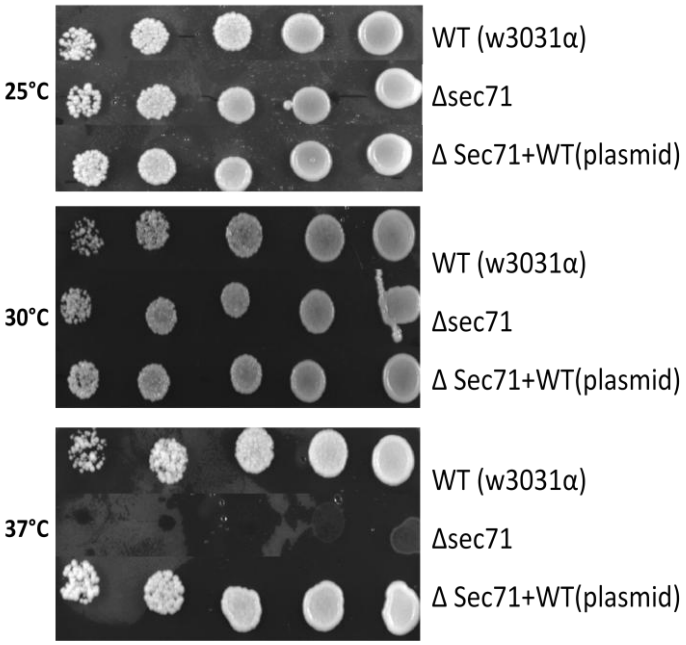

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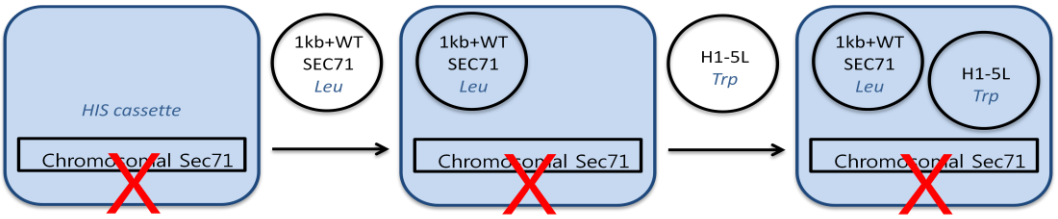


Negative control	Sec71 Δ + empty pRS415 + H1-5L
Test construct	Sec71 Δ + 1kb-sec71 + H1-5L
Positive control	W3031α + H1-5L

*Constructs are grown at 30°C and 37°C

Temperature		30°C			37°C		
Plasmid	strain	sec71del		W3031α	Sec71 del		W3031α
pRS415		Empty	1kb+ Sec71	-	Empty	1kb+ Sec71	-
p424		H1-5L	H1-5L	H1-5L	-	H1-5L	H1-5L

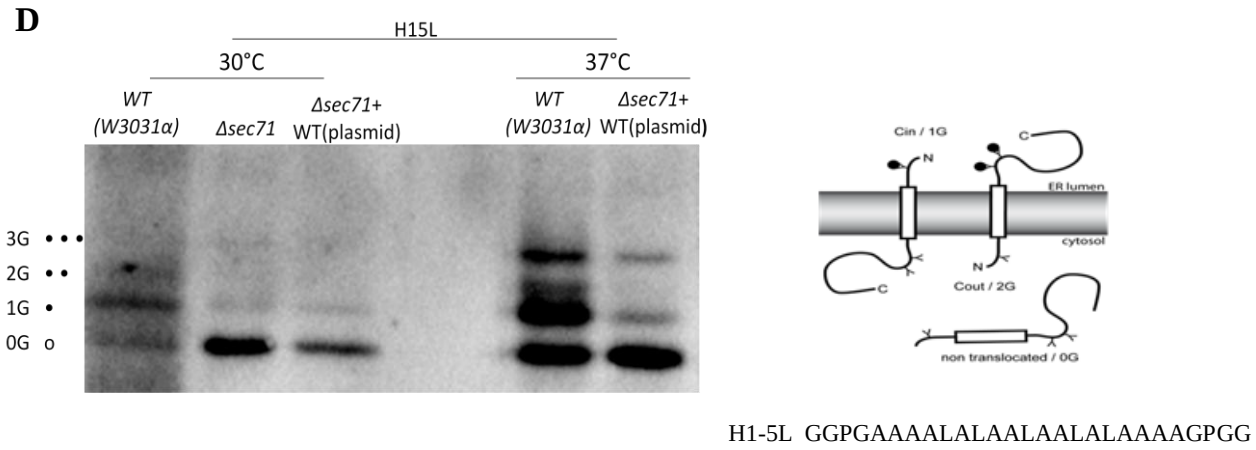


Figure4. Preparation of the yeast strains used in the study. (A) Verification of the substitution of *SEC71* gene with the HIS selection marker by Polymerase chain reaction (PCR). The PCR product was ran in 5% agarose gel. The HIS cassette amplifying primers are shown with arrows. The detected band corresponds to the expected size of 1.5KDa. (B) Wild type (*W3031α*), *sec71::HIS3 and sec71::HIS3* + WT (plasmid) strains were grown at different temperatures. (C) Schematic representation of the co-transformation method used to make the isogenic wild type Sec71. The table presents the translocation assay scheme plan.
(D) Translocation assay using H1-5L model protein. Cells expressing H1-5L were grown at either a permissive temperature 30°C or a non permissive temperature 37°C and the model protein translocation was detected via western blotting using the anti HA antibody.

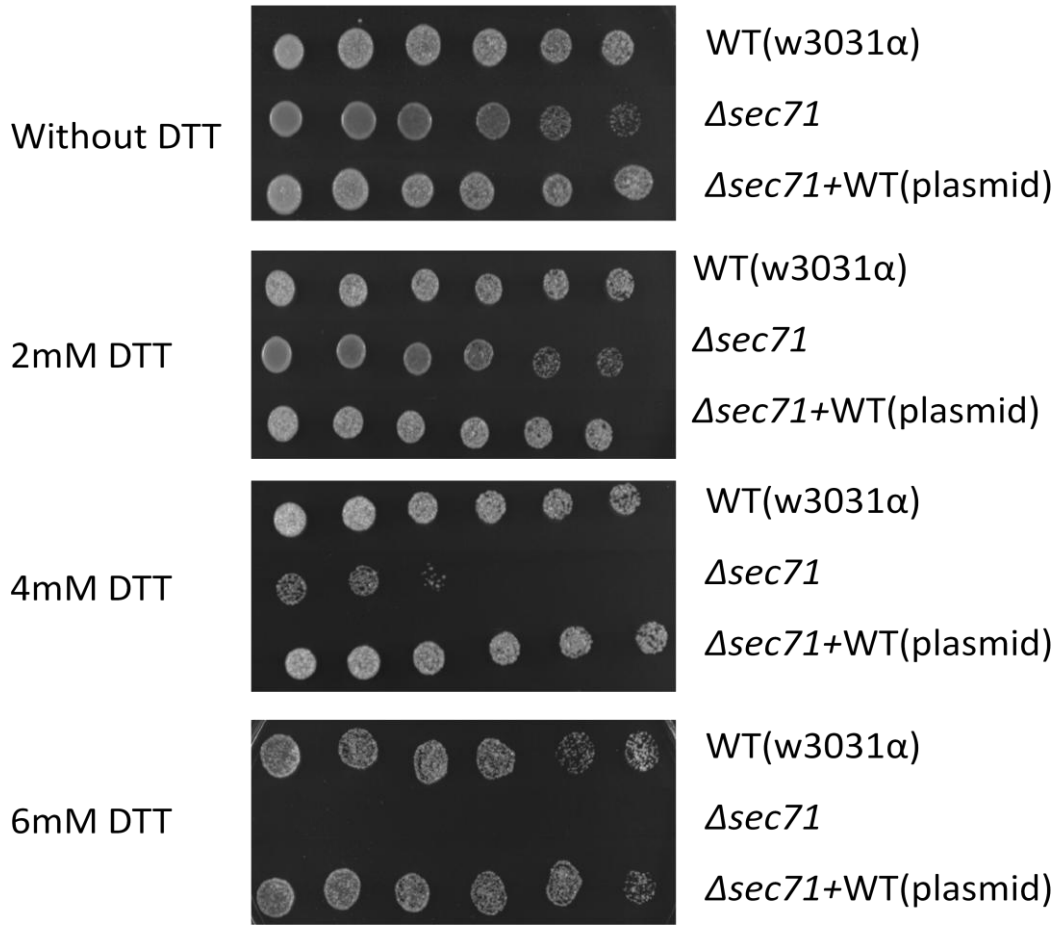


Figure5. DTT sensitivity test. Wild type (*W3031α*), *sec71::HIS3 and sec71::HIS3* + WT (plasmid) strains were grown at different DTT concentrations.

3. Translocation assay with presecretory proteins

Analysis of the effect of *sec71::HIS3* on the presecretory proteins

To investigate the effects of the deletion of Sec71p, translocation of a few secretory proteins into the ER lumen was tested. The protein translocation assay allows determining not only the translocation state of the tested proteins but also the intensity of the defect caused.
The translocation assay is based on the different states of the secretory proteins (precursor form and translocated form).

We tested three proteins, signal sequence which show different hydrophobicity. The hydrophobicity (ΔG) represents the quantity of energy required for a TM segment/signal sequence 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.

First protein we tested is carboxypeptidase Y (CPY) which is known to be a post-translationally translocated substrate with a hydrophobicity scale [1] of ΔG=1.471. Transformants containing CPY in either wild type or Sec71 defective strains were subjected to pulse chase labeling. This experiment allows us to investigate in greater depth the effect of *sec71*Δ on the translocation of our model protein because it allows following the protein state during time.
Comparing to the wild type strain, sec71 deletion strain showed a slower translocation of CPY as the unglycosylated form (pCPY) was predominant at 0min time point and the glycosylated form (CPY) appeared at later time point.
These results, in the absence of Sec71p from Sec62/Sec63 complex, may indicate that CPY takes a longer time to cross the ER membrane and has difficulties to get into the translocon and rather remains in the cytosol.
Therefore we suggest that the stability and the integrity of the Sec62/Sec63 complex in absence of Sec71p may be disrupted.

Hydrophobicity scales are values that define relative hydrophobicity of amino acid residues. The more positive the value, the more hydrophobic are the amino acids located in that region of the protein.

Another hypothesis can be raised is that the Sec71p may help recognizing and/or recruiting the proteins to the ER translocon. In addition, CPY is known to be translocated through the post translational translocation pathway in which the Sec62/Sec63 complex seems to be more involved than in the co-translational translocation pathway as it was shown from earlier studies. In this way the perturbation of the complex by deleting Sec71p may explain the difficulty that CPY gets to across the ER membrane.
This idea might be demonstrated more clearly by testing other various sets of proteins.

The second presecretory protein tested was dipeptidylaminopeptidase B (DPAP B) with $\Delta G = -0.025$. This protein was studied via pulse labeling and resolved by SDS-PAGE. When we compare the wild type strain to the *sec71::HIS3*, DPAP B showed two bands case of the Sec71 deletion strain: one corresponds to the glycosylated form and the other one corresponds to the unglycosylated form which means that there is a defect in the translocation of DPAP B in the absence of Sec71p. But the translocation defect in this case is not intense therefore the band intensity is quite low. From previous studies DPAP B is known to be a co translational translocated substrate so that the presence of the ribosome may help this substrate's translocation which explains the low effect of the absence of Sec71p. In addition, the hydrophobicity may also be an important factor allowing the translocation of DPAP B even in the absence of Sec71p, since DPAP B has a much more hydrophobic TMD than CPY that explains the different effect of sec71 deletion in both cases.

A third presecretory protein the pre pro alpha factor (ppαF) with signal sequence of a $\Delta G = 1.706$ was tested. The ppαF in the background of the wild type strain or sec71 deletion strain (sec71Δ) was radiolabeled and subjected to SDS-PAGE. The result did not show any defect in translocation of ppαF in the absence of Sec71p comparing to wild type strain. The profiles of the two strains show a similar result and the substrate is successfully translocated into the ER lumen that we could detect the glycosylated form of the protein referring to the endoglycosidase treatment.
Although this result was not expected because the *sec71::HIS3* strain showed a translocation defect of ppαF previously so we tried to compare the protein tested to find a plausible explanation of lack of effect in this case.

Comparing the ppαF to the CPY protein, we found a comparable hydrophobicity (both of them are moderately hydrophobic) but the difference resides in the body of the protein. The ppαF has a short body comparing to a longer one of CPY. So we thought that maybe the ppαF is translocated more easily without the help of Sec71p unlike of CPY and because of its length, which might require an additional help to cross the ER membrane.

For this reason and to confirm our hypothesis, we tested different constructs carrying the signal sequence of ppαF and the mature part of CPY and the other with the signal sequence of CPY and the mature part of ppαF.

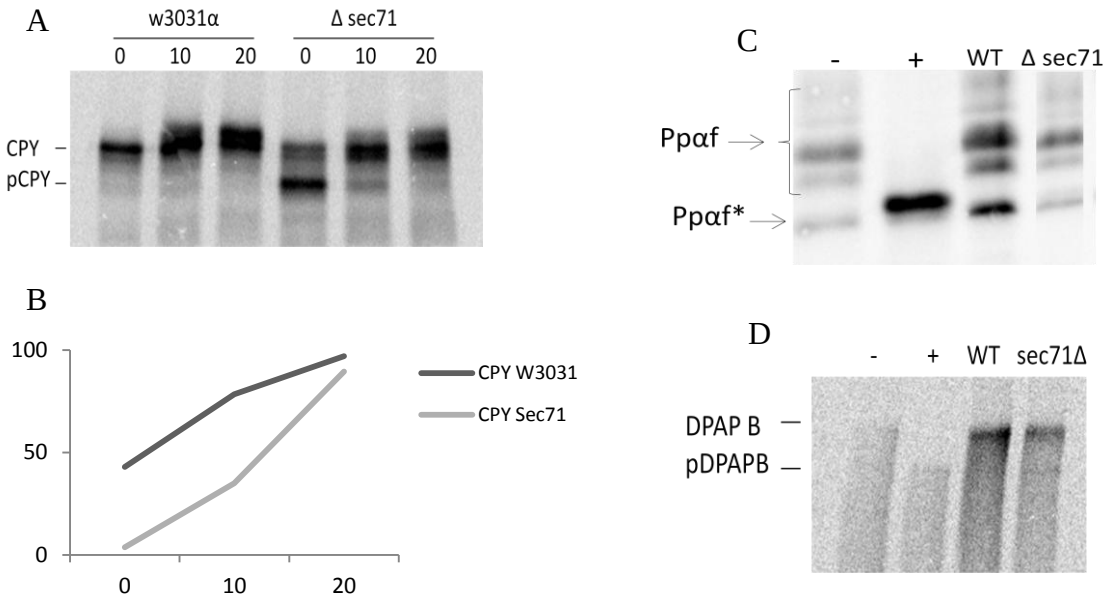


Figure6. The effect of *sec71::HIS3* on the presecretory proteins translocation. A.pulse chase experiment was carried out with wild type and *sec71::HIS3* (sec71Δ) expressing CPY at the indicated time. B. Quantification of the glycosylated CPY from in A. C. 5min pulse with wild type and Δsec71 expressing ppαF. D. 5min pulse with wild type and Δsec71 expressing DPAP B

We swapped the signal sequences between ppαF and CPY and tested their translocation by pulse labeling. As shown in figure 6, the signal sequence of CPY fused to ppαF body was successfully translocated into the ER lumen in both wild type strain and Sec71 deletion strain as we detected the glycosylated form of the protein referring to the EndoH treatment.

For the fused signal sequence of the ppαF to CPY body, we detected the glycosylated form of CPY in both wild type and deletion strain of Sec71, meaning that when the signal sequence of CPY was changed to that of ppαF, the protein could be translocated into the ER membrane whereas translocation with its own signal sequence was impaired in sec71 deletion strain.

These results show that the ppαF with its signal sequence or a foreigner one is still translocated however CPY showed a better translocation with the ppαF signal sequence.
So the recognition of these proteins on the ER membrane seems to be different.

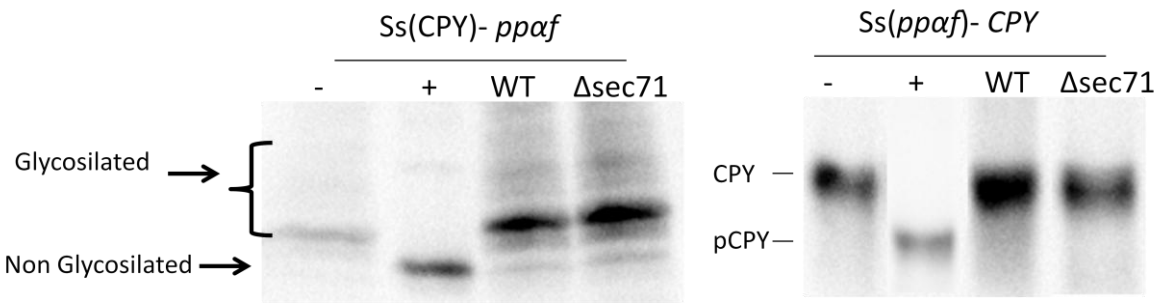


Figure7. Translocation assay after signal sequence swapping between CPY and ppαF. WT or Δ*sec71* cells expressing indicated proteins were radiolabeled for 5minutes, followed by IP with anti HA antibody and SDS-PAGE.

4. Translocation assay with membrane proteins

Previous studies investigated the effect of the deletion of the Sec71p only for the secretory proteins, thus we wondered how Sec71p affects translocation and/or insertion of membrane proteins. For this we varied two factors on the context of membrane proteins: the number of transmembrane segments and the hydrophobicity of the tested TM segment.

For translocation assay, we used the *E.coli* leader peptidase-based model membrane proteins harboring one, two or three transmembrane segments equipped with multiple glycosylation sites.

a) Single spanning membrane proteins

Construction of single spanning proteins and concept of translocation assay

For the construction of a single spanning model protein, Lep-H1, the first and second TM segment of original leader peptidase were substituted by H-segment (a hydrophobic segment), a 19 amino acid stretch composed of a varying number of leucines and alanines for a broad spectrum of hydrophobicity.(Figure7.A)

The hydrophobicity (ΔG) represents the quantity of energy 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. Lep-H1 has three glycosylation sites, one in N terminus and two in C terminus. In the case where the protein is fully translocated into the ER lumen, it gets glycans in all of its glycosylation sites (3G form corresponds to the fully translocated H1 protein). When H1 is inserted into the ER membrane, it can adopt two different orientations. Only its N terminal glycosylation site is modified when the protein is inserted with its N terminus in the ER lumen, whereas two C terminal glycosylation sites are glycosylated if the protein is inserted with its C terminus in ER lumen. When the protein remains in the cytoplasm, none of the glycosylation sites is modified and this is the untargeted form. The same concept is used for the other model proteins to investigate their translocation state with the different strains.

Therefore, the glycosylation status of these proteins allows assessing the targeting and translocation efficiency.

b) Translocation assay analysis

We tested a set of single spanning membrane proteins varying the hydrophobicity of their transmembrane domain in order to investigate the role of the Sec71p in their translocation into the ER lumen. The Lep-H1 model single spanning membrane proteins were labeled with [^{35}S]-Met and subjected to SDS-PAGE then the results were obtained by autoradiography. (Figure7.B). According to the obtained results with Lep-H1 labeling, we can distinguish two groups of Lep-H1 model proteins tested depending on the effect of *sec71p* on their translocation. The moderately hydrophobic Lep-H1 (H1-4L, H1-5L, and H1-6L) shows an intense defect in the translocation and insertion of the TM segment into the ER membrane where the amount of untargeted protein (0G) is dominant comparing to the wild type strain. However the translocation of Lep-H1 with higher hydrohobicity (H1-7L and H1-10L) is attenuated but less impaired compared to the less hydrophobic ones.

In case of H1-4L (containing the less hydrophobic transmembrane domain (TMD)) we can detect a dominant untargeted portion in the absence of Sec71p and also a less intense fully translocated version which migrates slowly on the gel because it is triply glycosylated. These results suggest that H1-4L protein may not insert stably to the ER membrane when Sec71 was deleted from Sec62/Sec63 complex.

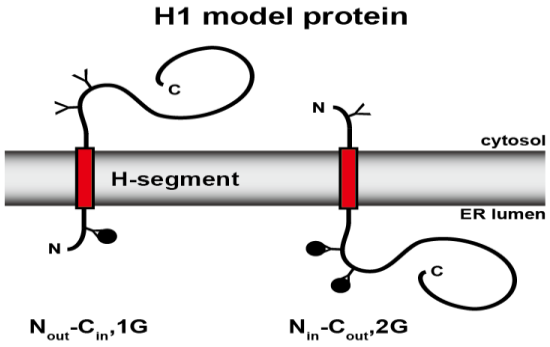
When the hydrophobicity of the TMD was slightly increased (H1-5L) around 70% of untargeted protein was detected and similar to H1-4L one small portion (about 15%) was totally translocated into the luminal side of the ER. In addition a new version appeared that the Nin-Cout form of H1-5L which is shown by the singly glycosylated form of the protein which means that the N-terminal of H1-5L enters the ER and the TMD was embedded into the membrane. Compared to the wild type which showed a dual insertion pattern of H1-5L, the deletion strain of Sec71 showed only the Nin-Cout form. Knowing that the N-terminal region of H1-lep protein is a lot shorter comparing to a long C tail, we may think that in the absence of Sec71p the Nin-Cout form is promoted because without Sec71p the long C-terminal cannot be translocated.

For that reason the dominant portion is untargeted and even the small portion which can enter the translocon could not be stable in the membrane environment so it goes to the lumen of the ER totally and by consequence we detect this triply glycosylated portion. In this case we suggest that Sec71p may help the less hydrophobic single spanning proteins to be targeted and inserted properly into the ER membrane, so that it may either bring these proteins to the translocon (pore) or it may stabilize them when they are in the pore.

Within a higher hydrophobicity of the TMD (H1-7L and H1-10L) the untargeted portion still detectable in case of the absence of Sec71p comparing to wild type strain. In the absence of Sec71p, increasing the hydrophobicity of the single TMD tested promotes more translocation even though the translocation defect still not totally recovered to the wild type level.

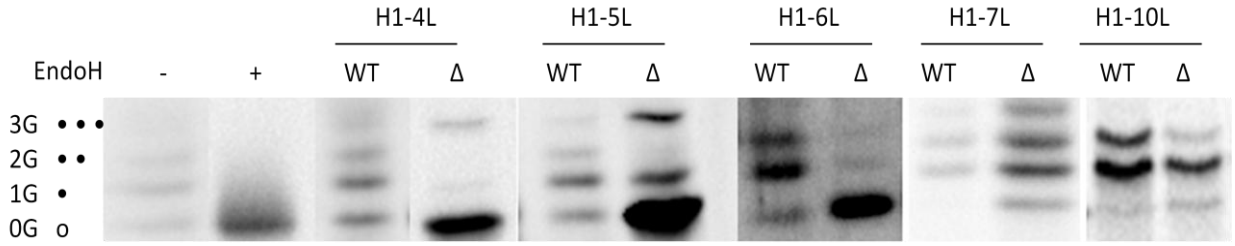
In this order, we think that Sec71p helps the Sec63 complex in the translocation of the single spanning membrane proteins in a hydrophobicity independent manner. in contrast of Sec62 which when deleted showed a translocation defect with only the moderately hydrophobic single spanning proteins, in case of Sec71p even the translocation of the higher hydrophobic H1 proteins (H1-7L and H1-10L) still reduced comparing to the wild type translocation level.

A



4L	GGPGAAAALALAAAAALALAAAAGPGG	-0.490 Kcal/mol
5L	GGPGAAAALALAALAALALAAAAGPGG	-0.999 Kcal/mol
6L	GGPGAAAALALALALALALAAAAGPGG	-1.540 Kcal/mol
7L	GGPGALAALALAALAALALAALAGPGG	-1.76 Kcal/mol
10L	GGPGLALALALALALALALALALGPGG	-3.115 Kcal/mol

B



C

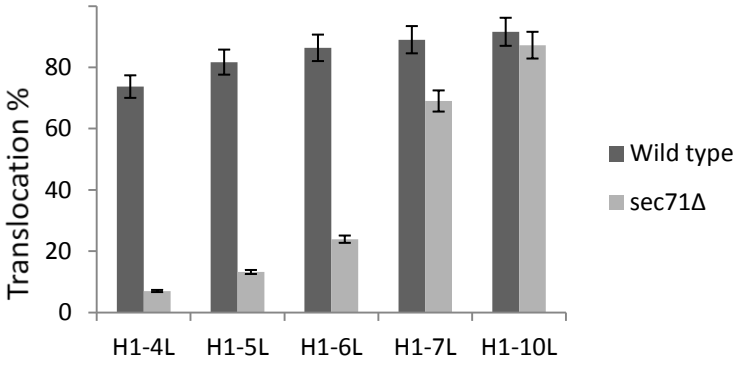


Figure8. Translocation of Lep-H1 model protein A.the Lep-H1 model protein has one TM domain. The sequences with their apparent free energy of insertion (ΔG) are indicated. N-linked glycosylation sites are indicated as Y and occupied sites are shown with close circles. B. WT and sec71 deletion cells expressing the different H1 proteins were labeled for 5min, subjected to immunoprecipitation, SDS-PAGE and analysis by autoradiography. C.Quantification of the data on B about the translocation efficiency of Lep-H1 model protein.

c) Multispanning membrane proteins

Multispanning model proteins were also tested in both wild type and *sec71::HIS3* strains varying the hydrophobicity of the last TM segment of each protein. We examined the translocation and insertion of the test segment in both cases to investigate in depth the function of Sec71p.

As shown in Figure8, H2 model protein was tested with two different range of hydrophobicity of the test segment (H2-3L and H2-5L). The two proteins were radio labeled and the whole cell lysate was analyzed by pulse labeling subjected to IP and SDS-PAGE and results were obtained with radiography. Using the EndoH treatment we could distinguish the glycosylated version and the non glycosylated version of the protein because the EndoH removes the glycans from the glycosylation sites. When the second TMD of H2 protein is inserted into the lumen of the ER it can be cleaved with the signal peptidase at its C-terminus as shown in the scheme (Figure 8A, black arrow). Because this cleaved products appear only when the test TMD is inserted into the membrane, it is regarded as membrane inserted (2G cleaved)..

Based on that we analyzed the data and we noticed that in case of H2-5L in the *sec71::HIS3* strain as the WT strain didn't show any defect in the translocation of the test segment (second TMD) and that the two strains showed the same profile of the 2G form or 1G cleaved form. These results mean that the test segment was efficiently inserted into the ER membrane with the C-terminus side facing the ER lumen which induced the glycosylation at its C-terminus.
In contrast, when the hydrophobicity of the TMD was lowered in H2-3L protein the *sec71::HIS3* strain showed a different translocation profile comparing to the wild type. Analyzing that we found when Sec71p was absent a small amount of the untargeted protein appeared. In addition the test segment showed less translocation and was not properly inserted to the ER membrane (1G form) comparing to wild type when sec71p was absent from the Sec62/Sec63 complex. This result was confirmed via Western blot and pulse labeling and the defect was reproducible. The same effect was not detected in the case of more hydrophobic test segment with H2-5L protein which is concomitant with the previous result of single spanning proteins. These results suggest that the moderately hydrophobic proteins need the help of Sec71p to be inserted properly into ER membrane therefore they are more affected with the deletion of the Sec71p.

At a low to moderated hydrophobicity the TMD seems to need the help of Sec71 to properly insert into the membrane.

Although the insertion is not totally defective in the *sec71::HIS3* that may be explained by the presence of a hydrophobic first TMD which may also help the second TMD to insert into the ER membrane.

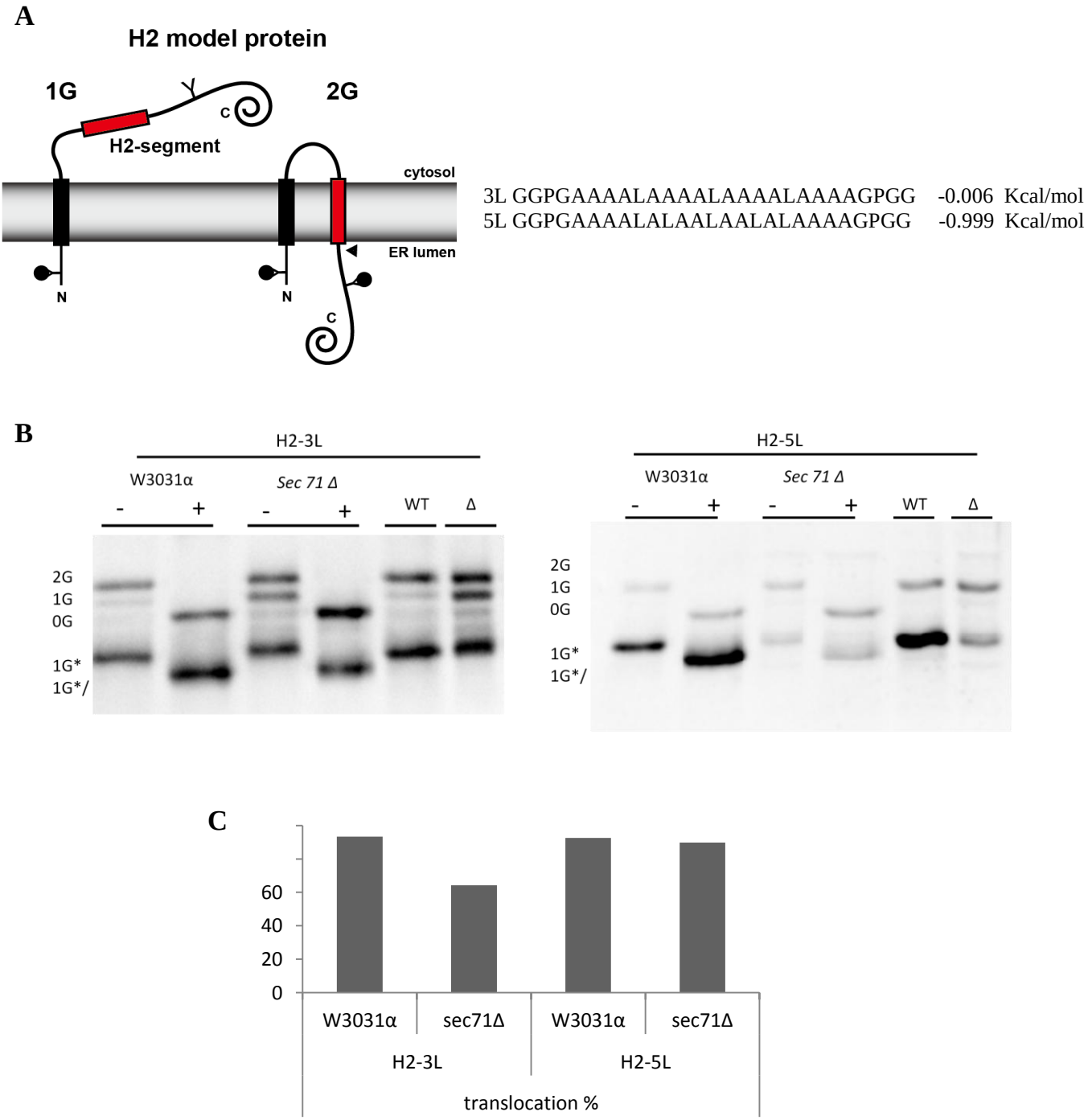


Figure9. Translocation of H2 model protein.A.the Lep-H2 model protein has two TM domains. The sequences with their apparent free energy of insertion (ΔG) are indicated. N-linked glycosylation sites are indicated as Y and occupied sites are shown with close circles. B. WT and sec71 deletion cells expressing the different H1 proteins were labeled for 5min, subjected to immunoprecipitation, SDS-PAGE and analysis by autoradiography. C.Quantification of the data on B about the translocation efficiency of Lep-H2 model protein.

Triple spanning model proteins derived from *E.coli* leader peptidase were tested in W3031α and *sec71::HIS3.* The first and second TM segments are very hydrophobic thus efficiently inserted into the ER 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 2.

We tested H3 model protein which is a truncated version of Lep4 protein (leader peptidase) lacking the fourth TMD and the C-terminal region. Truncated Lep4 (H3) contains three TM segments and three glycosylation sites. Triply glycosylated form represents C-terminal translocated proteins while doubly glycosylated form indicates that the third TMD is inserted into the ER membrane. (Figure 9.A)

H3 proteins varying the hydrophobicity of their third TMD were expressed in W3031α and *sec71::HIS3* and were subjected to pulse labeling and SDS-PAGE. The insertion of the Test segment is assessed by glycosylation status.

A less hydrophobic H3-4L showed less insertion of the test segment similarly in both wild type and sec71 deletion strain. The model protein was efficiently targeted to the ER therefore the unglycosylated portion was not significant. However the test segment insertion was defective in both wild type and *sec71::HIS3* and that is due to the low hydrophobicity of the third TMD in this case. To overcome the hydrophobicity effect and focus on the effect of Sec71p we increased the hydrophobicity of the test segment and we constructed H3-7L model protein. The data showed that the triply glycosylated form (3G) in case of *sec71::HIS3* strain was dominant and from the quantification we determine a less insertion of TM segment in absence of Sec71p. These results mean that the third TM segment is fully translocated into the luminal side of the ER. In this case we suggest that the insertion of the test segment into the ER membrane cannot be stable in the absence of Sec71p therefore we detect the C-terminal translocation of the H3.

All the previous results shown either with single spanning proteins (H1) or multispanning proteins (H2 and H3) lead us think about the stability of the Sec63 complex in absence of Sec71p because we detected every time, especially with the moderately hydrophobic proteins, a defect in their translocation or their insertion into the ER membrane. So we thought that maybe the Sec71 is responsible of the stability of the Sec63 complex or maybe another possibility can be also suggested that the Sec71p might stabilize directly the proteins which are less hydrophobic or long which may require energy to go through the membrane of the ER.

Sec71p might with some interactions get these proteins to the complex or push them at the complex pore.

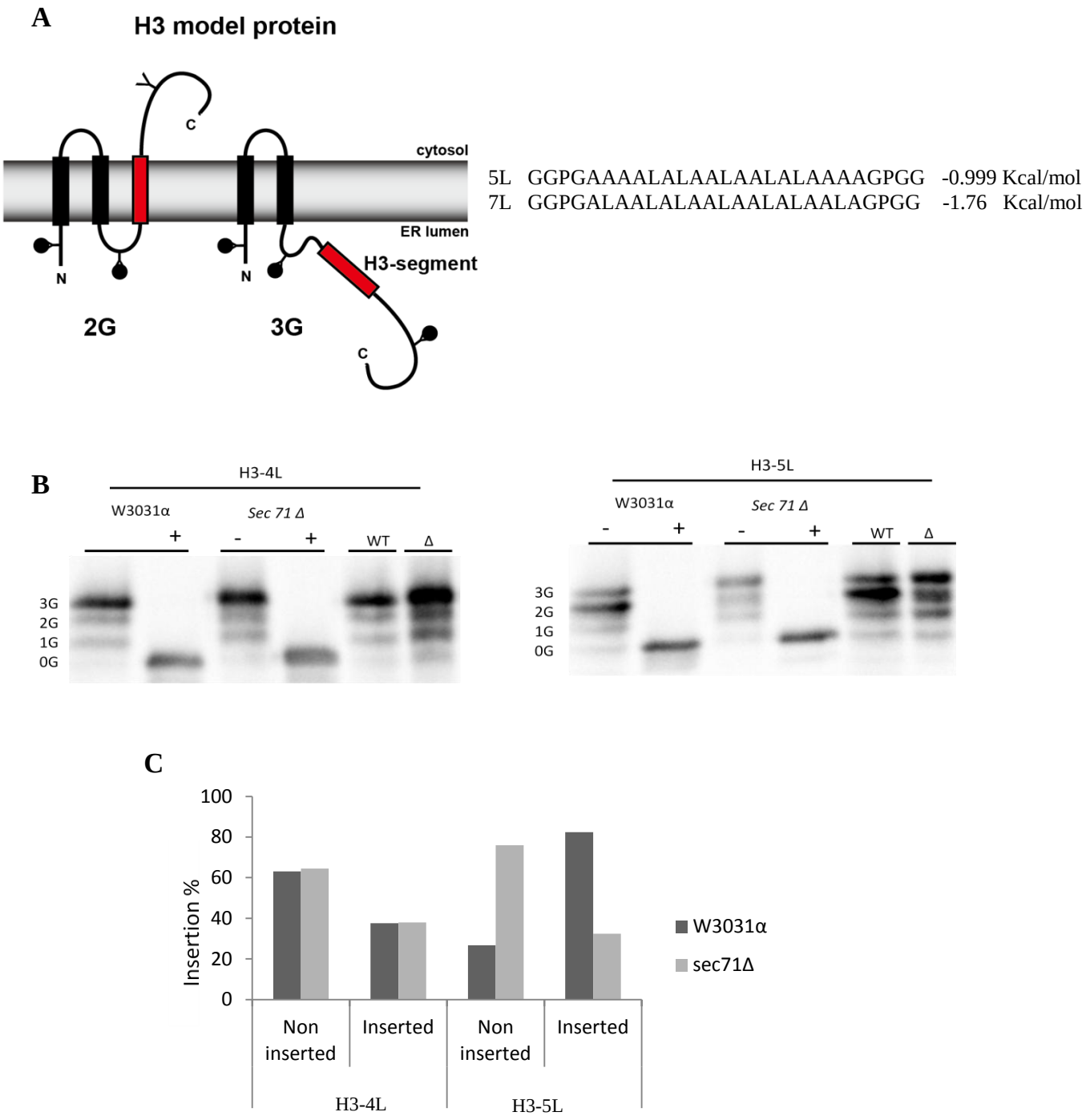


Figure10. Translocation of H3 model protein. A. The Lep-H3 model protein has three TM domains. The sequences with their apparent free energy of insertion (ΔG) are indicated. N-linked glycosylation sites are indicated as Y and occupied sites are shown with close circles. B. WT and sec71 deletion cells expressing the different H1 proteins were labeled for 5min, subjected to immunoprecipitation, SDS-PAGE and analysis by autoradiography. C.Quantification of the data on B about the insertion efficiency of Lep-H3 model protein.

VII Discussion

In our study we made the deletion strain lacking *SEC71* by homologous recombination and we confirmed that it is a temperature sensitive strain where the absence of Sec71p prevents growth at high temperature. The translocation defect also was more induced at the non permissive temperature for this strain. In this way Sec71 is named as an accessory protein because its deletion from the Sec62/Sec63 complex affect the growth at non permissive temperature and induce some translocation defect but the cell growth was not defective at a permissive temperature. Sec71 was not vital for the cells but impaired the translocation of some ER targeted proteins.

In case of secretory proteins tested, the absence of Sec71p showed a severe defect in translocation of the post translational translocated substrate (CPY) and a less defect for the co translational translocated substrate (DPAP B) which leads us to investigate that Sec71p seems to be more involved in the case of the post translational translocation pathway. This result can be explained by the fact that in the co translational translocation pathway the ribosome still attached to the protein while it enters the pore translocon so that these substrates may need less help from Sec71p to be stabilized or recruited to the translocon whereas in case of the post translational translocation pathway the ribosome is detached after the translation and the protein may need in this case more help of Sec71p to enter the pore and be stable there.
Comparing CPY to ppαF which didn't show any translocation defect in the background of *sec71::HIS3*, we may think that because of the length of CPY may need more help to enter the ER while the mature part of ppαF is a lot shorter translocating much easily even without Sec71 presence.

In case of membrane proteins, the effect of Sec71p was more intense in case of single spanning (Lep-H1 model protein) where sec71 deletion abolished the targeting of moderately hydrophobic proteins and still attenuated the targeting even for the ones having a higher hydrophobicity. Therefore without the help of other TMD, a single TMD has more difficulties to enter the translocation pore in the absence of Sec71p.
When we tested the multi spanning membrane proteins (H2 and H3) the effect of Sec71p was revealed with only the moderately hydrophobic TMD which had a decrease in their insertion into the ER membrane.

Our results on translocation and membrane insertion profiles of single spanning (H1) and multispanning membrane proteins (H2 and H3) in sec71 deletion cell suggest two possibilities: the Sec71 may be responsible for the stability of the Sec62/Sec63 complex or Sec71p may stabilize directly the translocating proteins which are less hydrophobic or large which may require more energy to be translocated across the ER. Sec71p may with some interactions with other translocation components help these proteins to position better for optimal translocation and membrane insertion.

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국문초록

막단백질과 분비단백질은 세포질에서 리보솜에 의해 합성된 뒤 두 가지 경로에 의해 소포체(endoplasmic reticulum, ER) 막으로 보내진다. 이 두 가지 경로는 번역동시 전좌와 번역후 전좌다. 번역동시 전좌의 경우 Sec61 복합체가 또 다른 이성 4 분자체 단백질 복합체인 Sec63 복합체와 함께 작용하는 것으로 보고되었다. Sec63 복합체는 세포생존에 필수불가결한 단백질인 Sec62, Sec63 더불어 가결적인 Sec71 과 Sec72 로 구성된다.

Sec62 단백질은 중간 정도의 소수성을 가지는 단일투과성 혹은 다중투과성 막단백질의 전좌에 작용하는 것으로 밝혀져 있다. Sec71 단백질과 Sec62 단백질은 동일한 Sec62/Sec63 복합체에 속하기 때문에, 막단백질의 전좌에 작용하는 Sec71의 고유한 기능이 있는지에 대한 의문이 남아있었다.

분비단백질과 막단백질이 전좌되거나 막으로 삽입되는 데 있어 Sec71 단백질의 기능을 연구하기 위해 *SEC71* 유전자가 결여된 돌연변이 세포주를 제작하였다. 일련의 모델 단백질이 *sec71::HIS3* (sec71 결실 세포주)에서 합성되었을 때, 일부 전분비단백질의 전좌가 정상적으로 일어나지 않았다.

SEC71 결실의 효과로 단일투과성 단백질(Lep-H1 모델 단백질)에서 중간 정도의 소수성을 가지는 경우 전좌가 완전히 가로막히는 한편 그보다 강한 소수성을 가지는 경우에도 단백질의 타겟팅이 약화되었다. 이는 다중투과성 막단백질을 모델로 이용하는 경우보다 강한 효과였는데, 다중투과성 단백질(H2, H3)은 Sec71 단백질이 없을 때 오직 중간 정도의 소수성을 지닌 막투과성 도메인을 가지는 경우에만 막 내 삽입이 감소하였다.

Sec71 이 없는 세포에서 단일투과성 단백질(H1)과 다중투과성 단백질(H2, H3)에 대하여 단백질 전좌와 막 내 삽입 경향을 분석한 본 연구결과는 두 가지 가능성을 제시한다. 첫째는 Sec71 이 Sec62/Sec63 복합체의 안정성에 유관하여 간접적인 전좌 조절을 한다는 것이다. 다른 하나는 소수성이 낮거나 단백질의 크기 자체가 커서 소포체를 통과해 전좌될 때 더 많은 에너지를 필요로 하는 단백질들이 전좌 중일 때 Sec71 이 직접적으로 작용하여 이들을 안정화시킨다는 것이다. 다시 말해, Sec71 은 복합체 내 다른 단백질들과의 상호작용 속에서 이들이 단백질의 전좌와 막 삽입을 할 수 있는 최적의 상태가 되도록 자리를 잡는 일을 도울 가능성이 있다.