Multiple functions of tail-anchor domains of mitochondrial outer membrane proteins

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Abstract Tail-anchored proteins form a distinct class of membrane proteins that have a single membrane anchor sequence at their C-terminus, the tail-anchor. Their N-terminal portion is exposed to the cytosol. We have studied the roles of tail-anchor domains of proteins residing in the mitochondrial outer membrane. Four distinct functions of the tail-anchor domain were identified. First, the domain mediates the targeting to mitochondria in a process that probably requires a net positive charge at the C-terminally flanking segment. Second, tail-anchor domains facilitate the insertion into the mitochondrial outer membrane. Third, the tail-anchor is responsible for the assembly of the respective protein into functional multi-subunit complexes; and fourth, tail-anchor domains can stabilize such complexes.
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Key words: Fis1; Mitochondrion; Protein targeting; TOM complex

1. Introduction

Proteins anchored to the membrane by their C-terminus form a distinct class of integral membrane proteins. These proteins consist of a hydrophilic N-terminal part of variable size which is exposed towards the cytosol and a tail-anchor containing a single transmembrane domain (TMD) which is flanked on both sides by short sequences usually containing charged residues [1]. Tail-anchored proteins are found in essentially all membranes abutting the cytosol [2].

In mitochondria tail-anchored proteins are present in the outer membrane, and like all proteins of this membrane, they are encoded by nuclear DNA, synthesized on cytosolic ribosomes and subsequently transported to their site of function. Tail-anchored proteins in the mitochondrial outer membrane include: Fis1, a protein involved in fission of mitochondria [3]; three small subunits of the TOM complex (Tom5, Tom6, Tom7) that interact with Tom40 to form the TOM core complex [4–7]; regulators of apoptosis belonging to the Bcl-2 family [8]; the mitochondrial form of cytochrome b_5 (cyt b_5) [9,10]; and an alternatively spliced isoform of vesicle-associated membrane protein/synaptobrevin (VAMP-1B) [11].

Tail-anchored proteins do not exhibit sequence similarity in their tail domains, rather the mitochondrial targeting information contained therein appears to be encoded in the special structural features of the tail domain. The TMDs of these tails are moderately hydrophobic, relatively short (<20 residues), and the flanking regions have positive charges on both sides [2].

The role(s) of tail-anchor domains and the structural features that allow them to fulfill their various roles are only partially understood. Open questions are: Is the only function of the tail-anchor domain to target the hydrophilic domain to the surface of the mitochondria and hold it at the mitochondrial outer membrane? Do tail-anchor domains interact in a sequence-specific fashion with other subunits with which they assemble into oligomeric complexes and do they have a role in the stabilization of such assemblies?

In this study we have used the yeast *Saccharomyces cerevisiae* to investigate the various functions of tail-anchor domains. Mutated versions of tail-anchored proteins were constructed and their correct intracellular sorting was verified by an in vivo functional complementation assay in combination with subcellular fractionation. Our results imply that a net positive charge in the C-terminal flanking region of the TMD is essential for a function as mitochondrial targeting signal. Remarkably, upon replacing the tail-anchor domain of Fis1 by that of Tom5 or Tom6 a functional Fis1 was obtained. Thus, the tail-anchor domain of Fis1 appears to play only a limited role, if any, in the particular function of this protein. A Tom6 variant in which the cytosolic domain was replaced by that of Fis1 could fulfill the function of authentic Tom6 in stabilizing the TOM complex. Moreover, a Tom5 variant with a similar exchange could partially replace the native Tom5 in promoting preprotein import. Hence, in addition to their functions in targeting the proteins to the mitochondria and anchoring the cytosolic domain in the outer membrane, tail-anchor domains form a structural element which is required for the assembly into and the stabilization of a multi-subunit complex.

2. Materials and methods

2.1. Yeast strains and growth methods

Standard genetic techniques were used for growth and manipulation of yeast strains [12]. Transformation of yeast was carried out using the lithium acetate method. Yeast cells were grown under aerobic conditions on YPD (1% [w/v] yeast extract, 2% [w/v] bactopeptone, 2% glucose) or on YPG (1% [w/v] yeast extract, 2% [w/v] bactopeptone, 3% glycerol) medium. The *Fis1* null strain, ADM552, and its corresponding parental strain ADM551 (a kind gift of Dr. J. Shaw) [3] as well as the *Tom5* null strain and its corresponding parental strain (a kind gift of N. Pfanner) [5] were used. The *Tom6* null strain and its corresponding parental strain BY4743 were obtained from Research Genetics (Huntsville, AL, USA).

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2.2. Recombinant DNA techniques

Fis1 variants and hybrid proteins were constructed by polymerase chain reaction amplification of the relevant DNA fragments and standard molecular biology techniques. DNA fragments encoding the protein of interest were introduced into the multicopy plasmid pYX132 (Invitrogen). All constructs were sequenced to ensure their correct DNA sequence.

2.3. Fluorescence microscopy

A *Fis1* null strain was co-transformed with plasmid pVT100U-mtGFP expressing mitochondria-targeted green fluorescent protein [13] and a plasmid with another genetic marker encoding a Fis1 variant. After selection on the appropriate markers, cells were grown for 16 h to exponential phase in liquid selective glucose medium at 30°C and analyzed by standard fluorescence microscopy. Classification and quantification of the morphology phenotypes were performed without knowledge of strain identity at the time of analysis. For quantification of the phenotype, at least 100 cells were analyzed in three independent experiments, and the average was calculated.

2.4. Biochemical methods

Mitochondria for in vitro import experiments were prepared by differential centrifugation as described [14]. Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine (Amersham) after in vitro transcription by SP6 polymerase from pGEM4 vectors containing the gene of interest. Import experiments were performed in a buffer containing 250 mM sucrose, 0.25 mg/ml bovine serum albumin, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS–KOH, 2 mM NADH, 2 mM ATP, pH 7.2. Protease treatment of mitochondria was performed by incubation with proteinase K for 15 min on ice, followed by addition for 5 min of 1 mM phenylmethylsulfonyl fluoride (PMSF). Import was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), autoradiography and phosphorimaging (Fuji BAS 3000).

2.5. Blue native gel electrophoresis (BNGE)

Mitochondria were lysed in 50 μl detergent-containing buffer (1% digitonin in 20 mM Tris–HCl, 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM PMSF, pH 7.4). After incubation at 4°C for 10 min and a clarifying spin (20 min, 36 700×*g*), 5 μl sample buffer (5% (w/v) Coomassie brilliant blue G-250, 100 mM Bis-Tris, 500 mM 6-amino-caproic acid, pH 7.0) was added, and the mixture was analyzed on a 6–13% gradient blue native gel [15].

3. Results

3.1. A net positive charge at the C-terminus of Fis1 is essential for mitochondrial targeting

Fis1 contains four positively charged amino acid residues in the segment located C-terminally to the TMD (Fig. 1A). Positive charges in this segment of another tail-anchored protein, Tom5, were suggested to play an important role in the targeting of the protein to mitochondria in mammalian but not in yeast cells [16]. To check whether this is a general property of tail-anchored proteins, we investigated the importance of the positive charges in the targeting and function of Fis1. To that end, two arginine residues, Arg154 and Arg155, were replaced

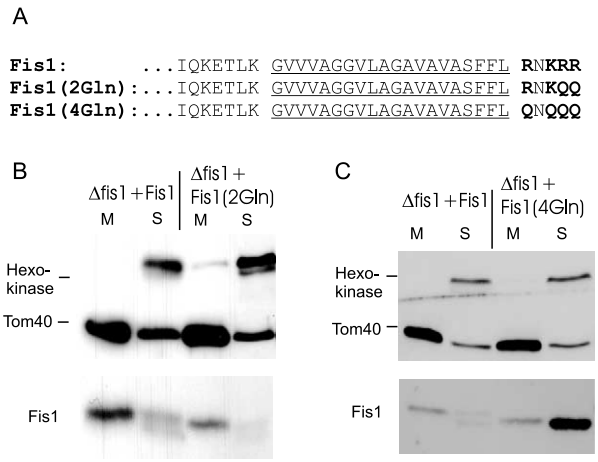

Fig. 1. A net positive charge at the C-segment of Fis1 is crucial for mitochondrial targeting. A: The sequences of the tail domains of native Fis1 and the two charge variants are presented. B,C: *Fis1* null cells transformed with a vector encoding either authentic Fis1 or the indicated variants of Fis1 were ruptured by vortexing in the presence of glass beads. A mitochondrial and a post-mitochondrial fraction were obtained by differential centrifugation (M and S, respectively) and were subjected to SDS–PAGE and immunoblotting. The antibodies used were directed against the cytosolic domain of Fis1, a control marker protein for the cytosol (hexokinase), and the mitochondrial outer membrane protein Tom40.

by glutamine residues reducing the net positive charge to +2 (Fig. 1A). The resulting Fis1 variant (Fis1-2Gln) was targeted to mitochondria in vivo as was demonstrated by subcellular fractionation (Fig. 1B). In addition, this variant was able to fully complement the morphology phenotype of *fis1* null strain (Table 1). When the net charge in the C-terminal segment was made neutral by mutating all four basic amino acids to glutamine residues (Fis1-4Gln, Fig. 1A) the vast majority of the resulting protein was in non-mitochondrial compartments (Fig. 1C). Hence, a net positive charge in the C-terminal segment appears to be crucial for mitochondrial targeting in yeast. The small amount of Fis1-4Gln molecules that were targeted to mitochondria was sufficient to complement the morphology phenotype of *fis1* null strain (Table 1). This suggests that the positive charge at the C-terminal segment is required for targeting to mitochondria, but not for the function of Fis1, once it is present in the membrane.

Note that the levels of expression of Fis1-4Gln are higher than those of wild type Fis1 or Fis1-2Gln. A recent publication on the characterization of the mammalian homologue of Fis1, hFis1, suggested that an increased level of mitochondrial hFis1 strongly promotes mitochondrial fission, resulting in an accumulation of fragmented mitochondria [17]. We speculate that the remarkable differences in the levels of expression

Table 1
Complementation analysis of Fis1 variants

Yeast strain: wild type or Δfis1 transformed with a plasmid encoding the indicated protein	% of cells with normal reticular mitochondrial morphology
Wild type	96
Δfis1+Fis1	96
Δfis1+Fis1-4Gln	95
Δfis1+Fis1-2Gln	89
Δfis1+Fis1(cyt)-Tom5C	79
Δfis1+Fis1(cyt)-Tom6C	69
Δfis1+empty plasmid	0

The percentage of cells with wild type-like mitochondrial morphology was determined using fluorescence microscopy.

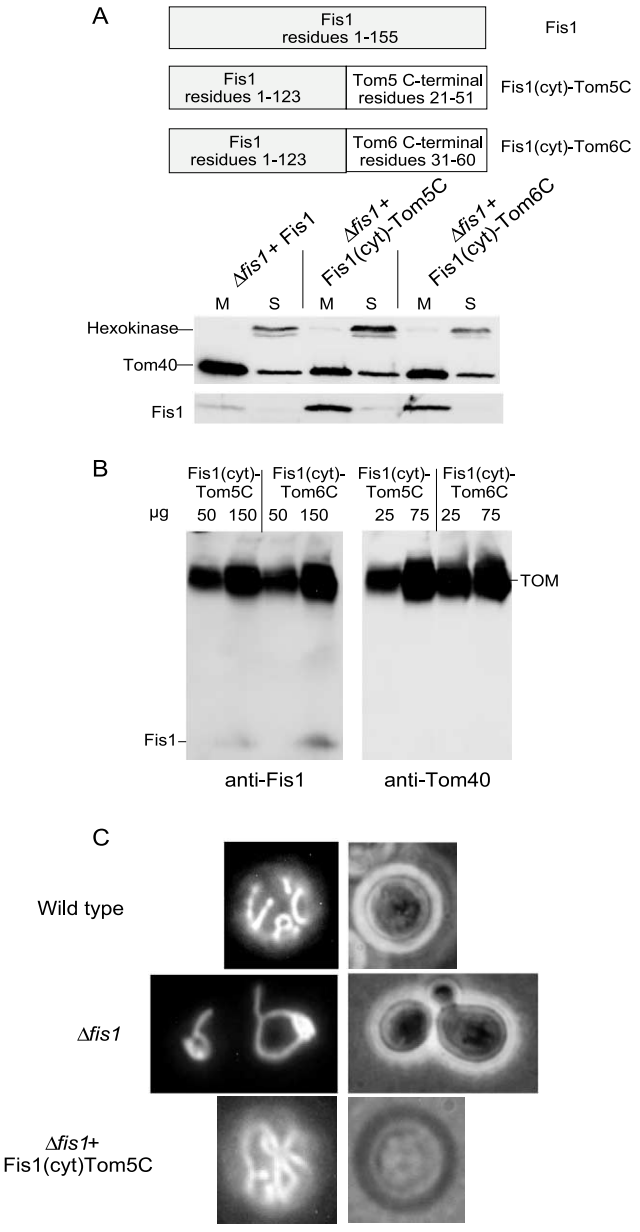

Fig. 2. The tail-anchor domains of Tom5 and Tom6 but not their cytosolic domains are required for targeting and assembly of the proteins. A: *Fis1* null cells transformed with native or with the indicated variants of Fis1 were sub-fractionated as described in the legend to Fig. 1. Fis1(cyt), the cytosolic domain of Fis1. B: Mitochondria isolated from a *fis1* null strain transformed with either Fis1(cyt)-Tom5C or Fis1(cyt)-Tom6C were lysed with digitonin and analyzed by BNGE. The amounts of mitochondrial proteins applied (μg) are indicated. Blots were immunodecorated with antibodies against either Tom40 or Fis1. The TOM complex (TOM) and the unassembled Fis1 variants (Fis1) are indicated. C: Fis1(cyt)-Tom5C can partially complement the mitochondrial morphology phenotype of the ΔFis1 strain. Cells of the indicated strains (containing mitochondria-targeted GFP) were analyzed by fluorescence (left) and phase contrast (right) microscopy.

among various Fis1 variants are the result of a tight regulation of the levels of functional Fis1 in the outer membrane. An over-expression of the functional wild type Fis1 or Fis1-2Gln might harm the cells and therefore their level of expression is regulated. In contrast, the mis-targeted Fis1-4Gln is not 'harmful' outside the mitochondria and hence, can be present in higher amounts.

3.2. The tail-anchor domain of Fis1 does not have a sequence-specific role

We investigated whether a tail-anchor domain of a particular tail-anchored outer membrane protein can functionally replace the signal-anchor domain of another one. Chimeric proteins were constructed, in which the cytosolic domain of Fis1 (amino acid residues 1–123) was fused to the tail-anchor domain of Tom5 (residues 21–51) or Tom6 (residues 31–60) (Fig. 2A). Both proteins, Fis1(cyt)-Tom5C and Fis1(cyt)-Tom6C, were targeted to mitochondria (Fig. 2A). Thus, tail-anchor domains of different proteins can be exchanged without losing their targeting and anchoring functions. Since the cytosolic domain of Fis1 does not contain a mitochondrial targeting signal [3], these results imply that the signal-anchor domains of both Tom5 and Tom6 are sufficient for mitochondrial targeting.

Tom5 and Tom6 are components of the TOM core complex [18,19]. We asked whether the tail-anchor domains of both proteins are sufficient to promote the assembly of the above fusion proteins into the TOM machinery. The TOM core complex isolated from strains harboring either Fis1(cyt)-Tom5C or Fis1(cyt)-Tom6C was analyzed by BNGE. The vast majority of both proteins assembled into the TOM complex as both proteins were present in the 410 kDa TOM complex together with Tom40 (Fig. 2B). Upon quantification of the immunodecoration we found that only about 6% of the expressed proteins migrated as unassembled low molecular weight species. Thus, the tail-anchor domains of both Tom6 and Tom5 are sufficient for the assembly of the fusion proteins into the TOM complex.

We tested whether expression of either Fis1(cyt)-Tom5C or Fis1(cyt)-Tom6C would restore the defective morphology of mitochondria in the *fis1* null strain. Based on quantitative Western blotting we estimated that mitochondria expressing Fis1(cyt)-Tom5C or Fis1(cyt)-Tom6C contain seven- to eight-fold more Fis1 molecules as compared to Fis1wt (our unpublished results). Although only minor amounts of these molecules were not assembled into the TOM complex (see above), both proteins were able to partially complement the morphology phenotype of Δ*fis1* strain (Fig. 2C and Table 1). Thus, we conclude that those molecules of Fis1 fusion proteins that were not assembled into the TOM complex can be as active as Fis1 wild type in mediating fission of mitochondria. The tail-anchor domain of Fis1 is probably not involved in sequence-specific interactions that are essential for the function of the protein. These conclusions are in line with a recent publication suggesting that the region mediating mitochondrial fission resides within the first 31 amino acids of the N-terminal cytosolic domain of human Fis1 [17].

3.3. The tail-anchor domain of Tom6 acts as a stabilizer of the TOM complex

Tom6 stabilizes the TOM core complex and forms a link between Tom22 and Tom40 [4,18,20]. Deletion of Tom6 resulted in a dramatic shift in the mobility of Tom40 upon analysis by BNGE [18]. About 80% of Tom40 molecules in the *tom6* null strain migrated as a 100 kDa complex (Fig. 3). Since Fis1(cyt)-Tom6C could assemble into the TOM complex, we asked whether this hybrid protein can functionally

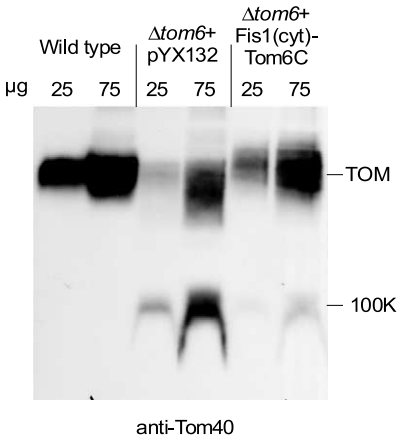

Fig. 3. The tail-anchor domain of Tom6 is sufficient for the TOM-stabilizing function of the protein. Mitochondria isolated from a wild type strain and from a *tom6* null strain transformed with either empty vector (Δtom6+pYX132) or vector encoding Fis1(cyt)-Tom6C (Δtom6+Fis1(cyt)-Tom6C) were lysed with digitonin and analyzed by BNGE. The amounts of mitochondrial proteins applied (μg) are indicated. Blots were immunodecorated with antibodies against Tom40. The native 410 kDa TOM core complex (TOM) and the 100 kDa form of Tom40 (100K) are indicated.

replace the native Tom6. When Fis1(cyt)-Tom6C was transformed into a *tom6* null strain most of the TOM core complexes regained their stability (Fig. 3). Thus, the tail-anchor domain of Tom6 is sufficient to fulfill the structural role of the protein.

3.4. The tail-anchor, but not the cytosolic domain of Tom5, is required for function of the protein

Deletion of the gene encoding Tom5 in yeast was reported to result in cells that cannot grow at the elevated temperature 37°C [5]. As a measure for functionality of the protein we asked whether expression of Fis1(cyt)-Tom5C in the *tom5* null strain is able to complement this growth phenotype. We observed a complete restoration of growth at 30°C, and a partial restoration of growth at 37°C (Fig. 4A).

Tom5 was suggested to link the surface receptors for preproteins with the general insertion pore [5,21]. Mitochondria lacking Tom5 were observed to be severely impaired in their ability to import precursor proteins in vitro [5]. The expression of Fis1(cyt)-Tom5C in *tom5* null cells led to partial alleviation of this import defect (Fig. 4B). Taken together, the cytosolic domain of Tom5 appears not to be essential for the function of the protein.

4. Discussion

We report here on multiple functions of the tail-anchor domains of mitochondrial outer membrane proteins. Initially, the domain mediates the targeting of the respective precursor protein to mitochondria. The importance of positive net charge in the C-terminal segment for correct mitochondrial targeting has been demonstrated before in mammalian mitochondria, but was questioned regarding targeting to yeast mitochondria [16]. An example in mammalian cells for the importance of positive charges at the C-terminal segment is provided by cyt b_5 and VAMP-1. Both of these proteins exist in two homologous isoforms that are localized specifically either to the mitochondrial outer membrane or to the endo-

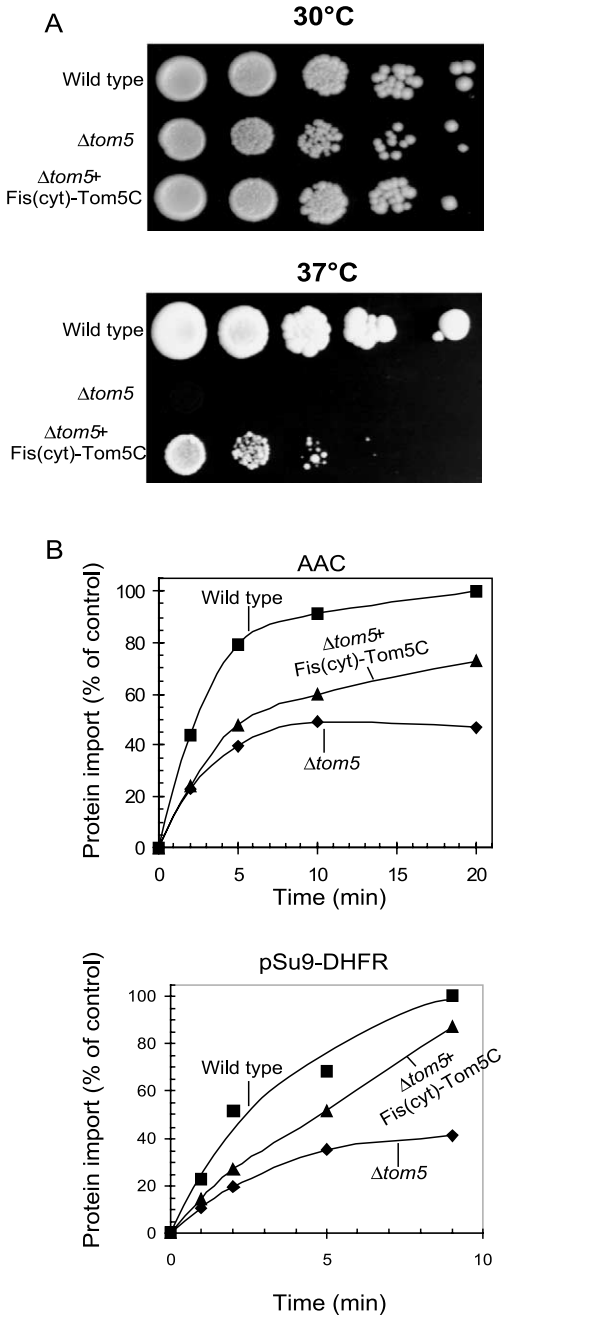

Fig. 4. The tail-anchor domain of Tom5 can partially complement the function of authentic Tom5. A: The fusion protein Fis1(cyt)-Tom5C can partially rescue the temperature-sensitive phenotype of a *tom5* null strain. Δ*tom5* cells, their isogenic wild type, and Δ*tom5* cells transformed with a plasmid encoding Fis1(cyt)-Tom5C were tested by dilution in 10-fold increments for their ability to grow on YPD medium at either 30°C or 37°C. B: Fis1(cyt)-Tom5C can partially rescue the import phenotype of *tom5* null strain. Radiolabeled precursor proteins of the ADP/ATP carrier (AAC) and of pSu9-DHFR (pSu9-DHFR) were incubated with isolated mitochondria at 25°C for the indicated time periods. After import the samples were treated with proteinase K and mitochondria were re-isolated. Imported proteins were analyzed by SDS–PAGE and autoradiography. The amount of precursor proteins imported into wild type mitochondria for the longest time period was set to 100%.

plasmic reticulum. In both cases it has been shown that the mitochondrial localization requires a lack of net negative charge at the C-terminal segment [2,11,22]. We could show that such positive charges are crucial for targeting of Fis1 to mitochondria in yeast cells.

The observation that tail-anchor domains of Tom5 and Tom6 are functionally interchangeable with that of Fis1 is quite surprising considering the lack of sequence similarity in these regions. Although the tail-anchor domain has a crucial role in topogenic signaling, it appears to play only a limited role in the specific function of Fis1.

Tom5 and Tom6 are unique in the sense that the tail-anchor domain can fulfill the function of the protein even when the cytosolic domain is replaced by an unrelated passenger domain. In the case of Tom6 this might have been expected considering the proposed function of the protein in linking membrane-embedded Tom components [4,18,20]. For Tom5, however, the ability of the tail-anchor domain to largely fulfill the function of the whole protein is quite surprising. The cytosolic domain of Tom5 has a net negative charge and was suggested to be part of an 'acid chain' that guides the sequential transport of positively charged mitochondrial presequences [5,21]. As Tom5 was found to be in the vicinity of preproteins in transit [5], it is tempting to speculate that the tail-anchor domain of Tom5 builds part of the preprotein translocation pore.

Recently, Horie et al. analyzed in detail the targeting signal of Tom5 in yeast [23]. It was shown that the positively charged C-terminal anchor segment of Tom5 was dispensable not only for targeting, but also for the integration into the TOM complex and for the function of the protein in yeast. Tom6, another tail-anchored protein, which plays a critical role in modulating the stability of the TOM complex [18], has a net negative charge in its C-segment. Thus, Tom5 represents a unique case among the tail-anchored family TOM components. In agreement with our results, proteins dispersed in the outer membrane, in contrast to the Tom complex component Tom5, do require a net positive charge to be inserted into the correct membrane.

In conclusion, we demonstrate that the tail-anchor domain can have four distinct functions. First, it is sufficient to target a passenger domain to the mitochondria in a process that probably requires a net positive charge at the C-terminus. Second, this domain can mediate the correct insertion of the protein into the outer membrane so that the N-terminal domain is facing the cytosol. Third, as shown for the TOM complex, the tail-anchor can act as an assembly signal or a domain that mediates assembly. Finally, it has a specific role either as a structural stabilization element or as a component of a preprotein conducting pore.

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References

[1] Wattenberg, B. and Lithgow, T. (2001) Traffic 2, 66–71.
[2] Borgese, N., Colombo, S. and Pedrazzini, E. (2003) J. Cell Biol. 161, 1013–1019.
[3] Mozdy, A.D., McCaffery, J.M. and Shaw, J.M. (2000) J. Cell Biol. 151, 367–379.
[4] Alconada, A., Kübrich, M., Moczko, M., Hönlinger, A. and Pfanner, N. (1995) Mol. Cell. Biol. 15, 6196–6205.
[5] Dietmeier, K., Hönlinger, A., Bömer, U., Dekker, P.J.T., Eckerskorn, C., Lottspeich, F., Kübrich, M. and Pfanner, N. (1997) Nature 388, 195–200.
[6] Allen, R., Egan, B., Gabriel, K., Beilharz, T. and Lithgow, T. (2002) FEBS Lett. 514, 347–350.
[7] Beilharz, T., Egan, B., Silver, P.A., Hofmann, K. and Lithgow, T. (2003) J. Biol. Chem. 278, 8219–8223.
[8] Cory, S. and Adams, J.A. (2002) Nat. Rev. Cancer 2, 647–656.
[9] Lederer, F., Ghrir, R., Guiard, B., Cortial, S. and Ito, A. (1983) Eur. J. Biochem. 132, 95–102.
[10] D'Arrigo, A., Manera, E., Longhi, R. and Borgese, N. (1993) J. Biol. Chem. 268, 2802–2808.
[11] Isenmann, S., Khew-Goodall, Y., Gamble, J., Vadas, M. and Wattenberg, B.W. (1998) Mol. Biol. Cell 9, 1649–1660.
[12] Sherman, F., Fink, G.R. and Hicks, J. (1986) Methods in Yeast Genetics: A Laboratory Course, Cold Spring Harbor Laboratory Press, New York.
[13] Westermann, B. and Neupert, W. (2000) Yeast 16, 1421–1427.
[14] Daum, G., Böhni, P.C. and Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033.
[15] Schägger, H., Cramer, W.A. and von Jagow, G. (1994) Anal. Biochem. 217, 220–230.
[16] Horie, C., Suzuki, H., Sakaguchi, M. and Mihara, K. (2002) Mol. Biol. Cell 13, 1615–1625.
[17] Yoon, Y., Krueger, E.W., Oswald, B.J. and McNiven, M.A. (2003) Mol. Cell. Biol. 23, 5409–5420.
[18] Dekker, P.J.T., Ryan, M.T., Brix, J., Müller, H., Hönlinger, A. and Pfanner, N. (1998) Mol. Cell. Biol. 18, 6515–6524.
[19] Ahting, U., Thun, C., Hegerl, R., Typke, D., Nargang, F.E., Neupert, W. and Nussberger, S. (1999) J. Cell Biol. 147, 959–968.
[20] Dembowski, M., Kuenkele, K.-P., Nargang, F.E., Neupert, W. and Rapaport, D. (2001) J. Biol. Chem. 276, 17679–17685.
[21] Schatz, G. (1997) Nature 388, 121–122.
[22] Kuroda, R., Ikenoue, T., Honsho, M., Tsujimoto, S., Mitoma, J. and Ito, A. (1998) J. Biol. Chem. 273, 31097–31102.
[23] Horie, C., Suzuki, H., Sakaguchi, M. and Mihara, K. (2003) J. Biol. Chem. 278, 41462–41471.