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A short C-terminal tail prevents mis-targeting of hydrophobic mitochondrial membrane proteins to the ER

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ABSTRACT

Sdh3/Shh3, a subunit of mitochondrial succinate dehydrogenase, contains transmembrane domains with a hydrophobicity comparable to that of endoplasmic reticulum (ER) proteins. Here, we show that a C-terminal reporter fusion to Sdh3/Shh3 results in partial mis-targeting of the protein to the ER. This mis-targeting is mediated by the signal recognition particle (SRP) and depends on the length of the C-terminal tail. These results imply that if nuclear-encoded mitochondrial proteins contain strongly hydrophobic transmembrane domains and a long C-terminal tail, they have the potential to be recognized by SRP and mis-targeted to the ER.

1. Introduction

Eukaryotic cells have evolved subcellular organelles containing distinct subsets of proteins. The vast majority of these proteins are translated by cytosolic ribosomes and targeted to the correct organelles. Protein targeting to the endoplasmic reticulum (ER) is mediated by N-terminal signal peptides (SPs), while most mitochondrial proteins have N-terminal mitochondrial targeting peptides (mTPs). However, many membrane proteins destined to either compartment lack a typical cleavable SP or mTP, but rather the transmembrane (TM) domain(s) function as a targeting signal [1–7].

TM domains of the ER-targeted membrane proteins are generally more hydrophobic than those of mitochondrially targeted membrane proteins [6,8]. The first hydrophobic TM domain of the ER-targeted membrane proteins is recognized by signal recognition particle (SRP) and mainly targeted co-translationally, whereas weakly hydrophobic TM domains of mitochondrial proteins such as carrier proteins interact with cytosolic chaperones and are targeted to mitochondria in a post-translational mode [9,10]. Tail-anchored membrane proteins that are post-translationally inserted into the ER or the mitochondrial outer membrane follow a similar pattern in that those with a more hydrophobic C-terminal TM domain are targeted to the ER whereas those with a less hydrophobic TM domain are targeted to mitochondria [11,12]. Thus, correct targeting of membrane proteins to the ER or to mitochondria seems to be, at least in part, ensured by differences in the hydrophobicity of their respective TM domains. Nevertheless, some cytosolically translated mitochondrial membrane proteins contain markedly hydrophobic TM domains, and it is still unknown by which mechanisms such proteins escape recognition by SRP and are correctly targeted to mitochondria.

Shh3 is a homolog of Sdh3, a subunit of the mitochondrial succinate dehydrogenase complex [13]. Both proteins contain 3 TM domains and reside in the mitochondrial inner membrane. In an earlier study, we found that Shh3 was mis-targeted to the ER when it has a long reporter domain fused to the C-terminus [14]. The hydrophobicity of TM1 and TM3 of Shh3 is unusually high, comparable to the hydrophobicity of typical ER signal sequences or TM domains in ER-targeted membrane proteins. Its homolog Sdh3 also has markedly hydrophobic TM1 and TM3 domains (Fig. 1A). These observations led us to speculate that the addition of a large C-terminal fusion domain to Shh3/Sdh3 might trigger recognition of the TM1/TM3 domains by SRP, resulting in co-translational mis-targeting to the ER.

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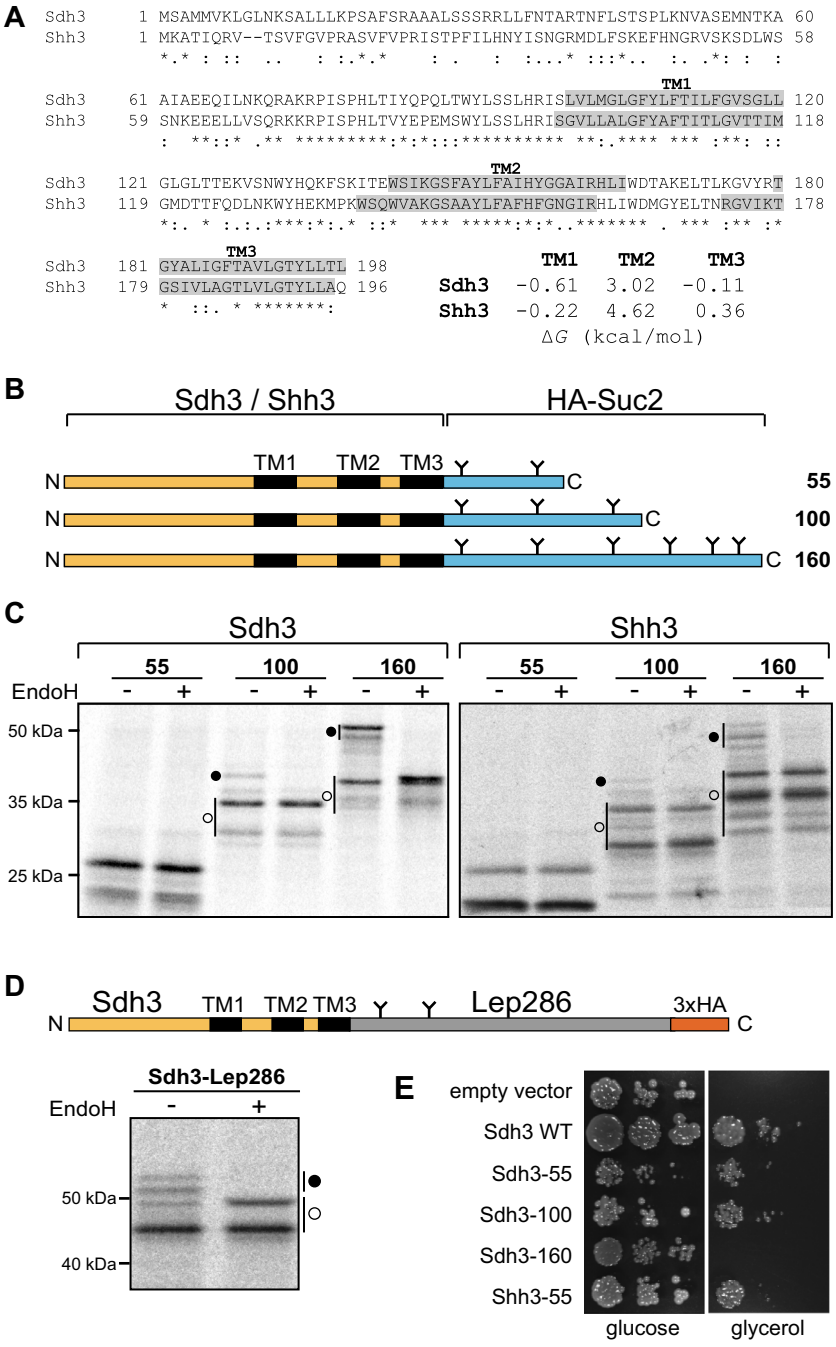

Fig. 1. Sdh3 and Shh3 can be targeted to the ER as the C-terminal fusion domain lengthens. (A) TM domains of Sdh3 and Shh3 are indicated with free energy of membrane insertion (ΔG) [23]. (B) Schematics of Sdh3 and Shh3 fusions. (C) Yeast cells expressing Sdh3 or Shh3 fusion proteins were radiolabeled with S^{35}[Met] and subjected to immunoprecipitation, prior to Endoglycosidase H (EndoH) digestion and SDS–PAGE analysis. Glycosylated and unglycosylated forms are indicated as black circle and open circle, respectively. (D) Yeast cells expressing Sdh3-Lep286 fusion protein were pulse labeled and treated as described above. (E) Δ*sdh3* strain expressing Sdh3 or Shh3 fusion proteins were grown in fermentable medium (glucose) or respiring medium (glycerol).

To test this idea, Shh3 and Sdh3 carrying C-terminal extensions of varying lengths were expressed in wild type and SRP-defective yeast strains. Our results show that targeting of Shh3/Sdh3 fusion proteins to the ER indeed depends on the length of the C-terminal fusion domain and is mediated by SRP. From these results we predict that nuclear-encoded mitochondrial membrane proteins containing TM segments with the hydrophobicity range for SRP recognition do not have long C-terminal tails.

2. Materials and methods

2.1. Strains

For protein expression, plasmids were transformed into the haploid yeast strain BWY46 (same as W303-α, *MAT*α, *ade*2, *can*1, *his*3, *leu*2, *trp*1, *ura*3) [15] or the isogenic strain BWY500 (*MAT*α, *sec*65-1, *ade*2, *can*1, *his*3, *leu*2, *trp*1, *ura*3) [15]. *sdh*3Δ (*MAT*α,

*ade*2, *can*1, *his*3, *leu*2, *trp*1, *ura*3, *SDH*3::*HIS*3) was derived from W303-1 α (*MAT*α, *ade*2, *can*1, *his*3, *leu*2, *trp*1, *ura*3) using the *HIS3* deletion cassette as detailed in [16]. Correct integration of *HIS3* cassette in the gene locus of *SDH3* was confirmed by PCR.

2.2. Plasmid construction

SDH3HASUC2HIS4C was subcloned into pJK90 as described in [14] and *SHH3HASUC2HIS4C* in pJK90 was obtained from [14]. These constructs were used as PCR templates to amplify various truncated versions of *SDH3/SHH3* fusions and subcloned into p424GPDHA [17] by homologous recombination.

The plasmid containing *SDH3-Lep286* was constructed by overlap PCR [18] using *p424SDH3HASUC2HIS4C-55* and p424GPD-Hl-5L [19] as templates and homologous recombination with p424GPDHA. Constructs p424-*SDH3*(TM1)-160, p424-*SDH3*(TM3)-160, and p424-*SDH3*(TM1)-Pho8 were generated by site-directed mutagenesis using p424-*SDH3HASUC2-160, p424-SHH3HASUC2*-160, and *pPHO8HA* [19] respectively. In p424-*SDH3*(TM1)-160, residues from 143 to 197 were deleted, so that after the TM1, the total length of the C-terminal tail is 182 (22 residue in Sdh3 plus 160 residues from the fusion domain). In p424-*SDH3*(TM3)-160, residues from 100 to 165, containg TM1 and TM2, were deleted; the total length of the C-terminal tail after TM3 remained 160. For the construction of p424-*SDH3*(TM1)-Lep and p424-*SHH3*(TM1)-Lep, a *SmaI* cleavage site was first engineered into the TM segment of Lep in p424GPD-H2 [17]. The TM segments 1 from *SDH3* and *SHH3* were amplified by PCR and cloned into *SmaI*-linearized p424GPD-H2.

Truncated *YTA10* were amplified from W303-1α strains by PCR and were subcloned into pJK90 [14] by yeast homologous recombination. The correct sequence of all plasmids was confirmed by DNA sequencing.

2.3. Pulse-labeling and immunoprecipitation

Pulse labeling experiments were carried out as described in [19]. For immunoprecipitation, protein G-agarose beads and anti-HA antibody were used. After overnight incubation and washing, beads were incubated with SDS–PAGE sample buffer at 60 °C for 15 min. Prior to SDS–PAGE, samples were subjected to Endoglycosidase H (EndoH) digestion with addition of 15 µl protein sample, 10.5 µl H_2O, 3 µl EndoH buffer, 1.5 µl EndoH (Roche) or 1.5 µl ddH_2O for the mock sample, and incubated for 2 h at 37 °C. Radiolabelled bands on SDS gels were visualized using a Fuji FLA-3000 phosphoimager and the Image Reader V1.8J/Image Gauge V 3.45 software.

2.4. Western blot analysis

Yeast transformants expressing fusion proteins were grown at 30 °C in 5 ml -Trp medium overnight. Temperature sensitive BWY500 cells were grown at 23 °C overnight and shifted to 37 °C for 4 h. Cells were harvested by centrifugation at 3000×*g* and washed with ddH_2O. Cell pellets were resuspended with 100 µl SDS–PAGE sample buffer and heated at 60 °C for 15 min. Yeast cells carrying Yta10 fusion constructs were grown at 30 °C in -Ura medium up to 1 A_{600} unit. Proteins were precipitated by trichloroacetic acid (TCA) as described in [20]. Prior to SDS–PAGE and Western blotting, samples were subjected to EndoH digestion as described above.

2.5. Growth complementation assay

Cells were grown in selective media containing glucose as carbon source overnight. After measuring A_{600}, each transformant was subjected to 8-fold serial dilution, and cells were grown on fermentable, glucose-containing or non-fermentable, glycerol-containing media at 30 °C for 2 or 4 days, respectively.

2.6. Bioinformatics analysis

All proteins annotated as mitochondrial, mammalian [21] and baker's yeast [22] were downloaded. They were homology-reduced to <30% identity using CD-HIT. From the remaining sequences the position and hydrophobicity of predicted transmembrane segments were obtained using the AG prediction server [23]. The protein structure dataset is composed of all mitochondrial proteins/chains found in OPM (107 proteins), the energy and membrane regions are the values and regions given in OPM [24]. The yeast data set is composed of 26 mitochondrial proteins. For protein sequence alignment of Sdh3 and Shh3 the ClustalW2 program was used [25].

3. Results

3.1. Shh3/Sdh3 fusion proteins with long C-terminal tails are mis-targeted to the ER

To determine whether the ER targeting of Shh3 (and its homolog Sdh3) depends on the length of the C-terminal tail, Shh3 and Sdh3 constructs with C-terminal extensions of varying lengths were expressed in the yeast *Saccharomyces cerevisiae*, and their subcellular localizations were assessed. The length of the C-terminal tail was adjusted to 55, 100, and 160 residues by shortening the originally fused topology reporter domain HASuc2His4C [14] from the C-terminal end. The C-terminal tails contained 2 to 6 N-linked glycosylation sites, so that the ER targeting could be monitored by glycosylation (Fig. 1B). Fusion constructs were transformed and expressed in the *S. cerevisiae* strain W303-1α. While Sdh3-55 was not glycosylated, increasing fractions of Sdh3-100 and Sdh3-160 became glycosylated (black circle), indicating that they were at least partially mis-targeted to the ER (Fig. 1C). A similar pattern was observed for Shh3 fusions (Fig. 1C). Minor bands detected by radiolabelling might be inefficient alternative translation products or proteolysis products of the full-length fusion proteins. To check whether ER targeting depends on the particular C-terminal fusion domain used, Sdh3 was also fused to the C-terminal periplasmic domain of the non-yeast protein *Escherichia coli* LepB [17]. Sdh3-Lep286 was also partially glycosylated (Fig. 1D), indicating mis-targeting to the ER.

3.2. Shh3 and Sdh3 fusion proteins with shorter C-terminal tails are targeted to mitochondria

Next, we determined whether Sdh3 and Shh3 fusion proteins are targeted to mitochondria, using a complementation assay. When the function of the succinate dehydrogenase complex is impaired in the *sdh3* deletion strain (Δ*sdh*3), yeast cells lose the capacity to respire [13]. Therefore, they cannot grow on medium containing glycerol, but can survive on glucose-containing fermentable medium. Sdh3 fusion constructs were transformed into Δ*sdh*3 strain, and cells were grown on plates containing glycerol. Shh3 is a functional homolog of Sdh3 and can rescue the Δ*sdh*3 strain [13]. Therefore, an Shh3-fusion protein was also assessed for functional complementation of the Δ*sdh*3 phenotype. Sdh3-55, Sdh3-100 and Shh3-55 rescued the growth defect of the Δ*sdh*3 strain on glycerol medium, suggesting that these fusion proteins are correctly targeted to mitochondria in sufficient amounts and associated with other subunits of the succinate dehydrogenase to form a functional complex (Fig. 1E). The longest Sdh3 fusion protein (Sdh3-160) failed to rescue the Δ *sdh*3 growth defect. This may be due to its mis-targeting to the ER or additionally, a failure

to fold or associate with other subunits of the succinate dehydrogenase complex in spite of mitochondrial targeting.

3.3. Sdh3 and Shh3 fusion proteins are targeted to the ER by SRP

We next assessed whether targeting of the Sdh3-160 and Shh3-160 fusion proteins to the ER is mediated by SRP. Sdh3-160 and Shh3-160 constructs were transformed and expressed in both a temperature-sensitive SRP-defective strain (BWY500) and its isogenic wild type strain (BWY46) [15]. While glycosylated product was observed in the wild type strain, it was not seen in the SRP-defective strain at the non-permissive temperature (Fig. 2A). These results suggest that the glycosylated products of Sdh3-160 and Shh3-160 resulted from the SRP-dependent targeting to the ER.

3.4. TM1 of Shh3 and Sdh3 can function as signal sequences and direct proteins to the ER

The hydrophobicity of TM1 and TM3 of Sdh3 are comparable to those of ER signal sequences (Fig. 1A). Thus, to test whether TM1 or TM3 alone can target Sdh3-160 to the ER, we prepared constructs that contain only TM1 (Sdh3(TM1)-160) or only TM3 (Sdh3(TM3)-160). With deletion of TM2-3 for Sdh3(TM1)-160, and TM 1-2 for Sdh3(TM3)-160 the C-terminal tails contain 182 and 160 residues, respectively. Since the full-length Sdh3-160, which contains 3 TM domains, was glycosylated, the protein was inserted with N_{in}-C_{out} topology; consequently, both TM1 and TM3 have N_{in}-C_{out} orientation. Hence, we reasoned that Sdh3(TM1)-160 and Sdh3(TM3)-160 may both attain a single spanning N_{in}-C_{out} membrane topology when targeted to the ER, such that the C-terminal reporter would be accessible for glycosylation. Sdh3(TM1)-160 became partially glycosylated, suggesting that the TM1 has the capacity to direct the fusion protein to the ER (Fig. 2B). However, no glycosylated product of Sdh3(TM3)-160 was detected, indicating that TM3 alone cannot target the fusion protein to the ER, albeit, we cannot exclude the possibility that Sdh3(TM3)-160 is inserted into the ER membrane with N_{out}-C_{in} orientation, with the C-terminal tail being located in the cytosol. It has been shown that the first TM segment in multi-spanning membrane proteins acts as a signal sequence [26]. Further, recognition by SRP arrests translation and recruits the ribosome–SRP–nascent chain complex to the ER, with translation resuming after ER targeting. Therefore, the TM1 of Sdh3 would be more likely the main TM segment responsible for the SRP-mediated ER-mistargeting.

To further determine whether TM1 of Sdh3 and Shh3 can act as an ER signal sequence independent of protein context, we replaced the signal anchor sequence of Pho8 with TM1 of Sdh3, and further replaced the TM domain of an engineered version of *E. coli* LepB [17] with TM1 of Sdh3 and Shh3 (Fig.2C). Pho8 [27] has two natural glycosylation sites in the C-terminal domain. If the protein is correctly targeted to the ER, it is inserted in an N_{in}-C_{out} membrane topology and becomes glycosylated. Endoglycosidase digestion showed that Sdh3(TM1)-Pho8 was completely glycosylated (Fig. 2C, black circle), indicating efficient ER targeting. The engineered *E. coli* Lep construct [17] contains two N-linked glycosylation sites flanking the TM domain, thus if it is targeted to the ER, it is glycosylated. The resulting constructs Sdh3(TM1)-Lep and Shh3(TM1)-Lep were expressed and the glycosylation status was

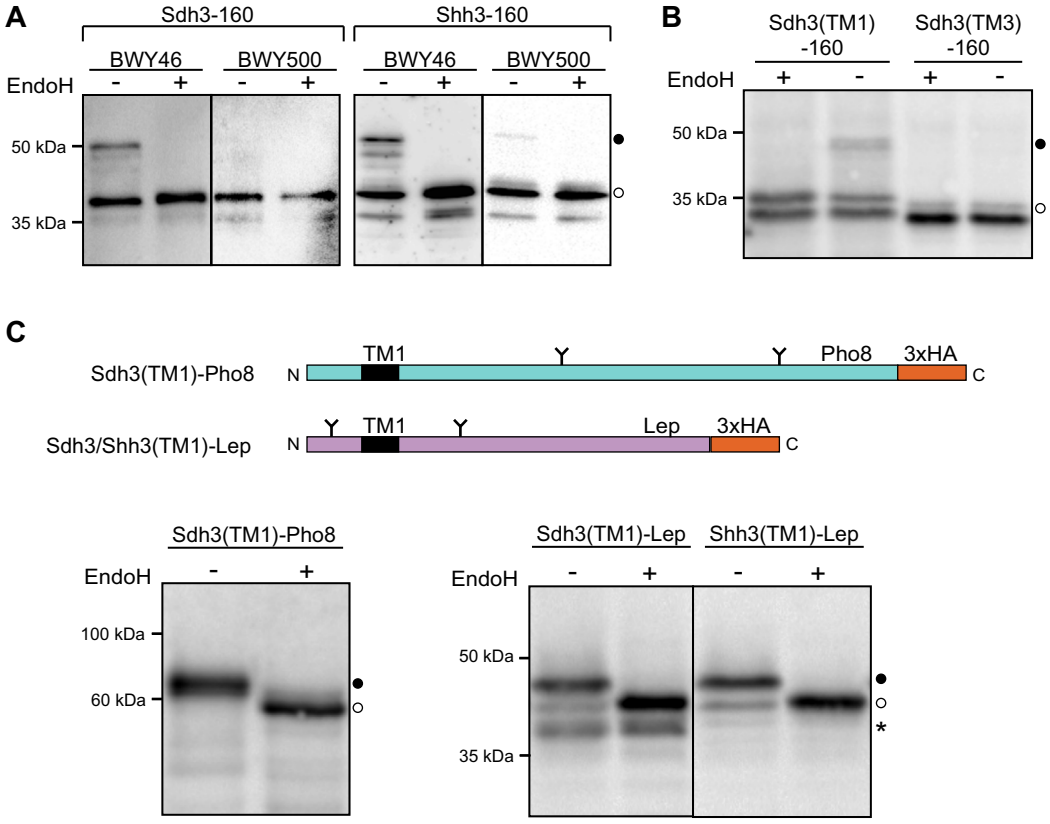

Fig. 2. The TM1 segment of Sdh3 and Shh3 can act as a signal sequence and target the protein to the ER by the SRP dependent translocation. Whole cell lysates of yeast cells carrying designated constructs were subjected to EndoH digestion and analyzed by SDS–PAGE and Western blotting. Glycosylated and unglycosylated forms are indicated as black circle and open circle, respectively. (A) Sdh3-160 or Shh3-160 construct was transformed into the wild type (BWY46) or SRP defective (BWY500) yeast strain, and whole cell lysates were analyzed. (B) Glycosylation status of Sdh3(TM1)-160 and Sdh3(TM3)-160 was assessed. (C) Glycosylation status of Sdh3(TM1)-Pho8 (left) and Sdh3(TM1)-Lep and Shh3(TM1)-Lep (right) was assessed. An asterisk indicates a cleaved form of the protein as previously detected [17].

again assessed by Endoglycosidase treatment. Sdh3(TM1)-Lep and Shh3(TM1)-Lep were mostly glycosylated, indicating that both proteins were efficiently targeted to the ER. These results demonstrate that TM1 of Sdh3 and Shh3 can function as signal sequences that direct cargo proteins to the ER.

3.5. Nuclear-encoded mitochondrial membrane proteins with highly hydrophobic TM domains have short C-terminal tails

Taken together, our results imply that if a mitochondrial membrane protein contains strongly hydrophobic TM segment(s) and a long C-terminal tail, it might be partially mis-targeted to the ER. If there are many such proteins in the eukaryotic cell, proper targeting to mitochondria could be severely compromised, thus the current mitochondrial proteome may not contain this type of proteins. We therefore examined the hydrophobicity of TM domains and the C-terminal lengths of known mitochondrial membrane proteins from *S. cerevisiae* (Fig. 3A), as well as all mitochondrial proteins whose three-dimensional structures are known (Fig. 3B). When the mitochondrial-encoded proteins are excluded, nuclear-encoded mitochondrial membrane proteins that contain sufficiently hydrophobic TM domains were found to have C-terminal tails of less than 100 residues (Fig. 3). These data show that the majority of mitochondrial proteins have moderately hydrophobic TM segments, but if they contain sufficiently hydrophobic TM segments, the C-terminal tail length is short.

3.6. Yta10 is prevented from mis-targeting to the ER by a strong mitochondrial targeting sequence

Only one protein, Yta10, a subunit of the *m-AAA* complex is an exception that contains very hydrophobic TM domains and a more than 500 residue-long C-terminal tail (Fig. 3A). To check whether Yta10 is also partially targeted to the ER, we fused the topology reporter domain HASuc2His4C (1143 residues) [14] with 13 N-linked glycosylation sites after TM1 or TM2 of Yta10 (Fig. 4A). The two variants were prepared to assure reporter localization to the lumen when targeted to the ER, since the first TM segment can theoretically adopt two different orientations in the membrane. Neither fusion construct was glycosylated, indicating they were not targeted to the ER (Fig. 4A). We reasoned that this may be due to a strong mitochondrial targeting sequence at the N-terminus, as Miyazaki et al. [28] have reported that a strong mitochondrial targeting sequence can override an ER targeting signal. When the Yta10 mitochondrial targeting sequence (residues 2–60 among the N-terminal 72 residues, predicted by MitoProtII [29]) was deleted, we found that the majority of the TM1 fusion protein was targeted to the ER as judged by its glycosylation status (Fig. 4B). These results suggest that in the presence of a strong mitochondrial targeting signal, proteins may be imported into mitochondria either co-translationally, or very quickly if post-translationally imported.

4. Discussion

The majority of cytosolically translated mitochondrial membrane proteins have significantly less hydrophobic TM domains compared to ER-targeted membrane proteins. This may be one way by which the eukaryotic cell can effectively sort membrane proteins between the ER and mitochondria [8]. Weakly hydrophobic TM domains of membrane proteins can be anchored via their charged flanking residues in the mitochondrial inner membrane [30].

In the present study, we demonstrate a way for eukaryotic cells to sort membrane proteins containing more strongly hydrophobic

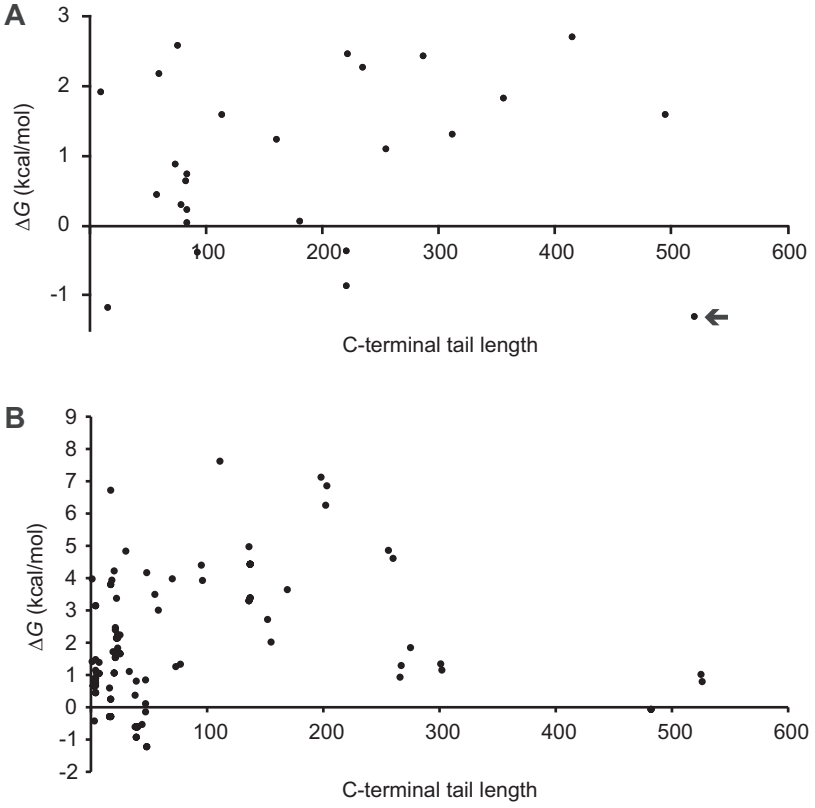

Fig. 3. The free energy of membrane insertion (ΔG) [23] is plotted against the distance to the C-terminus for all transmembrane helices of mitochondrial proteins found in *S. cerevisiae*(A) [22] and the OPM database (B) [24]. An arrow indicates Yta10.

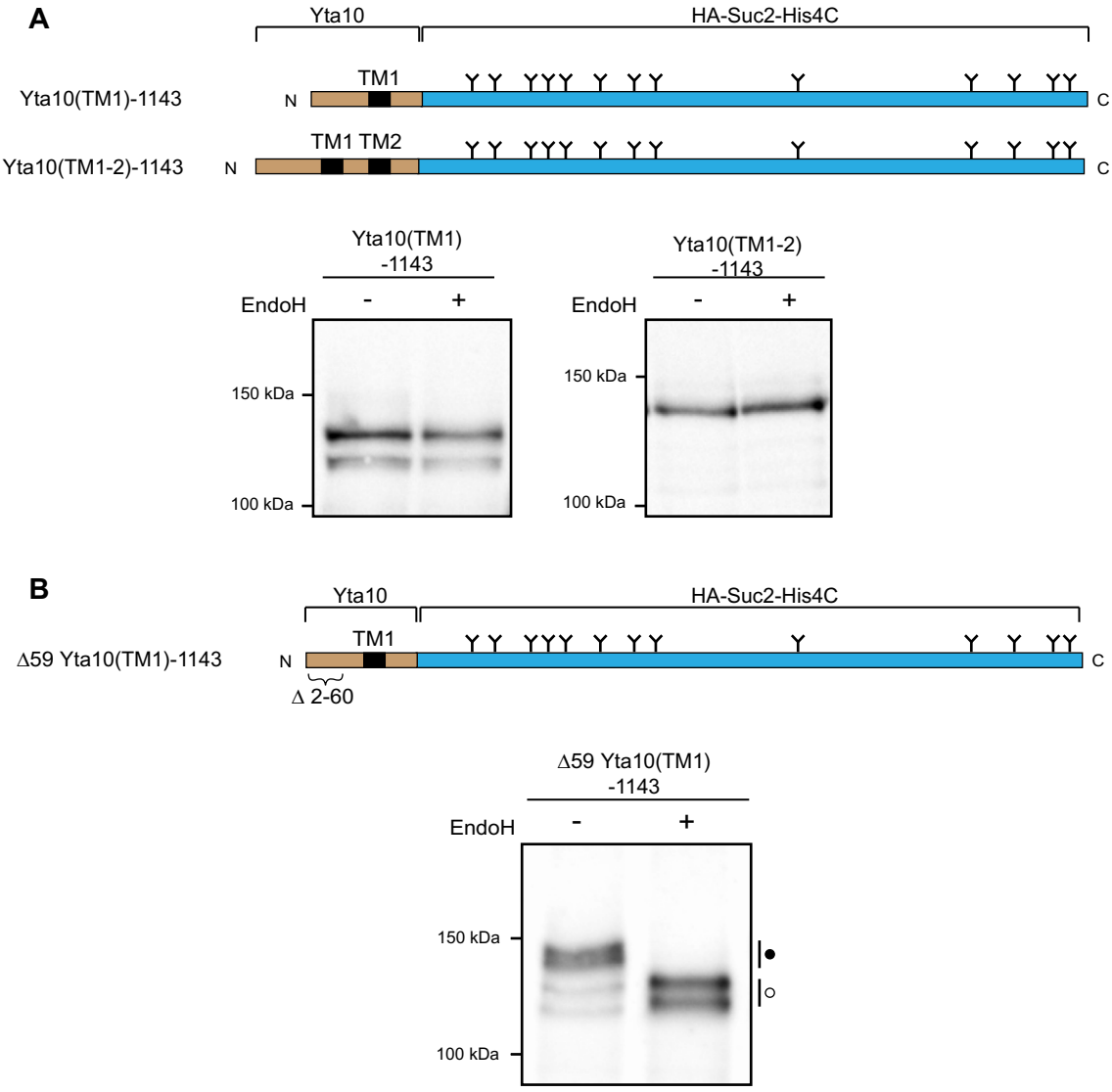

Fig. 4. N-terminally truncated Yta10(TM1) fusion is mis-targeted to the ER. (A) Schematic drawings of Yta10 fusion proteins. Samples prepared by TCA precipitation were subjected to EndoH digestion and analyzed by SDS–PAGE and Western blotting. (B) Construct Δ59 Yta10(TM1)-1143 with residues 2–60 deleted was expressed in W303-1α, and analyzed as described in (A). Glycosylated and unglycosylated forms are indicated as filled and open circles, respectively.

TM domains to mitochondria. Such TM domains, if recognized by SRP, would target the protein for co-translational insertion into the ER; however, if the C-terminal tail following the TM domain is short, the translated product will be released from the ribosome before being recognized by SRP and thus evade being mis-targeted to the ER. Indeed, we find that when two nuclear-encoded mitochondrial membrane proteins, Sdh3 and Shh3, both containing sufficiently hydrophobic TM domains for SRP recognition, are artificially extended, they are mis-targeted to the ER. By bioinformatics analysis, we further show that nearly all nuclear-encoded mitochondrial membrane proteins, both in *S. cerevisiae* and in mammalian cells, have C-terminal tails of lengths less than 100 residues. Recent work has shown that small proteins of less than 120 residues are post-translationally targeted to the ER in mammalian cells, indicating that effective SRP recognition may occur only when the translating sequences are longer than 120 residues [8,31]. Interestingly, in at least one case (Yta10), mis-targeting to the ER of a protein with a long C-terminal tail and a strongly hydrophobic TM domain is prevented by an efficient N-terminal mitochondrial targeting sequence. Finally, our results imply that one needs to be open to the possibility that C-terminal reporter-protein fusions to hydrophobic mitochondrial membrane proteins might trigger mis-targeting to the ER.

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