이학석사학위논문

TIM23 복합체를 통한 미토콘드리아 내막 단백질 삽입경로의 연구

Investigation of the TIM23 complex-mediated sorting pathways of mitochondrial inner membrane proteins

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이 논문을 이학석사학위논문으로 제출함

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Investigation of the TIM23 complex-mediated sorting pathways of mitochondrial inner membrane proteins

by

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ABSTRACT

Nucleus encoded mitochondrial inner membrane (MIM) proteins carrying a cleavable presequence in *Saccharomyces cerevisiae* are inserted into the inner membrane medicated by translocases of outer and inner membrane (TOM-TIM23 complex). A hydrophobic sorting signal within transmembrane (TM) domain of the protein is recognized and laterally transferred into the membrane from the TIM23 complex (stop-transfer pathway). Otherwise, entire protein is first imported into the matrix and exported into the inner membrane (conservative sorting pathway). In addition, a multi-spanning mitochondrial protein utilizes both pathways for its membrane integration. While mitochondrial inner membrane is the protein-richest membrane harboring various complexes for critical functions in the cell, only small number of MIM proteins' insertion mechanisms have been characterized owing to lack of robust experimental tools. Here, we established Mgm1 fusion protein (MFP) approach, which takes advantage of the rhomboid cleavage region in the C-terminal domain of Mgm1p, to elucidate the sorting pathways of MIM proteins *in vivo*. We validated this method with a set of proteins whose sorting pathways are well characterized and determined the membrane insertion mode of single or multi-spanning MIM proteins of which integration pathways were unknown. Our results suggested that Yta10p is inserted into the membrane not from matrix, but from intermembrane space (IMS). In addition, we found that a subunit of succinate dehydrogenase, Sdh4p and a mitochondrial inner membrane half-type ABC transporter, Mdl2p, are also integrated into the membrane via two different pathways. Furthermore, analyzing the sorting pattern of Mgm1 fusion proteins in various growth conditions and at different yeast mutant strains, we showed that insertion of proteins bearing moderately hydrophobic TM segments is more sensitive to intrinsic and extrinsic cellular factors and the presequence translocase-associated

import motor (PAM complex) is necessary for the translocation of Cox18p, Mdl1p, and Mdl2p.

Keywords: Mgm1p, Yeast mitochondria, TIM23 complex, Stop-transfer pathway, Conservative sorting pathway, Membrane insertion, *m*-AAA protease, PAM complex, multi-spanning inner membrane proteins

INTRODUCTION

In eukaryotic cells, mitochondria are crucial organelles and are involved in energy production, apoptosis, signaling and metabolic pathway of amino acids, lipids, and iron-sulphur clusters [1] [2]. As correct localization of mitochondrial proteins is essential for the normal functions, mislocalization leads to accumulation of dysfunctional mitochondria and it is associated with human diseases such as neurodegenerative disorder and cancer [3].

Whereas eight proteins are encoded by mitochondrial DNA in *Saccharomyces cerevisiae*, approximately 800~1000 mitochondrial proteins are encoded in the nucleus and are fully synthesized as precursors in the cytosol [4] [5]. The cytosolic precursors are recognized by the receptors on the mitochondrial membrane surface and sorted into one of the mitochondrial subcompartments in post-translational manner: outer membrane (OM), intermembrane space (IMS), inner membrane (IM), and matrix [6]. Several translocases and complexes facilitate the translocation of mitochondrial proteins. Translocase of the outer membrane (TOM complex) and the sorting and assembly machinery (SAM complex) mediate the sorting of outer membrane proteins. Mitochondrial intermembrane space assembly proteins (MIA) facilitate intermembrane space sorting proteins. Two translocases of the inner membrane (TIM22 and TIM23 complex) and OXA insertase are involved in the integration of inner membrane proteins. The TIM23 complex also mediates the translocation of all matrix proteins [2] [7] (Introduction Figure 1. A).

Metabolite carrier proteins containing multiple internal signals are guided to the TIM22 complex by small TIM proteins (Tim9p and Tim10p) and inserted into the

inner membrane. On the other hand, most inner membrane proteins have the cleavable N-terminal presequences (or matrix targeting sequence, MTS). It is enriched with positively charged residues and is able to form an amphipathic α-helix of variable length (10-80 residues) that is removed by mitochondrial processing peptidase (MPP) at the mitochondrial matrix [8]. Afterwards, proteins are integrated into the IM via either the stop transfer or the conservative sorting pathway [9] (Introduction Figure 1. B). If a protein contains a sorting signal within a single transmembrane (TM) segment, it would be recognized by the TIM23 complex and laterally released into the IM - the "stop-transfer route" [10]. In contrast, polytopic mitochondrial inner membrane (MIM) proteins, containing the presequecne but lacking stop-transfer signal, are completely translocated to the matrix by the presequence translocase-associated import motor (PAM) and then inserted into the IM mediated by the OXA1 insertase, Mba1p, Cox18p, or Bcs1p - the "conservative sorting route" [11] [12].

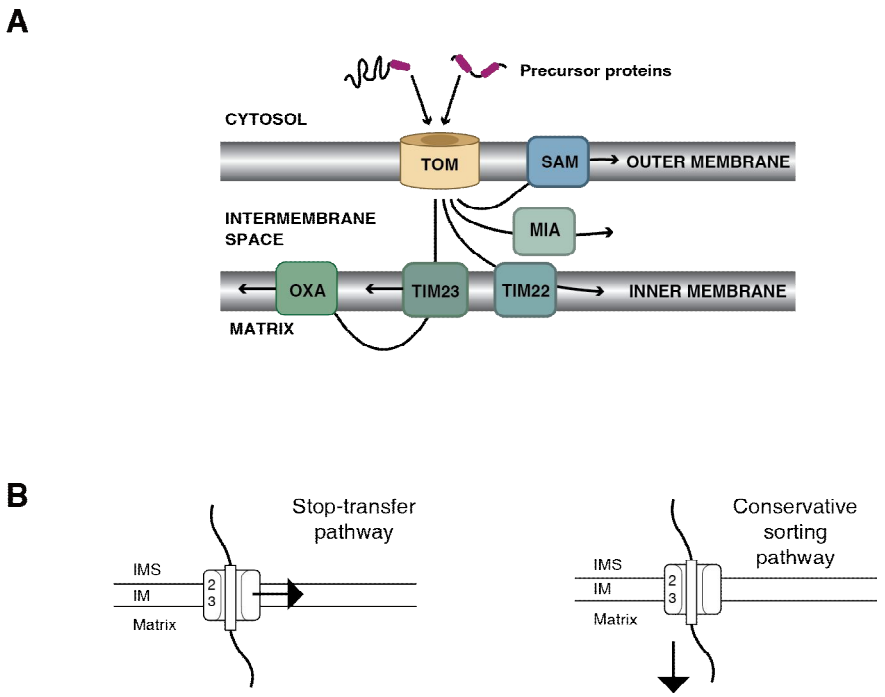
However, recently it has been revealed that not all multi-spanning IM proteins of mitochondria are sorted by the conservative sorting pathway. Their sorting pathways are apparently more complicated than we expected as Bohnert *et al*. [13] showed that the stop-transfer and conservative sorting pathway work together in the insertion of Mdl1p, a half-size ABC transporter. This protein has a presequence and six transmembrane segments. While TM1-2 and TM5-6 domains are sorted by the stop-transfer pathway, TM3-4 segments are conservatively sorted in OXA1 dependent manner.

Mitochondrial inner membrane is concentrated with proteins (80% is protein and 20% is lipid). It contains numerous complexes such as respiratory chain complexes, F_0F_1-ATPase, import machineries, and the AAA complex (ATPase Associated with

diverse cellular Activities). In order to study import, subcellular localization, and topology of MIM proteins, the *in vitro* mitochondrial protein import assay has been commonly used. After synthesized in a cell free system, MIM proteins are imported into the isolated mitochondria. This process is, however, highly susceptible to specific experimental conditions which may affect the protein's import and translocation and lead to conflicting results [14] [15].

In this study, we established a convenient method to investigate protein sorting mechanism *in vivo* by simply adding the carboxyl-terminal domain of Mgm1p to monotopic or polytopic MIM proteins. We found that the cooperative mechanism between stop-transfer and conservative sorting pathway is required for the proper insertion of Sdh4p and Mdl2p. Furthermore, we observed that internal and external factors are involved in the protein's import and translocation into the membrane, indicating the translocation of mitochondrial IM proteins is finely regulated by the dynamic cellular environments.

Introduction Figure 1



Introduction Figure1. Sorting of mitochondrial proteins

(A) Translocase of outer membrane (TOM) is general gate for the mitochondrial proteins. Depending on their sorting signal, cytosolic precursors are imported into different subcompartment of mitochondria: outer membrane, intermembrane space, inner membrane, or matrix. SAM, the sorting and assembly machinery; MIA, mitochondrial intermembrane space assembly; TIM22 and TIM23, translocase of inner membrane; OXA, insertase of the inner membrane [2] (B) Mitochondrial inner membrane (MIM) proteins are sorted via the TIM23 complex by two different mechanisms. Proteins are recognized and anchored at the level of TIM23 complex (stop-transfer pathway). Proteins are not arrested by the TIM23 complex and pass through the inner membrane. Afterwards proteins are integrated into the membrane by various insertases such as OXA insertase and Bcs1p (conservative sorting pathway) [9].

MATERIALS AND METHODS

Yeast strains

W303-1α *(MATα, ade2, can1, his3, leu2, trp1, ura3),* W303-1a *(MATa, ade2, can1, his3, leu2, trp1, ura3)*, Δ*yta10 (MATa, ade2-1, his3-11,15,* Δ*yta10::HIS3MX6, trp1-1, leu2,112, ura3-52)*, Δ*yta10*Δ*ccp1 (MATa, can1-100, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1,* Δ*yta10::HIS3MX6,* Δ*ccp1::kanMX4)*, Δ*ccp1 (MATa, can1-100, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1,* Δ*ccp1::kanMX4)* [16], temperature-sensitive pam16-3 mutant and the isogenic PAM16 wild type strain [17].

Plasmid construction

Each gene was amplified by PCR using genomic yeast DNA prepared from W303-1α with forward primers containing the start of the gene and reverse primers containing the specific region of the gene as described [18]. All plasmids will be prepared by homologous recombination in yeast as described [19]. For Mdl1/Mdl2 Mgm1 fusion proteins, the indicated number of TM segments was fused to the C-terminal domain of Mgm1p. To facilitate Western blot analysis, three copies of the hemagglutinin (HA) tag were fused to the C-terminus.

Western blot analysis

Yeast transformants of W303-1α, W303-1a, *m*-AAA mutant strain (Δ*yta10 or* Δ*yta10*Δ*ccp1*), Pam16 mutant strain (*pam16-3*) or their isogenic wildtype carrying each plasmid were grown overnight in 5ml of –Leu medium (2% glucose or 3% glycerol) at 25°C, 30°C, or 37°C and preparation of whole-cell lysates, SDS-PAGE,

and Western blotting conducted as described [18]. The relative amounts of L-MFP and *s*-Mgm1 were quantified with LAS-1000 or Image Lab system (Biorad).

Isolation of yeast mitochondria and protease K protection analysis

The transformants carrying MFP constructs were grown in 1 liter of -Leu medium containing glucose (2%, w/v) at 30 °C until reaching 1–2 A_{600} units/ml. Cells were harvested by centrifugation at 3,000 g for 5 minutes and treated with 100 mM of Tris-base, pH 11.0, and 10 mM dithiothreitol (DTT), for 20 min at 30 °C. Cells were then centrifuged at 2,000 g for 5 minutes and incubated with Zymolyase-100T (5 mg/g of cells) in 1.2 M sorbitol and 20mM potassium phosphate at 30 °C for 30 min (or up to 1 h). Cells were collected by centrifugation at 1,200 g for 5 minutes at 4 °C. Afterward, the pellet was resuspended in homogenization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% BSA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.6 M sorbitol), and cells were lysed by a glass homogenizer at 4 °C (13 strokes). The lysate was centrifuged at 1,200 g for 5 minutes at 4 °C to remove unbroken cells. The mitochondrial fraction was harvested by centrifugation at 12,000 g for 15 minutes at 4 °C. The pellet was resuspended in 500µl of suspension buffer (0.6 M sorbitol, 20mM HEPES-KOH, pH 7.4). Then 40 µg of prepared mitochondria were incubated with either 100 µl of suspension buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4) or suspension buffer with proteinase K (50 µg/ml) for 30 min on ice. To stop the proteolytic activity, 1µl of 0.1 M PMSF was added, and the suspension was incubated for 5 minutes on ice. Samples were centrifuged at 20,000 g for 10 minutes, and the pellets were precipitated with 12.5% (w/v) TCA as described [18], followed by SDS-PAGE and Western blotting analysis.

[^{35}S]-pulse labeling and chase experiment

Three A_{600} units of cells were harvested and resuspended in synthetic defined medium without ammonium sulfate and methionine. Cells were starved at 30°C or 37 °C for 15 minutes, and 30μCi (per A_{600} unit of cells) of [^{35}S]-methionine was added to the culture for 5 or 10 minutes at 30°C or 37 °C. The cells were more incubated with non-radioactive methionine (final concentration: 2mM) for indicated times. Labeling and chase were terminated by adding TCA to a final concentration of 10% (w/v). Later steps of the experiments were performed as described [18]. After radio-labeled samples were subjected to the SDS gels, protein bands were visualized in a Fuji FLA-3000 phosphor imager or Fujifilm BAS-2500 system.

RESULTS

Design of experiments

Mgm1p, a dynamin–related GTPase, is in the mitochondrial inner membrane [20]. It is required for the mitochondrial morphology and the inheritance of mitochondrial DNA (mtDNA) in *Saccharomyces cerevisiae* [21] [22] [23, 24] It contains an amino-terminal presequence of 80 amino acid residues long and two hydrophobic segments (HS1; residues 94~111, HS2; residues 156~169) (Figure 1A). Mgm1p is produced as two isoforms by the alternative topogenesis (*l*-Mgm1p, long-Mgm1p and *s*-Mgm1p, short-Mgm1p). When the presequence enters the matrix through the TIM23 import channel, it is cleaved by the MPP. The first HS is anchored into the inner membrane with an efficiency of 30~40%, resulting in *l*-Mgm1p [25]. However, at high level of matrix ATP, the first HS is not arrested by the TIM23 translocase but slips into the matrix by the PAM complex until the second HS reaches to the membrane. The mitochondrial rhomboid protease, Pcp1p, then cleaves between residue 160 and 161 or residue 162 and 163 within the second HS, known as the rhomboid cleavage region (RCR) [26] (Figure 1A).

Taking advantage of the RCR of Mgm1p, we fused the MIM proteins harboring a single spanning or multi-spanning TM domains to the C-terminal domain of Mgm1p (117-902 residues), and termed this chimera protein an Mgm1 fusion protein (MFP). In case of a monotopic MIM protein (e.g. Cox5ap), the truncated or full-length MIM protein were fused to the reporter domain (Figure 1B, upper panel). A polytopic protein (e.g. Yta10p) was systematically truncated to study the membrane insertion preference of distinct TM segments (Figure 1B, bottom panel). If an MFP

contains a stop-transfer sorting signal in a single TM segment, it is recognized by the TIM23 complex and laterally released into the membrane during its translocation. This generates long Mgm1 fusion protein (L-MFP) (Figure 1C, i). In contrast, if the stop-transfer signal is absent, an MFP goes into the matrix. Afterwards, Pcp1p processes the RCR and it gives rise to *s*-Mgm1p (Figure 1C, ii).

If an MFP contains double spanning TM segments with a stop-transfer sorting signal, *s*-Mgm1p would be generated as two TM segments get integrated into the membrane (Figure 1C, iii).

Figure 1

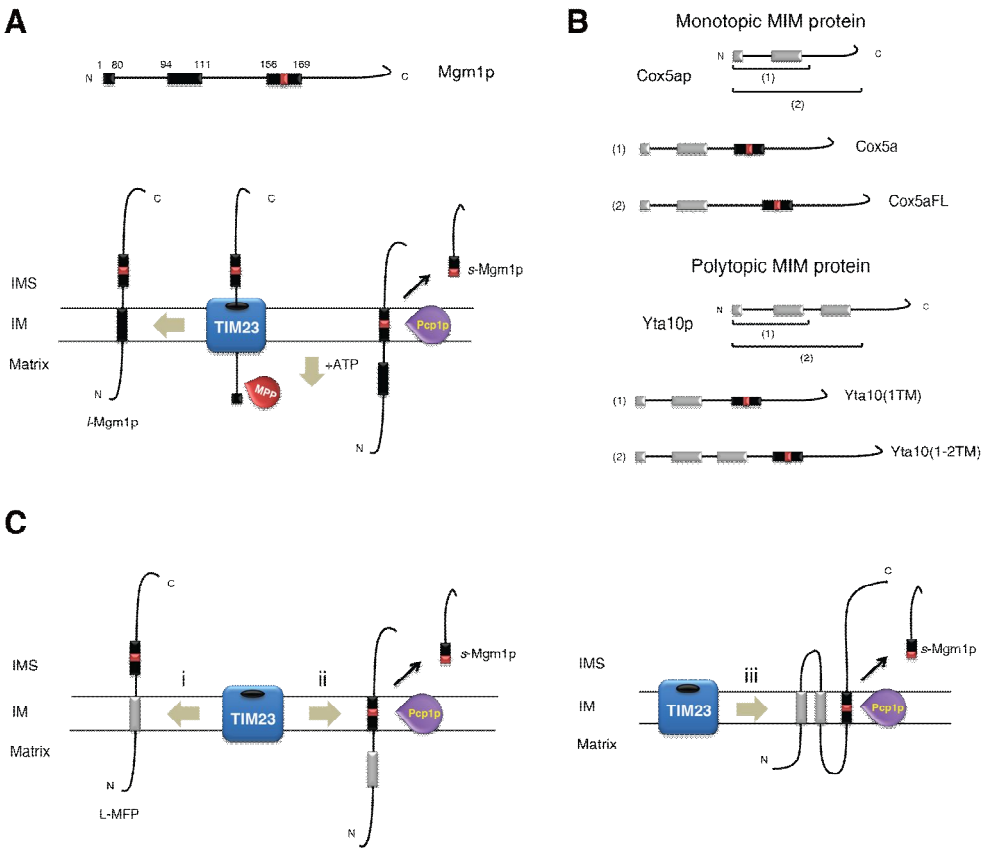


Figure 1. Mgm1 fusion protein method (A) Schematic drawing shows an alternative topology of Mgm1p in mitochondria [25]. It contains an N-terminal presequence, two hydrophobic segments (HS, *black rectangles*), and a large C-terminal domain. The rhomboid cleavage region (RCR, *red*) is present within the second HS. (B) The truncated or full length monotopic mitochondrial inner membrane (MIM) protein or sequentially truncated polytopic MIM protein were fused to the C-terminal part of Mgm1p. Full length is denoted as FL, and the number of transmembrane (TM) domains is denoted in the parenthesis. (C) Schematic represents the sorting patterns of Mgm1 fusion protein (MFP) harboring

single or multi-spanning TM segments (grey). TIM23, TIM23 complex; MPP, mitochondrial processing peptidase; Pcp1p, rhomboid protease; IMS, intermembrane space; IM, inner membrane; *l*-Mgm1p, long Mgm1 isoform; *s*-Mgm1p, short Mgm1 isoform; L-MFP, long Mgm1 fusion protein

MFPs inserted by the stop-transfer pathways mostly produce L-MFP

To test the feasibility of this approach with MIM proteins carrying cleavable presequences, a set of proteins was prepared whose insertion pathways were known (Table I). Yeast transformants harboring the indicated MFP constructs were grown upto A_{600} 1 in –Leu medium (2% glucose) at 30°C. Samples were prepared by TCA precipitation and analyzed by SDS-PAGE and western blotting with HA antibody. Mgm1p that were used as control showed the formation of *l*-Mgm1p and *s*-Mgm1p. The stop-transfer sorted MFPs mostly resulted in L-MFP except Cox5a and She9(1TM) because the TM segment of MFP was integrated into the IM, and thus, Pcp1p was not accessible to the RCR. In case of Cox5aFL, it generated long form up to 80%, but truncated Cox5a produced 30% of L-MFP, indicating the downstream of the TM might affect the proper insertion of the protein. Similarly the formation of L-MFP was increased in She9FL compared to the truncated version. Although She9p contains two predicted TM segments (Table II), the second TM domain may not anchor in the inner membrane but rather play an important role in insertion of the first TM domain into membrane. Therefore, the C-terminal downstream residues of the TM domain influence the correct sorting of the MIM proteins (Figure 2A).

To examine whether these fusion proteins are properly targeted to the mitochondria, we isolated mitochondria from the yeast transformants and proteinase was externally added to remove the untargeted proteins. While cytosolically exposed OM proteins (Tom20p or Tom70p) were degraded, IM marker (Tim54p), Matrix markers (Tim44p or Mge1p) and L-MFPs were protected under the proteinase K (PK) treatment as PK was unable to penetrate into the OM of intact mitochondria. This

indicated that all long-forms of MFP were in the mitochondria, not in the cytosol (Fig 2B). More *s*-Mgm1p was observed in the isolated mitochondria compared to whole cell lysates, possibly due to continuous activity of Pcp1p. In addition, *s*-Mgm1p of Dld1 and Oms1 were degraded in the presence of PK. They might be leaked out during the mitochondria preparation and be degraded under the PK treatment.

MFPs sorted by the conservative sorting pathways predominantly generate s-Mgm1p

When single or multi-spanning MIM proteins that do not have the stop-transfer signal fused to the C-terminal part of Mgm1p containing the RCR, the *s*-Mgm1p was largely formed except Rip1, Rip1FL, Yta10(1TM) Mrs2(1TM), and Mrs2(1-2TM) (Figure 2C). However, when cells were grown in the non-fermentable carbon source (3% glycerol), they also produced *s*-Mgm1p more than 80%, but the ratio of s-Mgm1p was not altered for Yta10(1TM) (Figure 6A). It suggested that TM domains of conservative sorted MFPs went into the matrix rather than inserted into the IM and processing of RCR led to *s*-Mgm1p. As Pcp1p is in the IM of mitochondria [25], a cleavage of RCR within the MFPs and the production of *s*-Mgm1p indicates that these proteins are efficiently imported into the mitochondria.

In sum, we could distinguish whether they are inserted into the IM by stop-transfer or conservative sorting mechanism *in vivo* by fusing a reporter domain, which does not interfere with proper sorting of proteins, to MIM proteins containing a cleavable presequence.

Figure 2

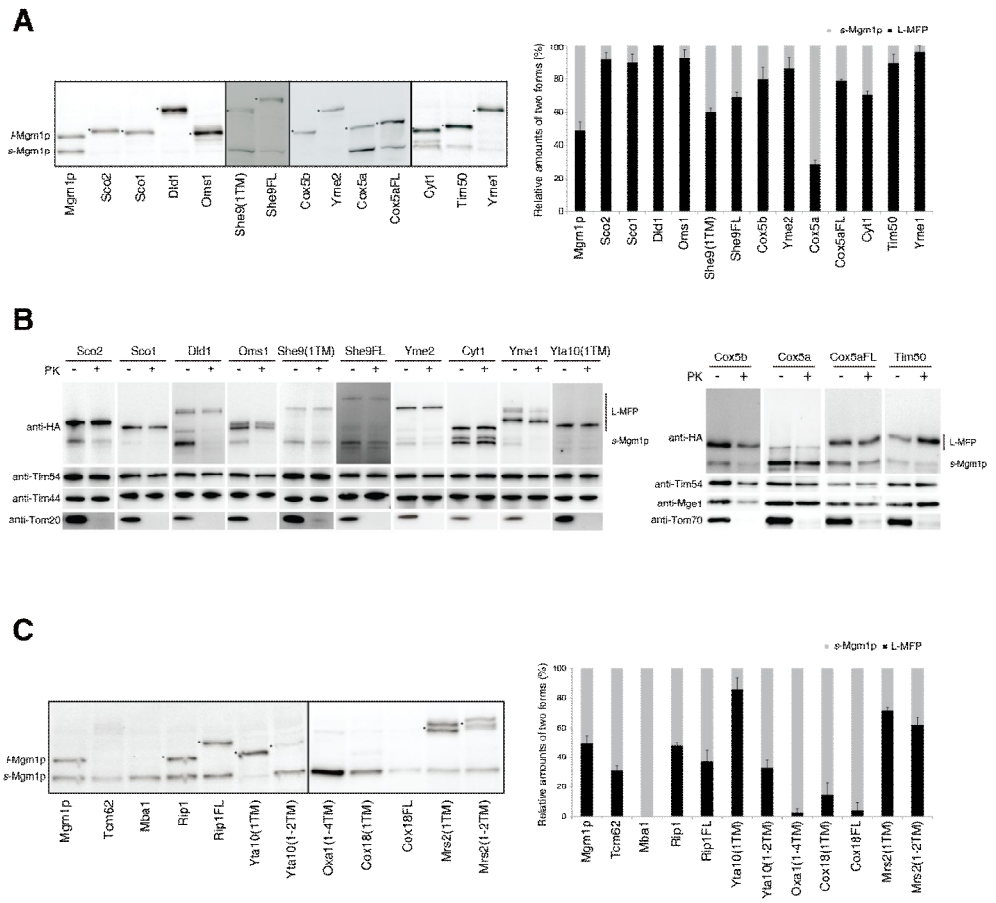


Figure2. Mgm1 fusion approach is used to determine membrane insertion pattern of stop-transfer or conservative sorted proteins.

Stop-transfer sorted MFP constructs (A) or conservative sorted MFP constructs (C) were transformed into W303·1α yeast strain and all cells were grown in fermentable carbon source medium (2% glucose) at 30°C. The total protein samples were prepared by TCA precipitation and were subjected to SDS-PAGE and Western blotting. Bands were detected by HA-antibody. The relative amounts of L-MFP and *s*-Mgm1p were quantified with more than three independent experiments and the average is shown with standard error. The sum of L-MFP and *s*-Mgm1p was set to

100%. L-MFP is denoted by *asterisks*. (B) Isolated mitochondria from yeast transformants carrying stop-transfer sorted MFPs or Yta10(1TM) MFP were incubated with or without proteinase K (PK) for 30 minutes at 4°C. Equal amounts of protein samples were analyzed by SDS-PAGE and immunodecorated with specific antibodies (OM makers, anti-Tom20 or anti-Tom70; IMS marker, anti-Tim54; Matrix markers, anti-Tim44 or anti-Mge1; anti-HA).

Yta10p is inserted into the inner membrane from the IMS

Interestingly, Yta10p, which is known as a conservatively sorted MIM protein, contains a presequence and two TM domains. It is still controversial how Yta10p is integrated into the IM of mitochondria. A recent study showed that Yta10p was fully translocated to the matrix and later reinserted into the lipid bilayer of the mitochondria in a membrane potential-dependent manner [27]. However, we noticed that its TM domains are more hydrophobic than those of conservatively sorted proteins (Figure 3A). We also found that Yta10(1TM) produced mostly L-MFP (Fig. 2C), indicating it was sorted by the stop-transfer pathway. We reasoned two possibilities for the insertion mechanism of Yta10p. 1) The second TM might interfere with anchoring of the first TM into the membrane, or 2) after first TM is inserted into the membrane, the following TM is imported from the IMS side. To address the second possibility, we first examined Cytochrome c1(Cyt1p) which is sorted from the IMS for its proper orientation (N_{IMS}-C_{Matrix}) [28] with the MFP assay. It contains two distinct sorting signals: N-terminal hydrophobic sorting signal and C-terminal internal signal (a stretch of positively charged amino acid residues following the TM). A MFP bearing N-terminal 70 residues of Cyt1p produced L-MFP, suggesting the HS of Cyt1p directed the protein into the membrane. However, Cyt1FL chiefly generated *s*-Mgm1p, indicating that after N-terminal HS integrated into the membrane, the TM is sorted from the IMS space into the membrane (Figure 3B).

We speculated that TM domains of Yta10p are also inserted into the IM similar to Cyt1p. Two positively charged residues (Arginine, R and Lysine, K) are present in the downstream of the TM2 of Yta10p. To investigate the role of these charged residues in the sorting of the TM2, two residues were changed to negatively charged

residues (Aspartic acid, D). The majority was L-MFP in Yta10(1-2TM)DD, implying that the insertion of TM2 into the membrane from the IMS depends on two positively charged residues (Figure 3C).

Figure 3

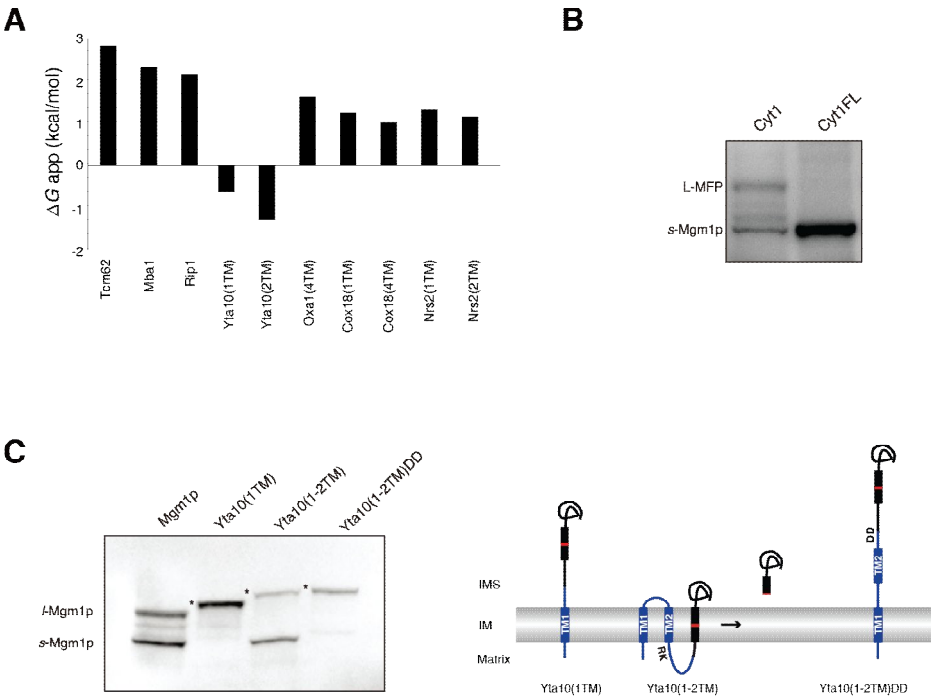


Figure3. TM2 segment of Yta10p is anchored into the membrane from the IMS. (A) The graph shows ΔG_{app} [29] of TM of conservative sorted MIM proteins. (B) Membrane integration of truncated or full length Cyt1 MFP was analyzed by SDS-PAGE and Western blotting. (C) Total protein samples were prepared from the yeast transformants carrying MFP constructs of Yta10(1TM), Yta10(1-2TM), and Yta10(1-2TM)DD as described in Fig 2(A). Schematic shows how MFPs of Yta10 variants are inserted into the membrane. *Asterisks* represent L-MFP.

Mdl1p is sorted into the inner membrane by stop-transfer and conservative sorting pathways

Next, to extend the usage of our MFP method for multi-spanning MIM proteins, we tested the feasibility of the assay with Mdl1p whose insertion mechanism had been determined by the *in vitro* protein import and proteinase fragmentation experiment. It contains a presequence and six predicted TM segments. Bohnert *et al.* showed TM1-2 segments are sorted by the stop-transfer pathway and the translocation of regions after TM3 domain is mediated by mtHsp60. While TM3-4 domains are reinserted into the membrane from the matrix in OXA1 complex-dependent manner (conservative sorting pathway), TM5-6 domains are likely to be anchored into the membrane by the stop-transfer pathway at the level of the TIM23 translocase [13].

TM domains of Mdl1p were sequentially truncated and fused to the C-terminal domain of Mgm1p. Mdl1(1TM) predominantly produced L-MFP in SDS-PAGE, suggesting it is sorted by the stop-transfer pathway. However, the rest of the constructs resulted in *s*-Mgm1p, indicating that TM2 segment is anchored into the membrane from IMS like Yta10p or Cyt1p, and subsequent portions after TM3 domain, are all translocated into the matrix (Figure 4A). Unlike previous study, we found that TM5-6 segments were not anchored by the stop-transfer mode. To determine this more in detail, we swapped the TM5 segment of Mdl1 (1-5TM) and Mdl1 (1-6TM) with natural TM domains which are sorted by the stop-transfer (Sco2p and She9p) or the conservative sorting (Mba1p) mechanism (Table I) (Figure 4B). When these proteins were expressed in the W303·1a, they all led to produce *s*-Mgm1p. However, Mdl1 (1-5TM) or Mdl1 (1-6TM) mutant carrying TM segment of the stop-transfer sorted proteins resulted in L-MFP in the *m*-AAA mutant strain (Δ*yta10*) (Figure 4C). It implies that *m*-AAA protease, which is

involved in the quality control of MIM proteins, sensed and dislocated these unnatural chimera proteins into the matrix, so that the RCR entered the membrane and processed. In case of Mdl1(1-5TM)[Mba1] and Mdl1(1-6TM)[Mba1], they generated s-Mgm1p similar to Mdl1(1-5TM) and Mdl1(1-6TM). It suggests that TM5-6 segments are likely to be translocated to the matrix rather than inserted into the membrane (Table II).

Figure 4

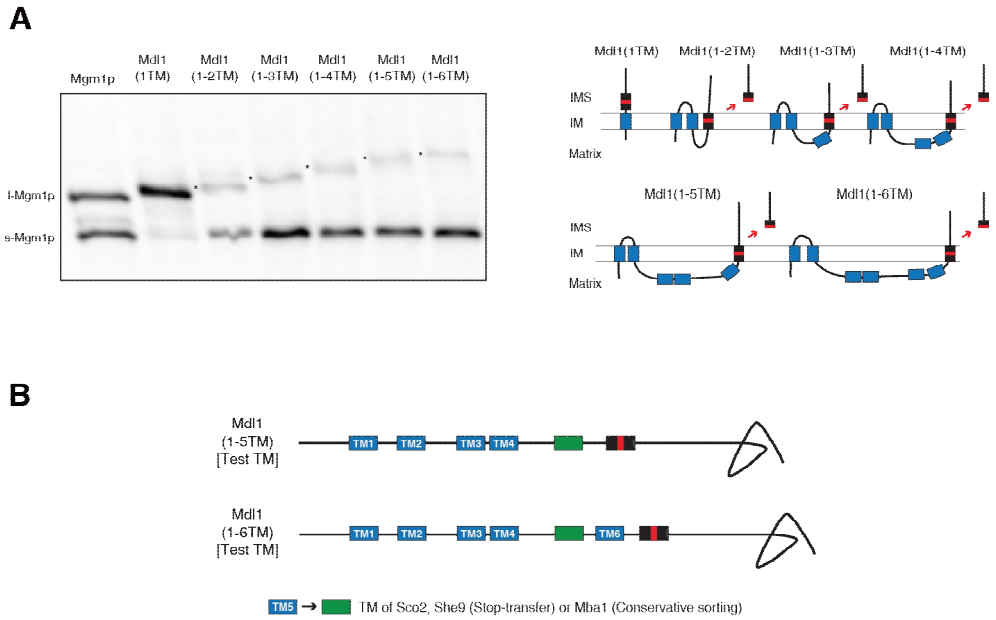


Figure 4. Mdl1p is integrated into the IM by both the stop-transfer and conservative sorting routes.

(A) Mdl1-MFP constructs were transformed into W303·1a strain and expressed in –Leu medium at 30°C. Whole cell lysates were prepared by TCA precipitation and analyzed by SDS-PAGE and Western blotting. Schematic of sorting mode of the individual TM segments (*blue*) of Mdl1p is shown. (B) TM5 (*blue*) of Mdl1(1-5TM) and Mdl1(1-6TM) were exchanged with a natural TM domain (*green*)

bearing either a stop-transfer sorting signal (Sco2 or She9) or a conservative sorting signal (Mba1). (C) Mdl1(1-5TM) or Mdl1(1-6TM) swapped TM domain constructs were transformed into W303·1a (wildtype) or Δ*yta10* strain, and insertion modes were analyzed as described in (A).

Insertion modes of MIM proteins with previously uncharacterized sorting mechanism is determined

We determined the sorting mechanism of MIM proteins carrying a presequence whose insertion pathways are not well characterized. We chose three subunits of cytochrome bc1 complex (Qcr8p [30], Qcr9p, and Qcr10p [31] [32] [33]) and a cytochrome oxidase assembly factor (Cox16p [34]) as single spanning MIM proteins which have an N_{Matrix}-C_{IMS} orientation. Moreover, Sdh4p [35, 36], a subunit of the succinate dehydrogenase complex, and Mdl2p [37], a homologue of Mdl1p, were selected as multi-spanning MIM proteins (Table II). The free energy of the membrane insertion of the TM domain is shown in Figure5A. Cox16 and Sdh4(3TM) fall into the range of stop-transfer pathway and consequently produced L-MFP more than 65% when they were tested with an MFP method (Figure 5B and Table III). It implied that these TM segments were integrated into the membrane by the stop-transfer pathway. Sdh4(1TM) and Sdh4(2TM) fall into the range of the conservative sorting pathway, and dominantly gave rise to *s*-Mgm1p indicating that TM1-2 of Sdh4 were imported into the matrix (conservative sorting route) (Figure 5B and Table III).

In case of Qcr8, Qcr10, and Qcr9, the ΔG presents within the overlapping region between stop-transfer and conservative sorting pathway. Previously, proline residue within the TM were shown to be an important determinant for the sorting of MIM proteins because it disfavors membrane integration [9]. Whereas Oxa1(3TM), Cox18(3TM) and Cox18(4TM) contain one or two proline residues, Yme1 and Cyt1 do not. Qcr9 and Qcr10 do not have any proline residues, but Qcr8 has one proline residue in its TM. However, positively charged residues were found near the TM of Qcr8 which may promote the TM insertion into the membrane [38]. Qcr8, Qcr9, and

Qcr10 generated L-MFP more than 70% (Figure 5B and Table III), indicating that if the hydrophobicity of TM segment is within the scope of overlap, proline residues and/or flanking charged residues influence membrane insertion of the protein. We also checked localization of L-MFPs by the mitochondria isolation experiment (Figure 5C).

Mdl2 is a homolog of Mdl1. Their protein length, the number of TM and hydrophobicity of TM are very similar to each other (Table II) (Figure 5A). Therefore, we hypothesized that the insertion pattern might not differ from Mdl1p. However, we observed that Mdl2(1TM) and Mdl2(1-3TM) produced predominantly L-MFP, whereas other constructs resulted in *s*-Mgm1p (Figure 5D and Table III). It indicated that TM1 and TM3 domains were inserted into the membrane at the level of the TIM23 import channel (stop-transfer pathway); TM2 and TM4 domains were anchored from the IMS. TM5-6 segments were sorted by the conservative sorting pathway. TM-exchanging experiment also supported that TM5 and TM6 were not anchored by the stop-transfer pathway (Figure 5E and Table III).

Figure 5

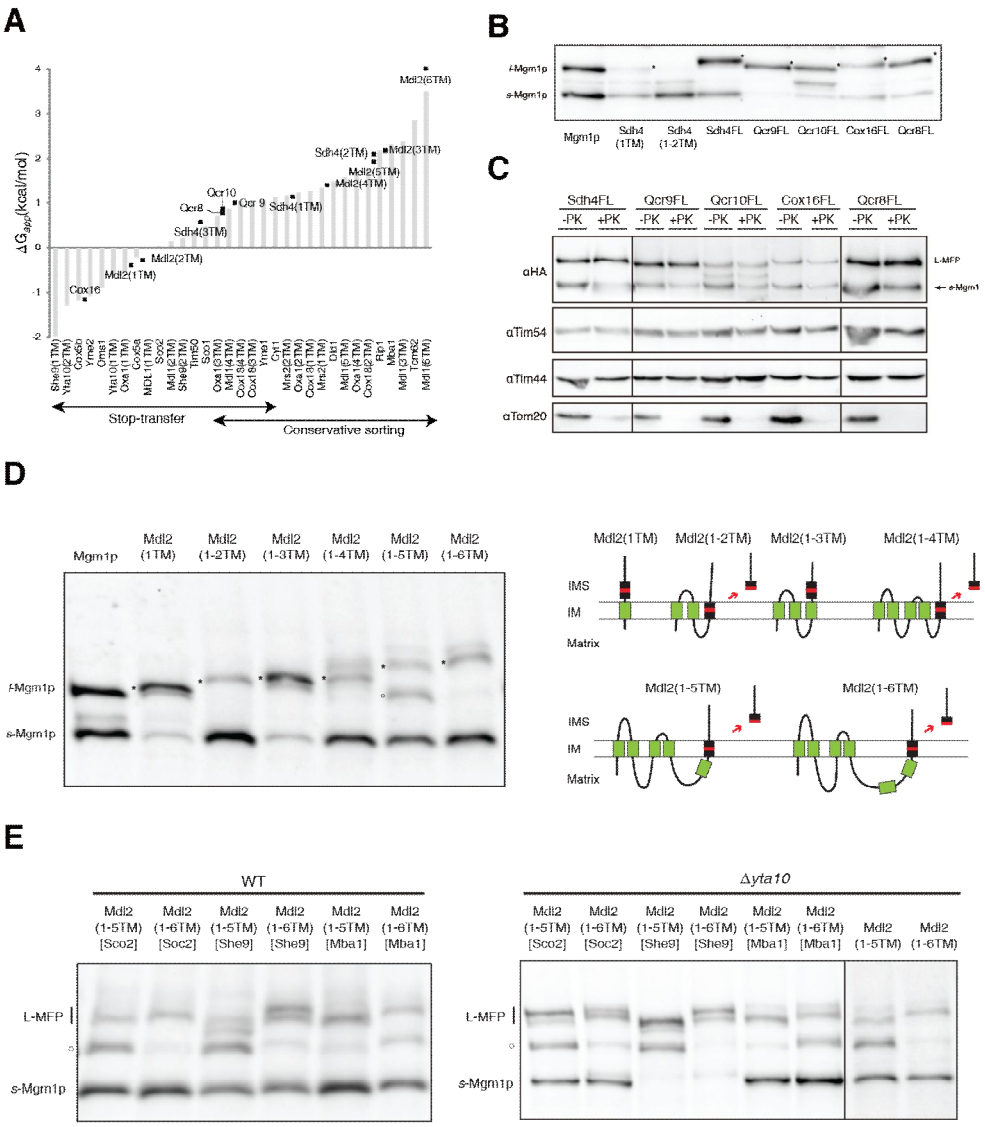


Figure 5. Membrane insertion of proteins whose sorting mechanism is not known is determined by the MFP assay.

(A) TM domains sorted by the stop-transfer or conservative sorting modes were arranged according to ΔG_{app} (*grey bars*) and the hydrophobicity of individual TM segment of MIM proteins whose membrane integration mechanism is unknown is indicated by *black* squares. (B) Indicated MFP constructs were transformed into W303·1α and proteins samples were prepared and accessed as described in Fig. 2A. (C) Localization of L-MFP was determined as described in Fig. 2B. (D), (E) Insertion pattern of the distinct TM domains of Mdl2p were determined as described in Fig. 4. Schematic representation of sorting mechanism of the distinct TM segments (*green*) of Mdl2p is shown.

Growth under respiring conditions increases the import of MFPs carrying conservative sorting proteins into the matrix

When yeast cells are grown under non-fermentable carbon source, such as glycerol, the cells depend on respiration which requires high activity of the mitochondria and thus a strong membrane potential is built across the IM. To check whether the membrane potential affects the sorting and translocation of MIM proteins, the transformants carrying different MFP constructs were grown in respiring or fermentable conditions. There is no significant effect on translocation of MFPs encoded stop-transfer pathway proteins except Dld1. The TM of Dld1 is less hydrophobic compared to the other tested TM segments of stop-transfer sorted MIM proteins (Figure 5A). Thus, the sorting of Dld1 might have been more sensitive to stronger membrane potential experienced with non-fermentable carbon source.

On the contrary, Tcm62, Rip1, Rip1FL, Yta10 (1-2TM), Mrs2 (1TM), and Mrs2 (1-2TM) produced more *s*-Mgm1p under the glycerol medium. It implied that they were efficiently translocated into the matrix under the non-fermentable carbon source (Figure 6A). Altogether, the sorting of the proteins containing moderated TM domains is influenced by the energetic states of mitochondria in the cell.

Growth at different temperatures shows varying membrane insertion efficiency of MFPs

When a precursor is unfolded at the higher temperature, its import into mitochondria is efficiently facilitated [39]. In addition, recent study reported different

submitochondrial localization of a GFP domain of GFP-tagged Tim23 depends on temperature [40]. These two studies imply that import and translocation of MIM proteins is correlated with the growth temperature. Nevertheless, the effect of various growth temperatures on membrane insertion is still unknown.

To test whether different temperatures affect the integration into the membrane of MIM proteins, yeast transformants carrying different MFP constructs were grown overnight in –Leu medium (2% glucose) at 25°C, 30°C, or 37°C. She9(1TM), Yta10(1-2TM), Cox5aFL , Cyt1 and Mrs2(1-2TM) produced more *s*-Mgm1p at 37°C compared to 25°C. It indicates that translocation of proteins to the matrix is increased with higher temperature. However, Rip1FL generated more L-MFP at 37°C compared to 25°C (Figure 6B) Recently, Cui *et al* [41] suggested that Mzm1p, Rip1p chaperone, stabilizes the Rip1p in the matrix by folding of the C-terminal domain at high temperature. The protein level of Rip1 was dramatically decreased at 37°C in Δ *Mzm1* cell compared to 30°C due to temperature-induced aggregation or protein degradation. It is conceivable that the C-terminal part of Rip1 may be folded or aggregated in the cytosol or IMS at elevated temperature during translocation, which would prevent further translocation into the matrix.

Altogether, these data indicate that translocation of MIM proteins and membrane insertion can be modulated according to various cellular environments, such as the growth temperature and composition of the growth medium.

Figure 6

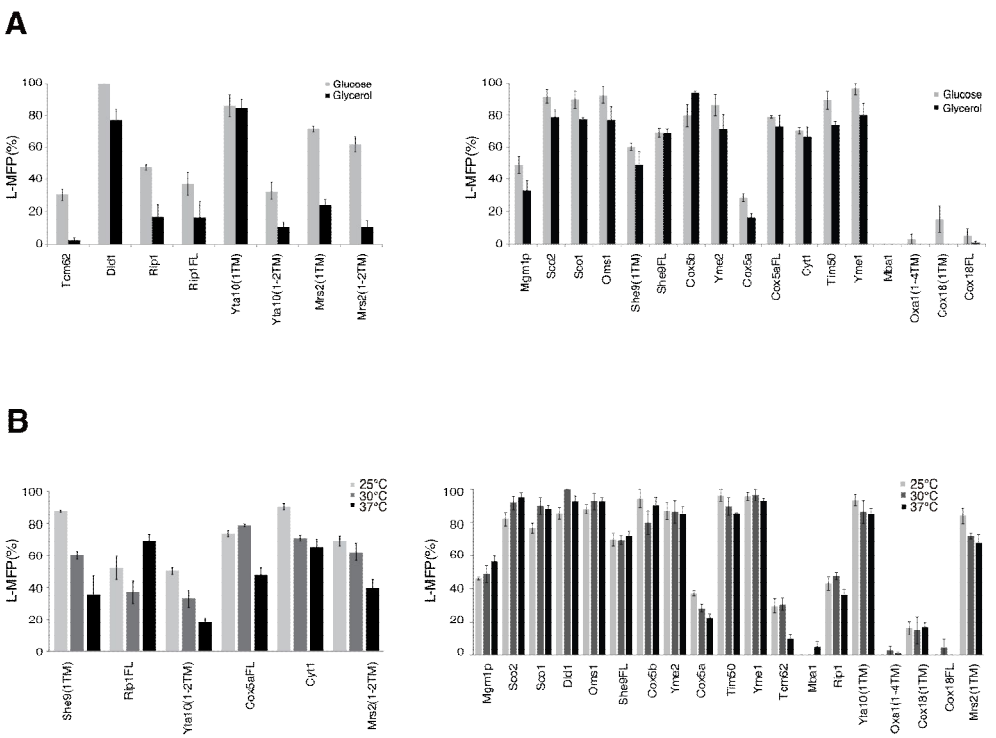


Figure 6. Import and translocation of Mgm1 fusion proteins into the mitochondrial membrane are influenced by different cellular environments.

(A) Import and membrane insertion of different Mgm1 fusion proteins grown in the medium supplemented with glucose (2%, w/v) or glycerol (3%, w/v), (B) in various temperatures (25°C, 30°C, or 37°C). Whole cell lysates were subjected to SDS-PAGE and Western blotting. The average ratio of L-MFP is shown with standard error. The experiments were conducted more than three times.

Impairment of PAM16 function inhibits translocation and membrane insertion of Cox18p, Mdl1p and Mdl2p.

The PAM complex consists of Pam16p, Pam17p, Pam18p, mtHsp70, Mge1p and Tim44p. It modulates translocation of proteins into the matrix by consuming ATP [17]. Therefore, impaired function of the PAM machinery not only hinders the translocation of matrix targeted proteins, but also results in increment of long isoform in Mgm1p [25] [42], because a functional import motor is critical for the production of *s*-Mgm1p. To demonstrate whether PAM complex is required for the correct translocation of other MIM proteins, we transformed various MFP constructs into temperature sensitive *pam16-3* mutant and its isogenic wildtype cell. Whereas L-MFP of Cox18FL Mgm1 fusion was significantly increased in *pam16-3* strain at non-permissive temperature, the sorting of Mba1, Cox18(1TM), Mrs2 was mildly affected (Figure 7A and B). This indicates that the import motor is essential for the translocation of Cox18p into the matrix.

A recent study showed that import of TM3-6 of Mdl1p was impaired in the defective mitochondria in mtHsp70 function [13]. To assess the effects of the PAM complex in translocation and membrane insertion of Mdl1p *in vivo*, systemically truncated Mdl1-MFPs were transformed into *pam16-3* strain and its isogenic wildetype strain. Relative amounts of L-MFP of Mdl1 (1-2TM) MFP, Mdl1 (1-3TM), Mdl1 (1-4TM), Mdl1 (1-5TM), and Mdl1 (1-6TM) MFP were all increased in *PAM16* mutant strain compared to the wildtype, suggesting that the PAM complex is required for the proper import of TM3-6 domains of Mdl1p consistent with previous results [13] (Figure 7C).

Next, we also examined the dependence of PAM complex on the sorting of individual TM domains of Mdl2p. Protein expression levels of Mdl2 (1-3TM), Mdl2 (1-4TM), Mdl2 (1-5TM) and Mdl2 (1-6TM) were very low in *Pam16* mutant cell and only small amounts of *s*-Mgm1p form was detected, compared to its wildtype (Figure. 7D). We suspected that membrane insertion of TM3-6 of Mdl2p is dependent on the PAM complex, and those inefficiently imported Mdl2p might undergo rapid degradation.

Figure 7

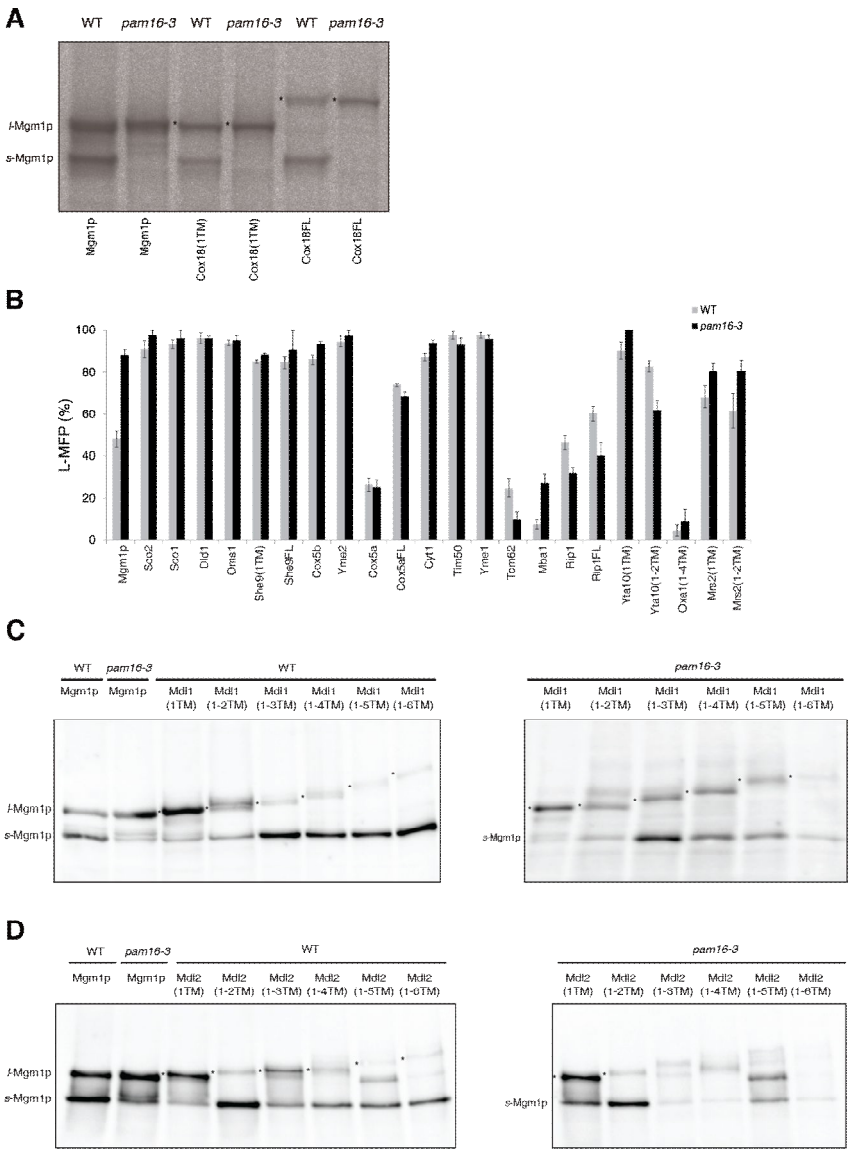


Figure 7. Inactivation of PAM complex increases the membrane integration of some multi-spanning MIM proteins.

(A) Mgm1p, Cox18(1TM), and Cox18FL in *pam16-3* or its isogenic wildtype yeast strain were expressed with [^{35}S]-methionine for 10 minutes at non-permissive temperature (37°C). Samples were immunoprecipitated and analyzed to SDS-PAGE

and autoradiography. Indicated MFP constructs (B), Mdl1 MFP constructs (C) or Mdl2 MFP constructs (D) were transformed into defective Pam16 function or its isogenic wildtype yeast strain, and protein samples were prepared and analyzed as described in Fig 2A. L-MFP was indicated by *asterisks*. The average ratio of L-MFP is shown with standard error. The experiments were conducted at least three times.

Inactivation of m-AAA protease results in increment of membrane insertion

The *m*-AAA protease is composed of two subunits, Yta10p and Yta1012p and forms hetero-oligomeric structure. It plays an important role in quality control of MIM proteins by degrading non-assembly, unfolded, and/or damaged proteins in the matrix side [43] [44] [45]. Furthermore, recent research reported that the *m*-AAA protease is also involved in the processing of pre-protein (the mitochondrial ribosomal subunit, MrpL32p [46]) and dislocation mitochondrial protein (Cytochrome c peroxidase, Ccp1p [16]) for the maturation of proteins.

To investigate if the *m*-AAA complex is also involved in the membrane integration of MIM proteins, we transformed various MFP constructs to the *m*-AAA deficient strain, which is one subunit of the *m*-AAA complex is deleted (Δ*yta10* or Δ*yta10*Δ*ccp1*), and its isogenic wildtype (W303-1a or Δ*ccp1*). The formation of L-MFP in the *m*-AAA deficient cell was increased more than 2-fold in Rip1, Rip1FL, and Yta10(1-2TM) and up to 80% in Cox5a (Figure 8A). The sorting of Rip1, Rip1FL, and Yta10(1-2TM) was sensitive to the growth conditions (Figure 6A).

Leonhard and his colleagues demonstrated that when the integral membrane proteins were loosely folded, they were highly degraded depending on the *m*-AAA protease [44]. We speculated that the *m*-AAA complex specially sensed the C-terminally truncated Cox5a, and dislocated it for the degradation. In our data, it produced more *s*-Mgm1p in wildtype, but not in the *m*-AAA deficient cell (Figure 8A). To elucidate this possibility we monitored the degradation or turnover of truncated Cox5a protein in the presence or absence of functional *m*-AAA protease. Truncated or full-length of Cox5a (Cox5aT-HA or Cox5aFL-HA, respectively) were HA-tagged for the

immunoprecipitation, radiolabeled with [^{35}S]-methionine and later incubated at 30°C for indicated times. Radiolabeled protein of Truncated Cox5a was completely degraded upon incubation; however, Cox5aFL was not (Figure 8B).

Figure 8

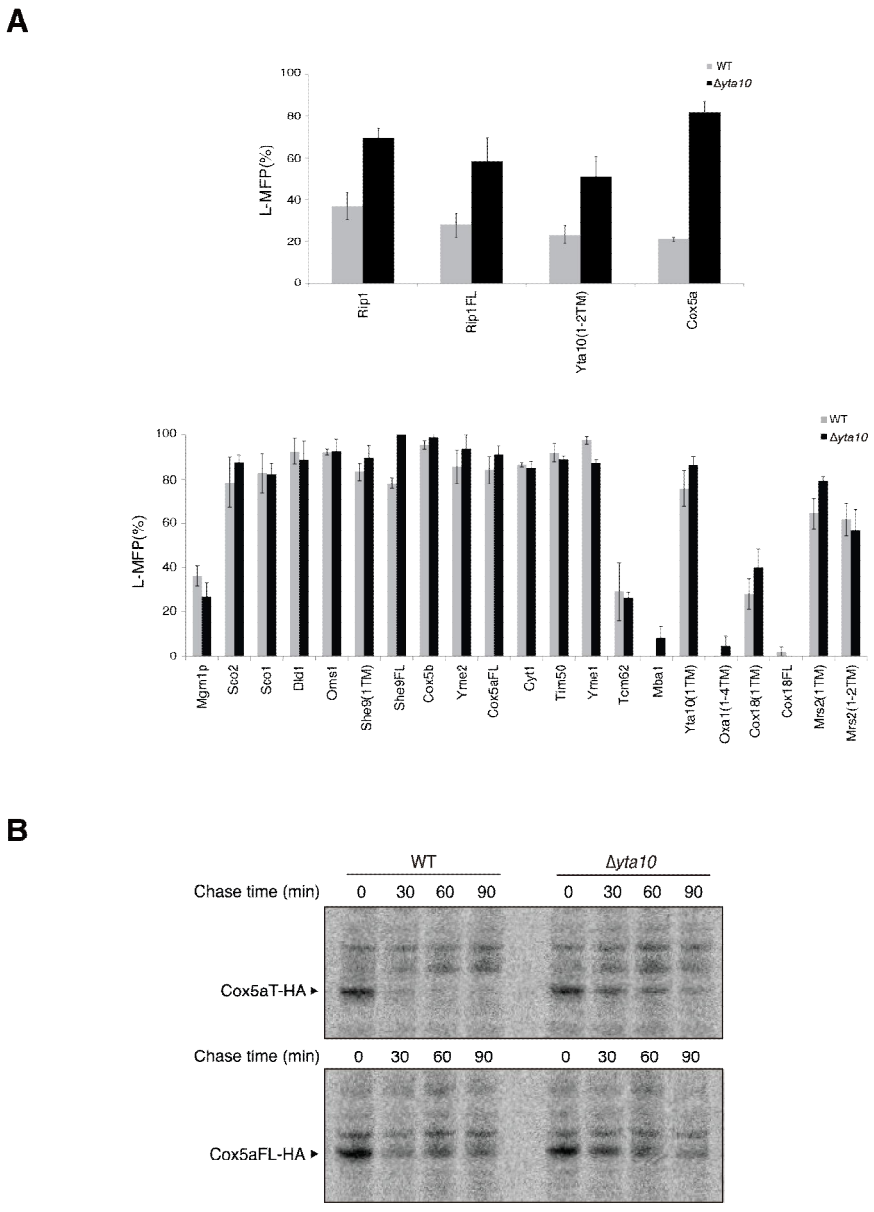


Figure 8. Membrane insertion of some tested proteins is increased in the *m*-AAA deficient strain.

(A) Mgm1 fusion proteins were expressed in the yeast strain defective *m*-AAA function (Δ*yta10*Δ*ccp1*) or wildtype (Δ*ccp1*). Total samples were prepared and

analyzed as described in Fig. 2A. (B) HA-tagged truncated or full-length of Cox5ap (Cox5aT-HA and Cox5AFL-HA, respectively) were expressed in Δ*yta10*Δ*ccp1 or* Δ*ccp1*, and radiolabeled with [^{35}S]-methionine for 10 minutes and further incubated at 30°C for indicated time points. Proteins were analyzed as described in Fig 7 (A).

DISCUSSION

In this study we developed the Mgm1 fusion protein (MFP) method to characterize the sorting mechanisms of the mitochondrial inner membrane (MIM) proteins by fusing the C-terminal domain of Mgm1p containing the rhomboid cleavage region (RCR). While *in vitro* import assay is performed under controlled environments, the MFP approach enables to investigate insertion mechanisms of MIM proteins in yeast cells and thus the outcomes would be more reliable compared to data derived from the *in vitro* experiment. In addition, we could study relations between the protein sorting and different cellular conditions.

We assessed the feasibility of this method with a number of the MIM proteins whose insertion modes were known and determined the sorting pathways of single or multi-spanning MIM proteins with previously uncharacterized sorting mechanisms. We suspected that downstream sequences of transmembrane (TM) domain in single-spanning MIM proteins are important for the proper membrane insertion/translocation (Figure. 2). In case of truncated Cox5a, the lack of the C-terminal region was sensed by the *m*-AAA complex and entire protein was dislocated into the matrix for its degradation (Figure. 8B)

It has been believed that there are two kinds of MIM proteins and they are integrated into the membrane by utilizing different sorting pathways (a stop-transfer or a conservative sorting pathway). However, we found that double spanning membrane proteins are inserted by two mechanisms: a stop-transfer mode and loop insertion by positively charged residues (Figure. 3C) Furthermore, membrane insertion of multi-spanning MIM proteins are facilitated by three sorting modes: a stop-transfer, a

conservative sorting and loop insertion mechanisms (Figure 4A, 5B and D). Our results show that membrane integration/translocation is more complex than previous thought.

We demonstrated the sorting mechanisms of Mdl1p and Mdl2p, half-size ABC transporters are different in spite of that they share high sequence homology. In particular we found that the mode of TM3 insertion differs between Mdl1p and Mdl2p although the predicted hydrophobicity of those TM segments is essentially no different (Table II). TM3 domain of Mdl2p was membrane inserted at the level of the TIM23 complex but not the TM3 segment of Mdl1p. Beasley et al [47] reported that presence of a proline or a glutamine residue in the hydrophobic stretch led to missorting of the cytochrome b_2 into mitochondrial matrix, indicating the introduction of proline or glutamine residues may change a specific conformation and it might disrupt the sorting signal. We found one glutamine residue in the TM3 domain of Mdl1p, but not in the Mdl2p. These observations imply that presence of glutamine within TM domains could be a crucial determinant for the sorting mechanism of MIM proteins. However, further studies are required to elucidate how this glutamine disrupts stop-transfer sorting.

Our data show that membrane insertion of proteins containing moderately hydrophobic segments was highly sensitive to different strength of the membrane potential upon the growth medium (Figure 6A). In addition, the sorting of MFPs was altered at elevated temperature (Figure 6B). Leonhard *et al.* reported that the *m*-AAA protease enables to recognize and degraded the unfolded domains of integral membrane proteins [48]. In this context, it is possible that even though MFPs were anchored into the membrane, solvent-exposed domains may be unfolded at high temperature and dislocated into the matrix mediated by the *m*-AAA complex.

We observed that the translocation of Cox18p, Mdl1p, and Mdl2p highly depend on the activity of the presequence translocase-associated import motor (PAM) (Figure 7). It indicates that these MIM proteins are required PAM complex for their translocation into the matrix. However, still it is unclear which specific regions are recognized and pulled by the PAM complex.

Our results show that the MFP approach is a useful and reliable experimental tool for determining the membrane insertion mechanisms of single-spanning as well as multi-spanning MIM proteins containing a cleavable N-terminal preseqeunce. In addition, our study suggests that the way the TIM23 complex mediates the stop-transfer membrane sorting may be different from the Sec61-mediated stop-transfer mechanism in the endoplasmic reticulum (ER). Furthermore, the sorting of MIM proteins is affected by different cellular conditions.

Table I. Information of sorting pathways of proteins tested in this study

	Systematic name	Gene name	Previously determined sorting pathway	References
Single spanning proteins	YBR024w	SCO2	Arrested	[9, 49]
	YBR037c	SCO1	Arrested	[9]
	YBR044c	TCM62	Conservative	[9]
	YBR185c	MBA1	Conservative	[9, 50]
	YDL174c	DLD1	Arrested	[9, 51]
	YDR316w	OMS1	Arrested	[9, 52]
	YEL024w	RIP1	Conservative	[9, 12]
	YIL111w	COX5B	Arrested	[9]
	YMR302c	YME2	Arrested	[9, 53]
	YNL052w	COX5A	Arrested	[9, 54]
	YOR065w	CYT1	Arrested	[9, 55]
	YPL063w	TIM50	Arrested	[9]
	YPR024w	YME1	Arrested	[9, 48]
Multiple spanning proteins	YDR393w	SHE9	Arrested	[9, 56]
	YER017c	YTA10	Conservative	[9, 27]
	YER154w	OXA1	Conservative	[9, 57]
	YGR062c	COX18	Conservative	[9]
	YLR188w	MDL1	Arrested + Conservative	[13]
	YOR334w	MRS2	Conservative	[9, 27]

Table II. Information of TM domains of proteins used in this study

Residues in the predicted TM sequence by the TOPCONS [58] and free energy of membrane insertion for the individual TM domain by the predictor [29].

Systematic name	Name	Length (a.a.)	Predicted TM domains	Predicted TM sequence	Length of MFP(a.a.)	ΔG_{app} (kcal/mol)
YBR024w	Sco2	115	1	^{79}RWKATIALLLLSGGTYAYL^{97}	944	0.023
YBR037c	Sco1	108	1	^{71}FSTGKAIALFLAVGGALSYFF^{91}	937	0.479
YBR044c	Tcm62	501	1	^{468}FMTKVGINAVLSAVILPSEVAF^{489}	1330	2.842
YBR185c	Mba1	107	1	^{74}VFAHPLIVANALIRRLYTF^{92}	936	2.321
YDL174c	Dld1	377	1	^{43}WLKYSVIASSATLFGYLFA^{61}	1206	1.479
YDR178w	Sdh4 (1TM)	98	1	^{69}WYMEKIFALSVVPLATTAMLTT^{90}	927	1.136
YDR178w	Sdh4 (1-2TM)	128	2	^{69}WYMEKIFALSVVPLATTAMLTT^{90} ^{93}LSTAADSFFSVMLLGYCYM^{111}	957	1.136 2.096
YDR178w	Sdh4FL	181	3	^{69}WYMEKIFALSVVPLATTAMLTT^{90} ^{93}LSTAADSFFSVMLLGYCYM^{111} ^{131}KYAMYMLGLGSAVSLFGIYKL^{151}	1010	1.136 2.096 0.578
YDR316w	Oms1	143	1	^{105}MTKYMIGAYVIFLIYGLFFTKKL^{127}	972	-0.89
YDR393w	She9 (1TM)	333	1	^{296}TWGTFILMGMNIFLFIVLQLLL^{317}	1162	-1.985
YDR393w	She9FL	456	2	^{296}TWGTFILMGMNIFLFIVLQLLL^{317} ^{438}FYLYSISLVSMTILVSGLI^{456}	1285	-1.985 0.215
YEL024w	Rip1	87	1	^{53}RSYAYFMVGAMGLLSSAGA^{71}	916	2.172
YEL024w	Rip1FL	215	1	^{53}RSYAYFMVGAMGLLSSAGA^{71}	1044	2.172
YER017c	Yta10 (1TM)	160	1	^{116}FANTMFLTIGFTIIFTLLT^{134}	989	-0.619
YER017c	Yta10 (1-2TM)	256	2	^{116}FANTMFLTIGFTIIFTLLT^{134} ^{225}FTFLFPFLPTIILLGGLYFITR^{246}	1085	-0.619 -1.295
YER154w	Oxa1 (1-4TM)	316	4	^{126}LPWWGTIAATTILIRCLMFPLYV^{148} ^{199}RWLAAPMLQIPIALGFFNALR^{219} ^{245}PYLGLQVITAAVFISFTRL^{263} ^{278}RLFTILPIISIPATMNLSSAVVL^{300}	1145	-0.549 1.234 0.714 1.644
YGR062c	Cox18 (1TM)	88	1	^{51}ASHIPWIVLVPLTTMTLRTLVTL^{73}	917	1.261
YGR062c	Cox18FL	316	4	^{51}ASHIPWIVLVPLTTMTLRTLVTL^{73} ^{167}ALLPMVQIPLWVTVSMGIRTLT^{188} ^{215}LVAMPLLAPILVGTLAVLNVEL^{236} ^{273}RLGCVVMLAMSSQAPFLLSLYWI^{295}	1145	1.261 1.796 1.050 1.029
YGR183c	Qcr9FL	66	1	^{10}FFKRNAVFVGTIFAGAFVFQTVF^{32}	895	1.002
YHR001w-a	Qcr10FL	77	1	^{30}LMLWGGASMLGLFVFTEGW^{48}	906	0.874
YIL111w	Cox5b	126	1	^{91}FITKGVFLGLGISFGLFGLVRLL^{113}	955	-1.163

YJL003w	Cox16FL	118	1	^{34}FLFFGLPFCATIVLGSFWLSSFT^{56}	947	-1.152
YJL166w	Qcr8FL	94	1	^{49}FRRFKSQFLYVLIPAGIYWYWWK^{71}	923	0.777
YLR188w	Mdl1 (1TM)	156	1	^{100}SKYIGLALLLILISSSVSMAV^{120}	985	-0.027
YLR188w	Mdl1 (1-2TM)	232	2	^{100}SKYIGLALLLILISSSVSMAV^{120} ^{155}FFTALGAVFIIGAVANASRII^{175}	1061	-0.027 0.144
YLR188w	Mdl1 (1-3TM)	254	3	^{100}SKYIGLALLLILISSSVSMAV^{120} ^{155}FFTALGAVFIIGAVANASRII^{175} ^{233}DGTRAIIQGFVGFGMMSFLSW^{253}	1083	-0.027 0.144 2.378
YLR188w	Mdl1 (1-4TM)	336	4	^{100}SKYIGLALLLILISSSVSMAV^{120} ^{155}FFTALGAVFIIGAVANASRII^{175} ^{233}DGTRAIIQGFVGFGMMSFLSW^{253} ^{255}LTCVMMILAPPLGAMALIYGR^{275}	1165	-0.027 0.144 2.378 0.88
YLR188w	Mdl1 (1-5TM)	372	5	^{100}SKYIGLALLLILISSSVSMAV^{120} ^{155}FFTALGAVFIIGAVANASRII^{175} ^{233}DGTRAIIQGFVGFGMMSFLSW^{253} ^{255}LTCVMMILAPPLGAMALIYGR^{275} ^{337}GLFFGSTGLVGNTAMLSLLLV^{357}	1201	-0.027 0.144 2.378 0.88 1.582
YLR188w	Mdl1 (1-6TM)	407	6	^{100}SKYIGLALLLILISSSVSMAV^{120} ^{155}FFTALGAVFIIGAVANASRII^{175} ^{233}DGTRAIIQGFVGFGMMSFLSW^{253} ^{255}LTCVMMILAPPLGAMALIYGR^{275} ^{337}GLFFGSTGLVGNTAMLSLLLV^{357} ^{373}SSFMMYAVYTGSSLFGLSSFY^{393}	1236	-0.027 0.144 2.378 0.88 1.582 3.507
YMR302c	Yme2	384	1	^{286}TRIAIPVLFALLSIFAVLVF^{305}	1213	-1.079
YNL052w	Cox5a	128	1	^{94}FIAKGVAAGLLFSVGLFAVVRMA^{116}	957	-0.228
YNL052w	Cox5aFL	153	1	^{94}FIAKGVAAGLLFSVGLFAVVRMA^{116}	982	-0.228
YOR065w	Cyt1	70	1	^{36}LVTAGVAAAGITASTLLYA^{54}	899	1.125
YOR065w	Cyt1FL	309	2	^{36}LVTAGVAAAGITASTLLYA^{54} ^{268}RLGLKTVIILSSLYLLSIWV^{287}	1138	1.125 -0.012
YOR334w	Mrs2 (1TM)	344	1	^{315}VTIYTLGFTVASVLPAFYGMNL^{336}	1173	1.334
YOR334w	Mrs2 (1-2TM)	421	2	^{315}VTIYTLGFTVASVLPAFYGMNL^{336} ^{345}WGFTSVAVFSIVSALYITK^{363}	1250	1.334 1.171
YPL063w	Tim50	151	1	^{112}YANWFYIFSLSALTGTAIYMAR^{133}	980	0.398
YPL270w	Mdl2 (1TM)	171	1	^{115}DWKLLLTAILLLTISCSIGMS^{135}	1000	-0.396
YPL270w	Mdl2 (1-2TM)	247	2	^{115}DWKLLLTAILLLTISCSIGMS^{135} ^{172}SFFTVALLIGCAANFGRFILL^{192}	1076	-0.396 -0.283
YPL270w	Mdl2 (1-3TM)	269	3	^{115}DWKLLLTAILLLTISCSIGMS^{135} ^{172}SFFTVALLIGCAANFGRFILL^{192} ^{248}DGVKALICGVVGVGMMCSLSP^{268}	1098	-0.396 -0.283 2.171
YPL270w	Mdl2 (1-4TM)	351	4	^{115}DWKLLLTAILLLTISCSIGMS^{135} ^{172}SFFTVALLIGCAANFGRFILL^{192} ^{248}DGVKALICGVVGVGMMCSLSP^{268} ^{270}LSILLLFFTPPVLFSASVFGK^{290}	1180	-0.396 -0.283 2.171 1.390
YPL270w	Mdl2 (1-5TM)	387	5	^{115}DWKLLLTAILLLTISCSIGMS^{135} ^{172}SFFTVALLIGCAANFGRFILL^{192} ^{248}DGVKALICGVVGVGMMCSLSP^{268} ^{270}LSILLLFFTPPVLFSASVFGK^{290} ^{352}AKFFTTTSLLGDLSFLTVLAY^{372}	1216	-0.396 -0.283 2.171 1.390 1.919
YPL270w	Mdl2 (1-6TM)	422	6	^{115}DWKLLLTAILLLTISCSIGMS^{135} ^{172}SFFTVALLIGCAANFGRFILL^{192} ^{248}DGVKALICGVVGVGMMCSLSP^{268} ^{270}LSILLLFFTPPVLFSASVFGK^{290} ^{352}AKFFTTTSLLGDLSFLTVLAY^{372} ^{388}TAFMLYTEYTGNAVFGLSTFY^{408}	1251	-0.396 -0.283 2.171 1.390 1.919 4.182
YPR024w	Yme1 MFP	330	1	^{230}VSRWVKWLLVFGILTYSFS^{248}	1159	1.063

Table III. Summary of membrane insertion mechanism of single TM and Multiple TM proteins of mitochondria

	Name		Sorting mechanism
Single TM Proteins	Qcr8		Arrested
	Qcr9		Arrested
	Qcr10		Arrested
	Cox16		Arrested
Multiple TM proteins	Sdh4	TM1	Conservative
		TM2	Conservative
		TM3	Arrested
	Mdl2	TM1	Arrested
		TM2	Inserted from IMS
		TM3	Arrested
		TM4	Inserted from IMS
		TM5	Conservative
		TM6	Conservative

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국문초록

*Saccharomyces cerevisiae*에서 절단 가능한 전서열을 가진 미토콘드리아 내막 단백질은 핵에 암호화되어 있고, 내·외막 트란스로카제 (TOM-TIM23 복합체)에 의해서 내막으로 삽입된다. 단백질 막관통영역 내의 소수성 분류 신호가 TIM23 복합체에 의하여 인식되어 내막으로 삽입된다 (stop-transfer 경로). 그 외 경우, 우선 전체 단백질이 기질로 수송되고, 그 이후 내막으로 보내진다(conservative sorting 경로). 또한 미토콘드리아의 다중막관통 단백질은 위 두 경로 모두를 이용하여 막으로 삽입된다. 미토콘드리아 내막은 가장 단백질이 풍부한 막이며, 세포 활동에 있어서 중요한 역할을 하는 다양한 복합체들을 포함하고 있다. 그럼에도 불구하고 효과적인 실험방법의 부재로, 아주 적은 수의 미토콘드리아 내막 단백질 삽입 메커니즘만이 밝혀졌다. 우리는 세포 내 미토콘드리아 내막 단백질의 삽입 기작을 연구하기 위하여, Mgm1 단백질의 카르복시말단에 위치한 롬보이드 절단 부위를 이용한 Mgm1 융합 단백질 실험방법을 고안하였다. 우리는 이 실험방법의 유효성을 내막으로의 삽입경로가 잘 알려진 여러 단백질을 이용하여 입증하였고, 삽입 메커니즘이 알려지지 않은 미토콘드리아 단일 혹은 다중 막관통단백질의 내막 삽입 기작을 밝혔다. 우리의 결과는 Yta10 단백질이 기질에서가 아닌, 막사이공간으로부터 내막으로 삽입됨을 시사한다. 또한, 숙신산탈수소효소의 소단위, Sdh4 단백질과 미토콘드리아 내막의 ABC 수송체, Mdl2 단백질이 두 가지 경로(종결이동 경로와 conservative sorting 경로) 모두를 통하여 삽입됨을 밝혔다. 더욱이, 다양한 성장 조건과 효모 변이 주에서의 Mgm1 융합 단백질의 분류 유형을 분석함으로써, 우리는 중간 정도의 소수성 막관통 분절을 가진 단백질의 내막으로의 삽입이 내인성 및 외인성 세포 요인에 매우 민감함을 밝혔다. 더 나

아가, PAM 복합체가 Cox18, Mdl1, 그리고 Mdl2 단백질의 전좌에 필요하다는 것을 밝혔다.

주요단어: Mgm1 단백질, 효모 미토콘드리아, TIM23 복합체, 정지-이동 경로, Conservative sorting 경로, 막삽입, *m*-AAA 단백질 가수분해 효소, PAM 복합체, 다중 막관통 내막 단백질