Bipartite Signals Mediate Subcellular Targeting of Tail-anchored Membrane Proteins in Saccharomyces cerevisiae*

Received for publication, December 13, 2002, and in revised form, January 3, 2003
Published, JBC Papers in Press, January 3, 2003, DOI 10.1074/jbc.M212725200

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Tail-anchored proteins have an NH₂-terminal cytosolic domain anchored to intracellular membranes by a single, COOH-terminal, transmembrane segment. Sequence analysis identified 55 tail-anchored proteins in Saccharomyces cerevisiae, with several novel proteins, including Prm3, which we find is required for karyogamy and is tail-anchored in the nuclear envelope. A total of six tail-anchored proteins are present in the mitochondrial outer membrane and have relatively hydrophilic transmembrane segments that serve as targeting signals. The rest, by far the majority, localize via a bipartite system of signals: uniformly hydrophobic tail anchors are first inserted into the endoplasmic reticulum, and additional segments within the cytosolic domain of each protein can dictate subsequent sorting to a precise destination within the cell.

Tail-anchored proteins have a single transmembrane segment at their carboxyl terminus, and many of the proteins that mediate subcellular traffic and programmed cell death are tail-anchored into select membranes of eukaryotic cells (1). In the Bcl-2 family of proteins, key regulators of the programmed cell death pathway in animal cells, 12 of the 16 known family members are tail-anchored to either the mitochondrial outer membrane or endoplasmic reticulum, and their membrane location is critical for function (2). The SNAP-receptors (SNAREs) are a family of proteins essential for intracellular membrane fusion, and 19 of the 23 SNAREs in yeast are tail-anchored proteins. Membrane fusion absolutely requires the participation of SNAREs in both the donor and acceptor membrane, and each of the known SNAREs has a restricted location at a defined membrane compartment of the endomembrane system (3, 4).

Tail-anchored proteins fold co-translationally and the single hydrophobic segment at their carboxyl terminus allows for post-translational insertion into membranes (5, 6). Once membrane is inserted, the amino-terminal domain of the protein is displayed in the cytosol. An elegant study on the tail-anchored SNARE synaptobrevin-1/VAMP-1a found that the protein is inserted into the endoplasmic reticulum and subsequently sorted to presynaptic vesicles (5). Cytochrome b₅₆, another tail-anchored protein, is inserted into the membrane of the endoplasmic reticulum and maintained there despite some escape to, and retrieval from, the cis-Golgi cisternae (7).

But tail-anchored proteins are also located in the mitochondrial outer membrane, and the precise signal that distinguishes these from those targeted to the endoplasmic reticulum is still not clear. Deletion mutagenesis has shown the signal is contained within the tail segment, and in the few different model proteins examined to date critical determinants have been either the presence of charged residues or in some cases the number of hydrophobic residues (8–14). An understanding of the precise targeting signals that direct the majority of tail-anchored proteins to the endoplasmic reticulum, but allow some to go exclusively to mitochondria, has been hampered by the relatively small number of model tail-anchored proteins that have been available for study.

We applied several bioinformatic approaches to identify tail-anchored proteins encoded in the genome of Saccharomyces cerevisiae. Fifteen novel tail-anchored proteins were discovered; we report here on the localization of the previously unrecognized tail-anchored proteins. Analysis of the targeting segments from the 55 tail-anchored proteins in yeast suggests a bipartite system of signals: hydrophobic character in the tail segment determines targeting to the endoplasmic reticulum instead of mitochondria and discrete sorting signals that then direct tail-anchored proteins to their correct subcellular destination.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains—DNA fragments corresponding to each open reading frame were amplified by PCR using primers that generated one in-frame restriction site immediately preceding the start codon and another following the stop codon (oligonucleotide sequences available on request). PCR products were cloned behind GFP-S6ST under the control of the MET25 promoter and expressed from a centromeric plasmid (15). In semisynthetic (SD) media, expression from the plasmid is partially repressed.

PCR-based mutagenesis was used to convert hydrophilic residues in the transmembrane segments of Tom22 and Fis1 to leucine residues. For the mutant described here, Fis1(4L), Gly₁₃⁶, Gly₁₃⁷, Gly₁₄¹, and Ala₁₄₂ were converted to leucine residues. Initial trials to visualize expression of the fusion proteins were made in the diploid strain JKB-3dava(ura2-3,122/lei2-3,122,ura3-52/ura3-52, rme1rme1 trp1trp1, his3his4 GAL+/GAL+, HMLa/HMLa). In these cases, failed to give discernible fluorescence but expression of fusion proteins constructed from YBL100c, YFL046w, and YPL200c was possible using a strain defective in proteasome function (Mata, trp1, ura3, his, leu2, cin5–1; Ghishin et al. (38)). To generate yeast mutants lacking the FIS1 gene or the PRM3 gene, PCR-mediated gene disruption (15) was employed with the plasmid p3xHA-His5 as template.

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Centrifugation at 16,000 g/110,000 Relative Centrifugal Force (rcf) was performed to separate soluble (82,000 D) and nonsoluble (100,000 D) proteins. Following centrifugation, 5 mM magnesium acetate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% Triton X-100, 150 mM potassium chloride, 50 mM magnesium acetate, 5 mM phenylmethylsulfonyl fluoride, 1.2 µg/ml leupeptin, 0.75 µg/ml antipain, 0.25 µg/ml chymostatin, 1 µg/ml pepstatin, 50 mM HEPES, pH 6.8) and disrupted by two bursts (each of 2-min duration) in a mini-beadbeater-8 (Biospec products) using silica/zirconia beads. Cell debris was removed by centrifugation at 500 × g for 5 min. A crude membrane fraction was collected by centrifugation at 16,000 × g for 10 min. Membranes were extracted by resuspension in either 1% Triton X-100 or 100 mM Na2CO3 and incubated for 30 min on ice with intermittent vortexing. Soluble and insoluble proteins were separated by centrifugation at 100,000 × g in a Beckman Airfuge.

Miscellaneous—Published procedures were used for isolation of mitochondria and trypsin shaving, SDS-PAGE, and immunoblot analysis (16). Detailed comparative hydrophylicity analyses of the targeting sequences in each tail-anchored protein made use of the ProtParam site at (expasy.proteome.org.au/cgi-bin/protparam) using a window of 5 amino acids to scan through the predicted transmembrane segment according to the Kyte-Doolittle algorithm.

RESULTS

In addition to the tail-anchored proteins known in yeast, sequence analysis revealed 15 open reading frames that could be expressed as GFP fusions that localize to discrete subcellular membranes. Fig. 1 shows that Fis1 and YFL046w are mitochondrial proteins (Fig. 1A), seven proteins localized generally to the endoplasmic reticulum membrane (Fig. 1B), and four are localized to specific subdomains of the endoplasmic reticulum. Yeast Tail-anchored Proteins

**Yeast Tail-anchored Proteins**

**Membrane Isolation and Analysis—Microsomal membrane fractions** contained 15 open reading frames that could be expressed as GFP fusions that localize to discrete subcellular membranes. Fig. 1 shows that Fis1 and YFL046w are mitochondrial proteins (Fig. 1A), seven proteins localized generally to the endoplasmic reticulum membrane (Fig. 1B), and four are localized to specific subdomains of the endoplasmic reticulum.
YOR238w and YOR324c are found in clusters within the bounds of the endoplasmic reticulum, and Prm3 is confined to the perinuclear (nuclear envelope) membrane. YPL206c, a protein showing sequence similarity to bacterial glycerophosphodiester phosphodiesterases, was found concentrated in lipid bodies, regions of endoplasmic reticulum specialized for lipid metabolism (Fig. 1C). YOR324c and YOR324tc are found in clusters within the bounds of the endoplasmic reticulum, and Prm3 is confined to the perinuclear (nuclear envelope) membrane. YOR324c and YOR324tc were found concentrated in lipid bodies, regions of endoplasmic reticulum specialized for lipid metabolism. Prm3 is localized to the perinuclear (nuclear envelope) membrane.

The Tail Segment Is Necessary for Targeting to the Endoplasmic Reticulum—Comparative sequence analysis of the carboxyl-terminal segments of the 41 tail-anchored proteins targeted to the endoplasmic reticulum (including the 17 proteins sorted to other membranes of the secretory pathway) revealed no obvious motifs in the primary structure that might serve as a common targeting signal. However, hydropathy analysis through the transmembrane segments suggests regions of high hydropathy score in each protein (Fig. 2A). Conversely, hydropathy analysis of the predicted transmembrane domain from Fis1 (Fig. 2B) and for YFL046w, Tom5, Tom6, Tom7, and Tom22 (data not shown) suggests this segment of each polypeptide is more amphipathic than for proteins targeted to the endoplasmic reticulum. Previous work has shown that the transmembrane segments of the translocase subunits Tom5, Tom6, Tom7, Tom22, and Fis1 are necessary and sufficient for targeting mitochondria (13, 14, 18).

To test whether the character in the transmembrane segment of Fis1 distinguished it as a protein destined for mitochondria, site-directed mutagenesis was used to replace hydrophilic residues with leucines, such that the hydrophobicity

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approached that of proteins targeted to the endoplasmic reticulum (Fig. 2B). When yeast cells expressing these mutant proteins as GFP fusions were analyzed by fluorescence microscopy, the Fis1 mutant, Fis1(L4), is targeted to the endoplasmic reticulum (Fig. 2C). Similar results were found with mutations made in the transmembrane segment of Tom22 (data not shown). Fluorescence is sometimes also observed in the lumen of the vacuole, perhaps reflecting turnover of the inappropriately targeted proteins (Fig. 2C, “V”).

Bipartite Signals for Targeting and Sorting Proteins in Intracellular Membranes—Tail-anchored proteins in the endoplasmic reticulum can display distinct patterns of localization.

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side of the furrow exhibiting mating type a and cells from the other side being of mating type a. 3

Transcript profiling has shown that the PRM3 gene is induced in response to both pheromone stimulation (22) and during sporulation (23), and both processes culminate in karyogamy (see review by Rose (24)). Because Prm3 localization is dependent on a NLS and the Ran-GTPase cycle, we propose Prm3 as the first component of the cellular karyogamy machinery to function in membrane fusion at the level of the inner membrane of the nuclear envelope.

DISCUSSION

In addition to the discovery and localization of a large set of novel tail-anchored proteins, analysis of the sequence data provided by these proteins suggests a general model for the targeting of tail-anchored proteins to each intracellular membrane.

From analysis of the 41 tail-anchored proteins now known to localize to membranes of the secretory pathway, the only common property we could identify was the uniformly hydrophobic nature of the residues within the transmembrane segment. Previous studies on individual tail-anchored proteins have each suggested that the tail-segment contains targeting information (8–14). The hydrophobic tail segments of Bos1, Prm3, and YOR324c are sufficient to target each of these proteins to the endoplasmic reticulum, and these truncated fusions remain uniformly distributed through the perinuclear endoplasmic reticulum (i.e. the outer membrane of the nuclear envelope) and peripheral endoplasmic reticulum. A segment of lower hydrophobic moment, containing glycine, serine, and threonine residues, is found in the six tail-anchored proteins targeted to the mitochondrial outer membrane. Replacement of these residues with leucines, to alter the hydrophobicity and perhaps other structural features such as the rigidity of the helix it can form, prevents targeting to mitochondria.

The TOM translocation machinery mediates protein insertion into the outer membrane (25–27), as well as the import of soluble proteins into mitochondria (28–31). The recent three-dimensional structure of the TOM complex receptor subunit, Tom20, suggests that it has only a very shallow binding groove on its surface, into which binds the hydrophobic face of the targeting sequences found attached to soluble proteins targeted to mitochondria (32, 33). Tom20 has been implicated as the import receptor for tail-anchored proteins, and it will be of interest to determine the structural details of how the receptor binds the targeting segments of tail-anchored proteins.

In contrast to insertion of tail-anchored proteins into mitochondria, the nature of the machinery that mediates tail-anchored protein insertion into the endoplasmic reticulum is still unclear. Kutay et al. (5) suggested that the general post-translational Sec machinery might mediate insertion of tail-anchored proteins like VAMP-1a. Whatever the machinery, it is able to insert tail-anchored proteins with hydrophobic tail-segments; even a simple, artificial tail-segment consisting entirely of leucine residues was a suitable substrate for in vitro insertion into membrane vesicles derived from the endoplasmic reticulum (34), and the same tail-segment directs GFP to the endoplasmic reticulum when expressed in yeast cells. 3

Distinct regions in the cytosolic domain of VAMP-1a assist its subsequent transport from the endoplasmic reticulum to the presynaptic vesicles. Interactions mediated by distinct regions in the cytosolic domains are also required for Bos1 trafficking to the Golgi, Prm3 targeting to the nuclear envelope, and YOR324c clustering in punctate zones of the endoplasmic reticulum. Thus two independent processes, targeting to the endoplasmic reticulum and subsequent sorting to the correct membrane, are mediated by bipartite targeting and sorting signals. For the Golgi and post-Golgi membranes, sorting would be mediated through vesicular traffic. In the case of Prm3 and any other proteins that might exist in the inner membrane of the nuclear envelope, we suggest that this occurs via the lipid rivulets of pore membrane present in the nuclear pore complex (35, 36) that would link the outer and inner membrane bilayers and is catalyzed by Ran and the karyophilins that also drive the import of soluble nuclear proteins.

Acknowledgments—We thank Ben Blick, Tina Junne-Bieri, and Jeff Schatz for plasmids and antisera; Peter Walsh, Diana Macasev, and Ross Waller for critical suggestions on the manuscript; and Binks Wattenberg and David Huang for comments on the manuscript and critical discussions throughout the course of the project.

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*J. Biol. Chem. 2003, 278:8219-8223.*
doi: 10.1074/jbc.M212725200 originally published online January 3, 2003

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