Targeting of tail-anchored proteins to yeast mitochondria in vivo

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Abstract Tail-anchored proteins are inserted into intracellular membranes via a C-terminal transmembrane domain. The topology of the protein is such that insertion must occur post-translationally, since the insertion sequence is not available for membrane insertion until after translation of the tail-anchored polypeptide is completed. Here, we show that the targeting information in one such tail-anchored protein, translocase in the outer mitochondrial membrane 22, is contained in a short region flanking the transmembrane domain. An equivalent region is sufficient to specify the localisation of Bcl2 and SNARE proteins to the secretory membranes. We discuss the targeting process for directing members of this protein family to the secretory and mitochondrial membranes in vivo.

Key words: Tail anchor; Mitochondrion; Membrane protein; Transmembrane domain; Protein translocation

1. Introduction

Tail-anchored proteins are displayed on the cytosolic surface of membranes by virtue of a single, carboxy-terminal transmembrane domain. This class of membrane proteins includes most of the SNAREs involved in vesicle-mediated transport and many of the key proteins regulating programmed cell death [1–3]. Studies on the targeting of SNAREs to their respective membranes suggest a model whereby the nascent SNARE polypeptides would be synthesised in the cytosol, inserted into the endoplasmic reticulum and subsequently sorted to the Golgi, vacuole or plasma membrane [4–6]. Insertion of SNAREs into the endoplasmic reticulum must be a post-translational process, since the membrane anchor is the final segment of the polypeptide and this segment would have to leave the ribosome in order to insert into the target membrane.

Recently, it has become clear that several proteins found in the mitochondrial outer membrane are also tail-anchored proteins. These include translocase in the outer mitochondrial membrane (Tom) 5 [7], Tom6 [8] and Tom22 [9,10], all of which are components of the Tom complex. The insertion of the SNAREs into the membranes of the secretory system is mediated by interaction with protein components of the endoplasmic reticulum and includes at least one ATP-dependent reaction [4]. Similarly, insertion of Tom22 into the mitochondrial outer membrane is probably mediated by the Tom complex [11]. The fact that tail-anchored proteins themselves are required for vesicle targeting and in protein targeting to mitochondria underscores that the localisation of this class of proteins must be precise and tightly regulated. We are working to understand the initial sorting steps that ensure delivery of the various tail-anchored proteins from the ribosome to their target membrane.

At some stage during or soon after translation, the polypeptide chain must be specifically targeted to the secretory membranes (in the case of the SNARE proteins) or the mitochondria (in the case of Tom22 and the other tail-anchored Tom proteins). In order to define the sorting information required to direct tail-anchored proteins to the correct intracellular membrane, we constructed a series of fusion proteins with the green fluorescent protein (GFP) from *Aequoria victoria*. The carboxy-terminal region of each of the tail-anchored proteins tested, including the transmembrane segment and up to 13 flanking amino acids, is necessary and sufficient to direct correct intracellular targeting in vivo.

2. Materials and methods

2.1. Plasmid constructs

All GFP-tagged protein constructs were based on the centromeric plasmid p416MET25 (a kind gift from Johannes Hegemann) that carries the GFP($S^{65}T$) isoform. The open reading frame from the Bcl2 cDNA was amplified with the primers 5′-GACTAGATCTGCGCACGCTGGGAGAAC-3′ and 5′-GACTGAATTCTCACTTGTGGCCCAGATAG-3′ into the *Bgl*II/*Eco*RI site of the expression plasmid. GFP-synaptobrevin was constructed by amplification of the entire encoding region of human synaptobrevin 1A from pAct-VAMP-1A [17] with 5′-GACTAGATCTTCTGCTCCAGCTCAGC-3′ and 5′-GACTGAATTCTTTCAAGTAAAAAAGTAGATTAC-3′ into the same vector. The open reading frame from the *TOM22* gene was amplified from yeast genomic DNA with the primers 5′-GGC CGG GAT CCA TGG TCG AAT TAA CTG-3′ and 5′-GGG GCG AAT TCC CTA CTT CAT AAG TAT AGA TAA GGA TAG TGG-3′ and cloned into the yeast expression vector. The C-terminal *trans* domain of Tom22 is a receptor-like domain in the intermembrane space, but does not include targeting information [13–16]. We made use of the *tom22-9* allele, which lacks this small intramitochondrial domain, but duplicate deletion constructs made with the intermembrane space domain intact gave identical results for targeting and complementation of the Δ*tom22* phenotype.

GFP-Tom22-bcl was constructed by amplifying a DNA fragment encoding the transmembrane tail from Bcl2 (amino acid sequence F_{210}–K_{239}) with oligonucleotide primers 5′-GGGCGAGATCTCCATGGTTTGATTTCTCCTGGCTG-3′ and 5′-CGCTCGAATTCTCACTTGTGGCCCAGATAGG-3′ into the *Bgl*II/*Eco*RI site of the expression plasmid encoding a GFP-Tom22(M_1–A_{91}) fusion. GFP-Tom22-syn was constructed by amplification of amino acids K_{93}–T_{118} of human synaptobrevin 1A from pAct-VAMP-1A [17] with 5′-GATCGGATCCAAAAACTGCAAGATGATGAT-3′ and 5′-GACTGAATTCTTTCAAGTAAAAAAGTAGATTAC-3′ and cloned

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Abbreviations: mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; $F_1\beta$-, the β-subunit of the F_1F_0-ATPase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein

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into the *Bgl*II/*Eco*RI site of the expression plasmid encoding a GFP-Tom22(M_1–A_{91}) fusion.

To construct a version of GFP targeted to the lumen of the endoplasmic reticulum (p416MET25sec), the $S^{65}T$ version of GFP was amplified by PCR using an oligonucleotide primer that modified the open reading frame to introduce a sequence encoding the amino acids RSRAHDEL immediately before the stop codon. A *Bgl*II-*Bam*HI fragment of genomic DNA from the 5′ end of the yeast gene YIL169c was cloned immediately upstream of the GFP open reading frame. This DNA fragment encodes the first 48 amino acids of the protein encoded by YIL169c, which functions as a secretion sequence (T. Beilharz, S. Gratzer and T. Lithgow, unpublished).

2.2. Yeast strains, culture and microscopy

Freshly transformed wild-type cells (JK9-3d: *Mat***a**/*Mat*α, *ura3*/*ura3, leu2*/*leu2, his4*/*his4, trp1*/*trp1*) were grown on synthetic glucose media without uracil until the midlog phase in preparation for microscopy and observed with an Olympus BX50 microscope. Vital staining with Mitotracker and Arg-CMAC was according to the suppliers protocol (Molecular Probes).

Each of the various GFP-Tom22 constructs were transformed into the heterozygous diploid YTJB74 to test for complementation. YTJB74 is isogenic to JK9-3d and was derived by replacing one copy of the *TOM22* gene with the auxotrophic marker *LEU2*, as previously described [10]. After sporulation and dissection of the transformed YTJB74, only the plasmids encoding the full-length GFP-Tom22 could readily complement *LEU2*::*tom22* haploid progeny. The isogenic Δ*egd2* diploid strain YRLG1 (*Mat***a**/*Mat*α, *ura3*/*ura3*, *leu2*/*leu2*, *his3*/*his3*, *trp1*/*trp1*, *ade2*/*ade2*, *egd2*::*ADE2*/*EGD2*) has been described previously [12].

Subcellular fractionation was as previously described [18], but cells were grown on semi-synthetic medium without uracil to select for plasmid-based expression. Under these conditions, there is some contamination of the mitochondria by endoplasmic reticulum even after purification of the organelles on Nycodenz gradients buffered to pH 6.0.

Yeast cells were labelled with [^{35}S]methionine (NEN, DuPont) as previously described [19].

3. Results

The *TOM22* gene of yeast encodes a 17 kDa polypeptide, where a carboxy-terminal domain spans the mitochondrial outer membrane and an amino-terminal domain of about 13 kDa is exposed to the cytosol [10,13–16]. Fusion of GFP to the amino-terminus of Tom22 yields the bright fluorescence characteristic of mitochondrial staining (Fig. 1A), indistinguishable from the staining obtained when untransformed cells were incubated with Mitotracker (Fig. 1D). Similarly, expression of GFP fusions incorporating the mammalian tail-anchored proteins synaptobrevin and Bcl2 were measured. Fig. 1B shows the SNARE protein synaptobrevin localised to the vacuole in living yeast cells, which can be recognised as an apparent depression in the Nomarski view of the cells (upper panel) and can be specifically stained with the proteolytically activated dye Arg-CMAC (Fig. 1E). The GFP-Bcl2 fusion protein is localised to the endoplasmic reticulum, which in yeast corresponds to predominantly perinuclear staining, with the nucleus situated beside the vacuole (Fig. 1C). The localisation of GFP-Bcl2 is indistinguishable from the localisation of GFP carrying an amino-terminal signal sequence and carboxy-terminal HDEL sequence (Fig. 1F).

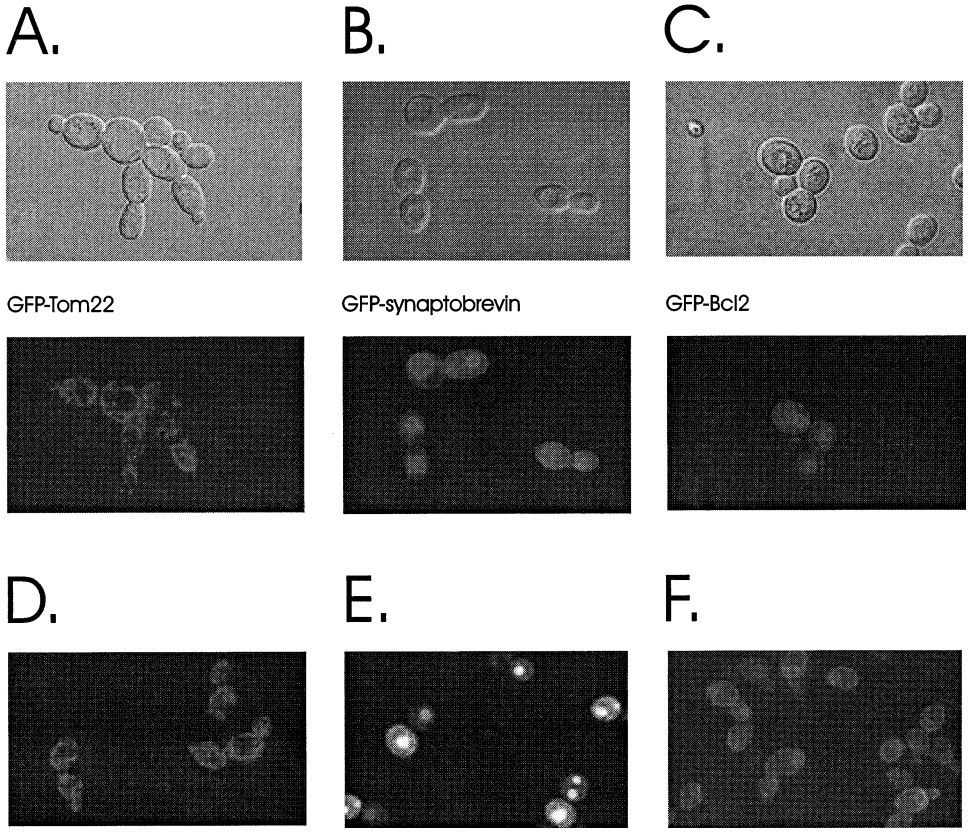

Fig. 1. GFP-tail anchor fusion proteins can be targeted to distinct intracellular membranes. Wild-type yeast cells transformed with a plasmid encoding (A) GFP-Tom22, (B) GFP-synaptobrevin or (C) GFP-Bcl2 and viewed with Nomarski optics (upper panels) or fluorescence microscopy (lower panels). (D) Wild-type yeast cells were incubated with Mitotracker for fluorescent staining of mitochondria. (E) Wild-type yeast cells incubated with Arg-CMAC for fluorescent staining of vacuoles. (F) Wild-type yeast cells transformed with a plasmid encoding GFP with a N-terminal signal sequence and C-terminal HDEL sequence to observe the endoplasmic reticulum.

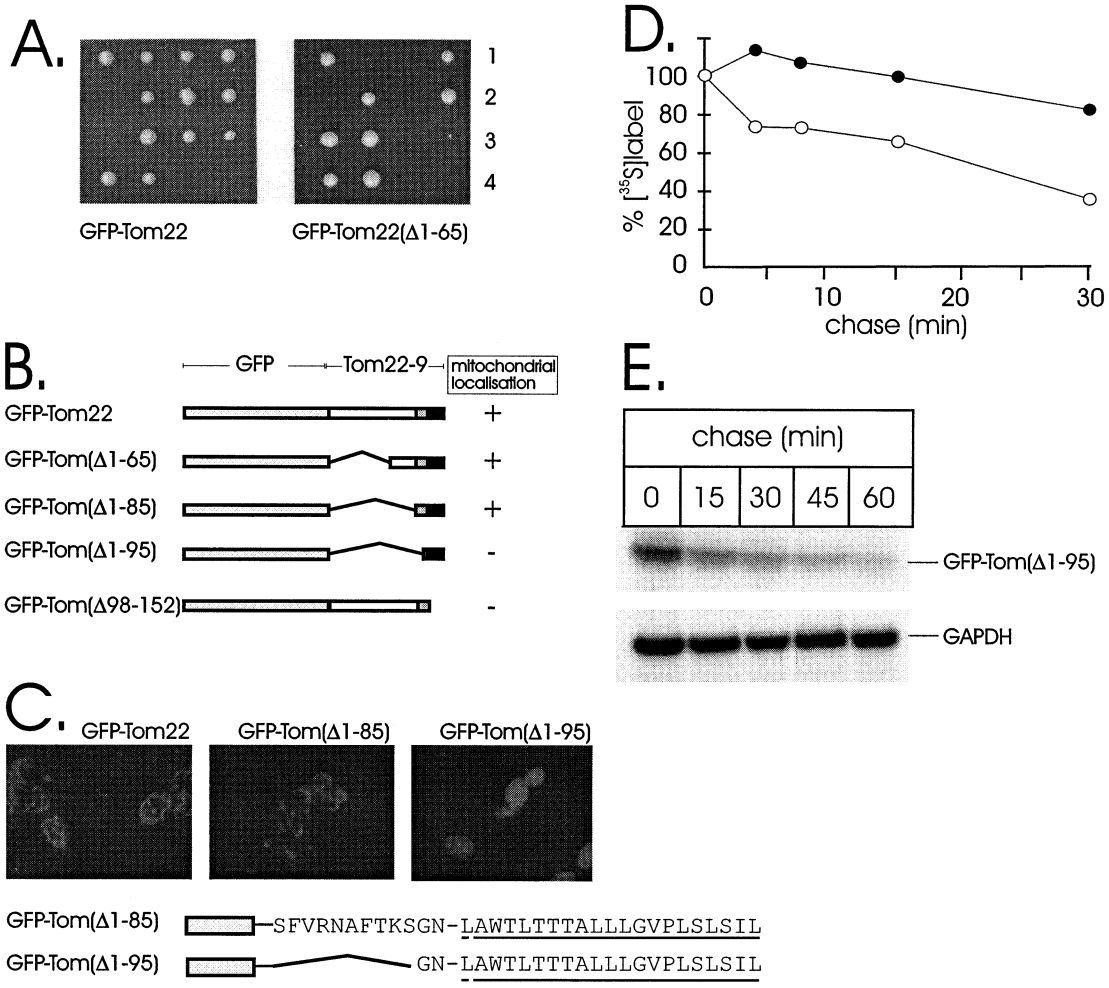

Fig. 2. The targeting information in Tom22 is associated with the tail domain. (A) To measure complementation, *TOM22*/Δ*tom22* yeast mutants were transformed with a plasmid encoding GFP-Tom22 or GFP-Tom22(Δ1–65), induced to sporulate and haploid progeny dissected. The products of four tetrads from each strain are shown. (B) Various truncation mutants of Tom22. Localisation of GFP-Tom22 domain constructs was assayed by fluorescence microscopy in living cells. Tetrad analysis revealed that none of the deletion constructs can complement the fatal defects of Δ*tom22* yeast mutants. (C) The amino acid sequence of two of the constructs, GFP-Tom22(Δ1–85) and GFP-Tom22(Δ1–95). The transmembrane domain of Tom22 (underlined) is predicted to begin at Leu_{98} or Ala_{99}. Fluorescent micrographs of yeast cells expressing the full-length and truncated fusion proteins are shown. (D) Yeast cells expressing either GFP-Tom22 (●) or GFP-Tom22(Δ1–95) (○) were incubated with [^{35}S]methionine for 15 min and then transferred to medium containing unlabelled methionine for the indicated times (min). After immunoprecipitation with anti-GFP antibodies and protein A-Sepharose, samples were analysed by SDS-PAGE and phosphorimaging. (E) For the cells expressing GFP-Tom22(Δ1–95), the protein turnover is compared to the relatively stable soluble protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The samples were split in half at each timepoint of the chase and analysed by immunoprecipitation with anti-GFP antibodies or anti-GAPDH antibodies. The recovery of the relatively stable GAPDH serves as the internal control for protein synthesis and recovery from the transformed yeast.

At least three proteins, Tom5, Tom6 and Tom22, are predicted to be tail-anchored in the mitochondrial outer membrane [7–10], though none of these proteins shares an obvious sequence similarity and none carries classical basic amphipathic targeting sequences found on most mitochondrial precursor proteins. In order to define the region of Tom22 responsible for targeting the mitochondria, we fused discrete regions of Tom22 to GFP and expressed the proteins in yeast. Tom22 is targeted exclusively to the mitochondria and is functional in the outer membrane. A GFP-Tom22 fusion protein is inserted into the mitochondrial outer membrane and complements the lethal defects in Δ*tom22* cells, demonstrating that the fusion protein is correctly integrated and functional within the Tom complex (Fig. 2A). The smallest deletion mutant we constructed, Tom22(Δ1–65), fails to complement the Δ*tom22* cells, supporting suggestions that this region of the protein is essential for the Tom22 function [13,20,21].

The failure of Tom22(Δ1–65) to complement Δ*tom22* cells is not due to mislocalisation of the fusion protein, since truncation of up to 85 amino acids from the amino-terminus of Tom22 did not diminish targeting to the mitochondria (Fig. 2B). The remaining 13 amino acids of Tom22 flanking the transmembrane domain specify delivery of the fusion to the mitochondria. However, the transmembrane domain alone cannot mediate targeting of GFP to the mitochondria: cells expressing the Tom22(Δ1–95) fusion protein show only a very weak fluorescence through the cytosol (Fig. 2C) and the fusion protein is degraded more rapidly than GFP-Tom22 as judged by pulse-chase analysis (Fig. 2D, E). Precise deletion of the predicted transmembrane region leaves GFP-

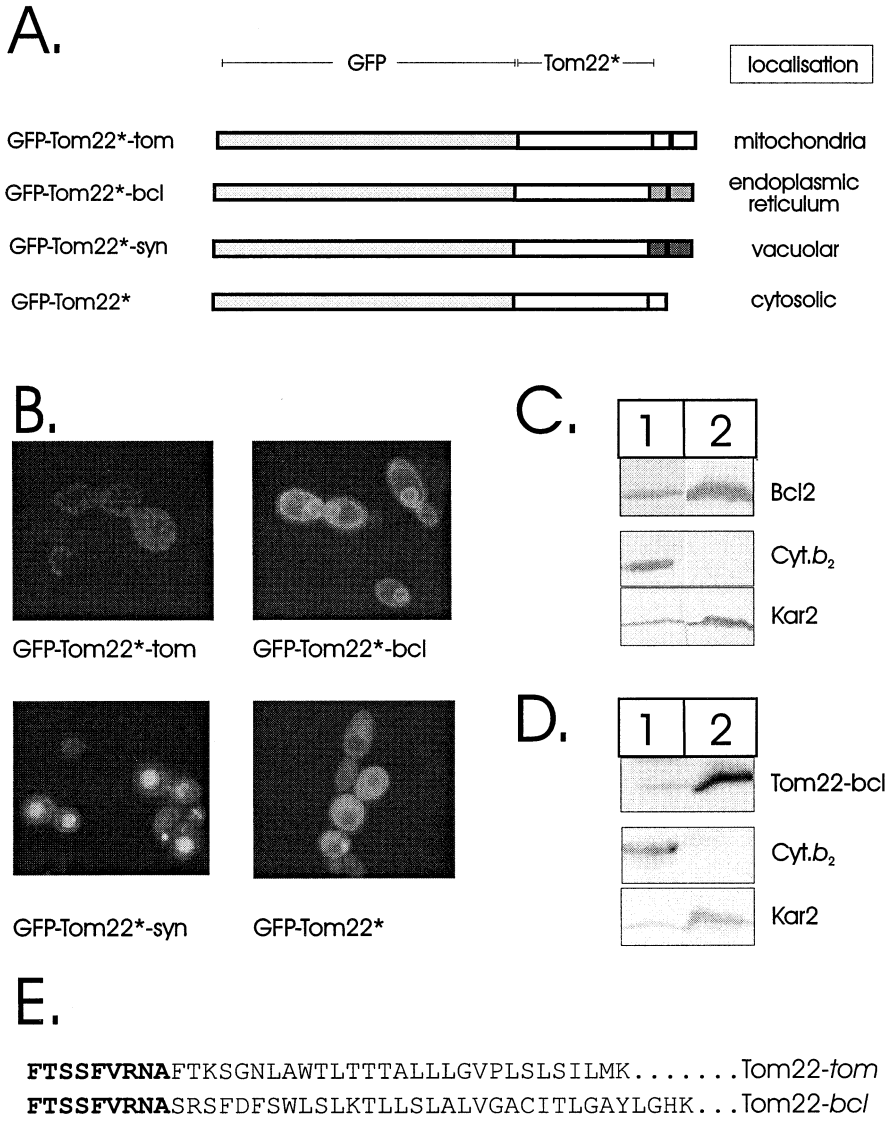

Fig. 3. The amino-terminal domain of Tom22 does not influence protein targeting. (A) Plasmid-based constructs expressing fusion proteins in which the entire cytosolic domain of Tom22 has been fused to the flanking region of synaptobrevin (GFP-Tom22-syn), Bcl2 (GFP-Tom22-bcl), Tom22 (GFP-Tom22-tom) or no flanking region (GFP-Tom22*, this represents Tom22Δ98–152) were transformed into wild-type yeast cells and the localisation of the GFP fusion proteins was determined by fluorescence microscopy. (B) Fluorescence micrographs of each of the fusion constructs. (C) Subcellular fractionation of cells expressing GFP-Bcl2. Immunoblotting of mitochondria (lane 1) and microsomal membranes (lane 2) with antisera recognising the mitochondrial protein cytochrome b_2 and Kar2, which is localised to the endoplasmic reticulum, reveal slight contamination of the mitochondria with membranes from the endoplasmic reticulum. (D) Cells expressing GFP-Tom22*-bcl were fractionated into mitochondria (lane 1) and microsomes (lane 2) and analysed by immunoblotting with antisera to GFP, cytochrome b_2 and Kar2p. (E) Sequence alignment of Tom22-*tom* and Tom22-*bcl*. The sequence in common (bold) extends to A_{91} of Tom22.

Tom22(Δ98–152) distributed throughout the cytoplasm (Figs. 2B and 3B). We conclude that the transmembrane domain plus 13 amino acids of the flanking sequence represent sufficient targeting information for this mitochondrial tail-anchored protein.

To test whether there is dominant mitochondrial targeting information in the cytoplasmic domain of Tom22, fusion constructs were made between the extramembrane domain of Tom22 (including the flanking region up to and including A_{91}, but not the transmembrane region) and the carboxy-terminal region of either Bcl2 or synaptobrevin (see Section 2 for details). Fig. 3 shows that the carboxy-terminal 26 amino acid residues from synaptobrevin or the carboxy-terminal 33 amino acid residues from Bcl2 are sufficient to relocate the amino-terminal region of Tom22 from the mitochondria to the membrane of the vacuole or endoplasmic reticulum, respectively. Two pieces of evidence further support the non-mitochondrial localisation. Firstly, the Tom22 fusions fail to complement the defects of Δ*tom22* yeast mutants (data not shown, see Section 2). Secondly, subcellular fractionation also shows that the GFP-Tom22-bcl construct has an identical localisation to GFP-Bcl2 (Fig. 3C and D), the microsomal fraction is enriched for the endoplasmic reticulum protein Kar2 and also for GFP-Bcl2. Although in mammalian cells a population of Bcl2 is also found in the mitochondria [22,23], the small amount of Bcl2 (and Tom22*-bcl) present in the yeast mitochondria is easily explained by the minor contamination of mitochondria with endoplasmic reticular membranes. The targeting regions from the Tom22-*tom* and Tom22-*bcl* fusion proteins are shown in Fig. 3E.

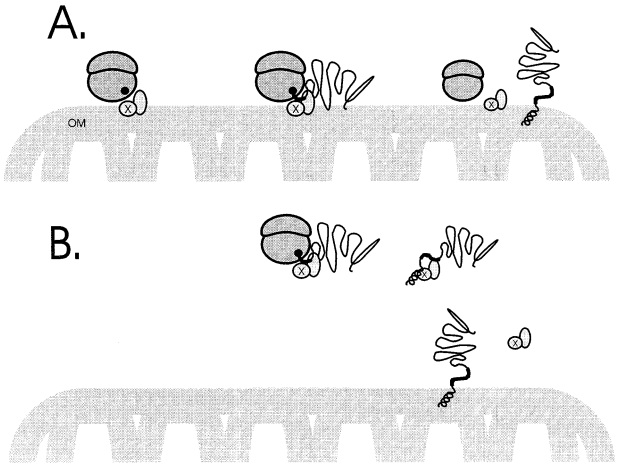

Fig. 4. Model describing the co-translational events that might influence the efficiency of tail-anchored protein targeting. As the C-terminal targeting sequence emerges from the ribosome, it would become available to be recognised by specific targeting factors (X) that might be located (A) on the surface of the mitochondria or (B) in the cytosol. Model A would require that the translating ribosome has been pre-localised to the mitochondria. Model B is supported by the observations that soluble forms of tail-anchored proteins are observed for mutants carrying small deletions in the targeting sequence and that the localisation of chimaeric constructs is determined solely by the final sequences in the polypeptide. In both models, insertion of the tail-anchored protein in its target membrane is a post-translational process.

4. Discussion

4.1. Synthesis and targeting of tail-anchored proteins

Given that protein folding occurs co-translationally in vivo [25], the amino-terminal domains of tail-anchored proteins should be able to fold even before the carboxy-terminal transmembrane domain leaves the ribosome. Since the targeting information in these tail-anchored proteins is at the carboxy-terminus, their insertion into the target membrane must occur post-translationally.

The nascent polypeptide-associated complex (NAC) is a ribosome-associated factor and yeast Δ*egd2* mutants, lacking the NAC function, are defective in the targeting of GFP fusion proteins bearing N-terminal signal sequences to both the endoplasmic reticulum and mitochondria [12,24]. We also find that Δ*egd2* mutants show an accumulation of tail-anchored proteins in the cytoplasm, GFP-Tom22, GFP-Bcl2 and GFP-synaptobrevin accumulate throughout the cytosol of cells lacking NAC (data not shown). Thus, even though the insertion of tail-anchored proteins into their target membrane must be a post-translational process, co-translational events effect the efficiency of targeting to the mitochondria and other organelles in vivo.

This suggests two possible models for the targeting of tail-anchored proteins in vivo. In the scenario depicted in Fig. 4A, ribosomes synthesising the polypeptide to be inserted would be pre-positioned at the target membrane and as the carboxy-terminal targeting domain leaves the ribosome, it could be recognised by membrane-located components (X) and then inserted into the membrane. However, we favour the second scenario (Fig. 4B) where a soluble targeting factor (X) is recruited to the nascent polypeptide during the synthesis on free ribosomes, to maintain the tail-anchored protein in a soluble form prior to insertion into the target membrane. In cells lacking NAC, the targeting efficiency is decreased either because NAC is required directly for targeting factor binding or because the polypeptides made on ribosomes lacking NAC have folding defects that indirectly effect targeting.

The crystal structure and NMR analysis of $BclX_L$, a close paralog of Bcl2, reveal a compact, globular, amino-terminal domain defined by residues 1–203, with the subsequent 13 amino acids (S_{204}–T_{218}) disordered in the crystal structure and shown to be highly flexible by NMR [26]. This segment corresponds to the flanking region we have defined as being necessary and sufficient for targeting Bcl2 to the endoplasmic reticulum in yeast. We anticipate that targeting factors would bind this flexible flanking domain co-translationally, shielding the emergent hydrophobic carboxy-terminal segment from the cytosol until the protein can be correctly positioned in the target membrane. We suggest that the targeting of other tail-anchored proteins like Tom22 and the SNAREs would also be initiated on the ribosome.

4.2. The sorting sequence and the import sequence in Tom22

In vitro assays using either isolated mitochondria or purified outer membranes revealed what might be called an 'insertion sequence' in the cytoplasmic domain of Tom22: deletion of discrete sequences anywhere between residues 45–75 of the *Neurospora crassa* isoform (corresponding to residues 57–82 from *Saccharomyces cerevisiae*) dramatically reducing the correct assembly of Tom22 into the oligomeric Tom complex [11]. Our observation that Tom22(Δ1–65) cannot complement the phenotype of Δ*tom22* yeast mutants is in accord with these previous findings in *N. crassa*.

However, the in vivo data presented here show that both Tom22(Δ1–65) and Tom22(Δ1–85) are still delivered to the mitochondria in intact cells. Reconciling this data with the previous work undertaken with isolated mitochondria [11] suggests that targeting from the ribosome to mitochondria is an independent process from assembly into the outer membrane. Each process would be promoted by independent targeting signals and insertion signals. When the flanking region (corresponding to amino acids T_{84}–A_{91}) is deleted, it prevents targeting to the mitochondria in vivo, but if equivalent proteins are synthesised in vitro, they can be assembled into the Tom complex of isolated mitochondria [11]. Taken together, the data gathered in vitro and in vivo are complimentary and are best described by the import model presented in Fig. 4B.

4.3. A consensus for targeting sequences?

While we have identified the region containing the mitochondrial targeting information in Tom22, there is no obvious sequence similarity to the corresponding regions of the other mitochondrial tail-anchored proteins Tom5 or Tom6. Similarly, our preliminary sequence analysis of the corresponding region of SNARE proteins targeted to the secretory membranes has not revealed any obvious common features (data not shown).

Targeting factors might recognise and bind the flanking region alone or the flanking region might dictate a polypeptide structure in the more distal hydrophobic segment. One approach to precisely define the consensus sequence for tail-anchored protein sorting is through extensive mutagenesis to try to switch the localisation from the secretory to mitochondrial membranes or vice versa. However, our initial studies suggest that tail-anchored proteins not effectively targeted to

their correct membrane in wild-type yeast cells remain in the soluble phase of the cytoplasm and are rapidly degraded. The alternative approach is to identify specific targeting factors from the cytosol, to address the nature of the sorting information by solving the three-dimensional structure of the sequences bound to these cognate factors.

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