A Signal-Anchor Sequence Selective for the Mitochondrial Outer Membrane

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Abstract. pOMD29 is a hybrid protein containing the NH$_2$-terminal topogenic sequence of a bitopic, integral protein of the outer mitochondrial membrane in yeast, OMM70, fused to dihydrofolate reductase. The topogenic sequence consists of two structural domains: an NH$_2$-terminal basic region (amino acids 1-10) and an apolar region which is the predicted transmembrane segment (amino acids 11-29). The transmembrane segment alone was capable of targeting and inserting the hybrid protein into the outer membrane of intact mitochondria from rat heart in vitro. The presence of amino acids 1-10 enhanced the rate of import, and this increased rate depended, in part, on the basic amino acids located at positions 2, 7, and 9. Deletion of a large portion of the transmembrane segment (amino acids 16-29) resulted in a protein that exhibited negligible import in vitro. Insertion of pOMD29 into the outer membrane was not competed by import of excess precursor protein destined for the mitochondrial matrix, indicating that the two proteins may have different rate-limiting steps during import. We propose that the structural domains within amino acids 1-29 of pOMD29 cooperate to form a signal-anchor sequence, the characteristics of which suggest a model for proper sorting to the mitochondrial outer membrane.

The mechanism of protein insertion into mitochondrial membranes is not well understood, other than the observations that deletion of predicted transmembrane segments in a limited number of such proteins can lead to relocation of the protein to soluble mitochondrial compartments (Liu et al., 1988; Glaser et al., 1990) and, conversely, that introduction of a heterologous transmembrane stop-transfer segment can result in membrane insertion of an otherwise soluble matrix protein (Nguyen and Shore, 1987; Nguyen et al., 1988). Studies in this area have been complicated by a number of issues. For example, the mitochondrion contains two translocation-competent membranes; the problem of membrane insertion, therefore, is also a problem of protein sorting. Also, two pathways may have evolved for protein sorting to mitochondrial membranes: the conservative sorting pathway, in which cytoplasmically-synthesized precursor proteins may be routed first to the matrix and then exported to the inner membrane (Hartl et al., 1986; Hartl and Neupert, 1990; Mahlke et al., 1990), and the stop-transfer sorting pathway, in which proteins are inserted into either the outer or inner membrane during unidirectional import into the organelle (Blobel, 1980; Liu et al., 1990; Mahlke et al., 1990; Glick and Schatz, 1991). Finally, progress has been limited by the fact that some of the best-studied examples of mitochondrial membrane proteins exhibit relatively complex structures. For example, the major protein of the outer membrane, porin, is devoid of uniformly hydrophobic transmembrane segments but, like the bacterial porins, may intercalate into the membrane as a β barrel (Jap, 1989).

To avoid many of these complications, we have focused on a simple bitopic integral protein of the outer mitochondrial membrane in yeast, OMM70 (Hase et al., 1984) (also called MAS70, Hines et al., 1990). The topogenic information in OMM70 resides within a stretch of 29 amino acids at the NH$_2$ terminus, resulting in a protein that is anchored in the outer membrane via a predicted 19 amino acid transmembrane segment (amino acids 11-29) in the N$_{N_{m}}$-C$_{m}$ orientation, leaving a large COOH-terminal fragment exposed to the cytosol (Hase et al., 1984; Nakai et al., 1989). Evidence has been obtained that the Neurospora homolog of OMM70, MOM72, employs the same import receptor as proteins destined for the matrix compartment (Söllner et al., 1990). However, proteins that are inserted into the mitochondrial outer membrane do not require an electrochemical potential across the inner membrane (Freitag et al., 1982; Mihara et al., 1982), indicating that routing to the outer membrane does not occur via a conservative sorting pathway.

Earlier studies suggested a model in which OMM70 is directed to mitochondria by a matrix-targeting signal located at the extreme NH$_2$-terminus (amino acids 1-12), with translocation to the matrix being arrested at the outer membrane by a stop-transfer sequence (amino acids 11-29) (Hurt et al., 1985). The efficiency of import to the matrix of reporter proteins carrying amino acids 1-12 of OMM70, however, was very weak and, as emphasized by Glick and Schatz (1991), such findings might arise indirectly from the fact that a high percentage of random, positively-charged sequences can function as weak matrix-targeting signals. Also, this region of OMM70 is replaced by a very different uncharged, proline-rich sequence in MOM72 (Steger et al., 1990).

Here, we present evidence that the basic NH$_2$-terminus of...
OMM70 cooperates with the transmembrane segment to form the requisite topogenic sequence for selection of the mitochondrial outer membrane, which we term a signal-anchor sequence. Comparisons to the functionally analogous signal-anchor sequences of type II and type III proteins (von Heijne, 1988) that are inserted into the membrane of the endoplasmic reticulum (Blobel, 1980; Wickner and Lodish, 1985) suggest a mechanism for correct sorting to the mitochondrial outer membrane.

**Materials and Methods**

**General**

Previous articles describe the routine procedures employed for recombinant DNA manipulations (Skerjanc et al., 1990; Sheffield et al., 1990), transfection of pSP64 constructs (Nguyen and Shore, 1987), translation of pSP64-derived mRNA in a rabbit reticulocyte cell-free system in the presence of [35S]methionine, and isolation of mitochondria from rat heart by standard techniques. This created the plasmid pSP (pOMD29) GTAGCCACGGATGATAATAAIGCG). Part of the CTACTATTATTACGCTGCA and 3'-ACGTCAACGACGATGTCCATGACGAAAACCG; adaptor II: 5'-GTqGCTGCTACAGGTACTC~CATCGGTGC- GGCTGCA and 3'-TACTTCTCGAAGTAAI~TTCCTTGTTCTGTCGGTAAAACCCG; adaptor II: 5'-AC-CTA~ATTA~CAAGACAGCCATTTTGGCTGCA and 3'-ATAC GTC TCGAAGTAATGTGTC ~TCTGTCGGTAAAACCG, to yield pOMD29A16-29 was created by only employing adaptor II in the manipulations described above. pSP(pOMD29A16-29) was created by using the single-letter code in Materials and Methods are shown, using the single-letter code for amino acids. The predicted transmembrane segment (amino acids 11-29, Hase et al., 1984; Nakai et al., 1989) is underlined; e, alanine substitution for threonine at residue 15 of OMM70; (dashes) deletions; (asterisks) substitutions of lysine and arginine by glutamine; (DHFR) marine dihydrofolate reductase (see Materials and Methods).

**Recombinant Proteins**

A 650-bp TaqI-BgIII fragment was excised from pSV2DHFR and inserted between the AccI and BamHI sites of pSP64 (Skerjanc et al., 1990). The resulting plasmid was digested with HindIII and PstI, and two adaptors containing HindIII and PstI overhangs were inserted between these sites (adaptor I: 5'-AGCTATGACCGTTCATACGAAAGAAACAGACCCATTTTGGCTGCA and 3'-TACCTTCGAAATAGTCTTCTGCTTGCTGCTGGTAAAACCG; adaptor II: 5'-GTGCTGCTACAGGTACTC~CATCGGTGCG- CTACTATTATTACGCTGCA and 3'-ACGTCAACGACGATGTCCATGACGAAAACCG). Part of the PstI site was removed by standard PCR techniques. This created the plasmid pSP (pOMD29) which encodes amino acids 1-29 of yeast OMM70 (Hase et al., 1984) connected via a glycine (position 30) to amino acids 4-186 of dihydrofolate reductase (DHFR), and in which amino acid 15 was changed from threonine to alanine. The plasmid pSP(pOMD29A16-29) was created by employing only adaptor I in the manipulations described above. pSP(pOMD29A16-29) was formed by deleting adaptor I from pSP(pOMD29) and replacing it with an adaptor (5'-AGCTATGACCGTTCATACGAAAGAAACAGACCCATTTTGGCTGCA and 3'-TACCTTCGAAATAGTCTTCTGCTTGCTGCTGGTAAAACCG) that encodes amino acids 1 and 11-15 of pOMD29. Finally, adaptor I of pSP(pOMD29) was replaced with the adaptor, 5'-AGCTTATGCAGGTGC- CTACTATTATTACGCTGCA and 3'-ATACGCTTCGAAATAGTCTTCTGCTTGCTGCTGGTAAAACCG, to yield pSP(pOMD29A16-29), in which amino acids 2, 7, and 9 of pOMD29 were replaced by glutamine.

**Mitochondrial Import**

Reaction mixtures contained 10% (v/v) rabbit reticulocyte lystate transcription-translation products labeled with [35S]methionine, mitochondria (0.5 mg protein/ml), 0.125 M sucrose, 40 mM KCl, 1.0 mM MgAc2, 100 mM Hepes, pH 7.5, 0.5 mM dithiothreitol, 0.5 mM ATP, 2.5 mM sodium succinate, 0.04 mM ADP, and 0.1 mM potassium phosphate, pH 7.5. After incubation at 30°C, aliquots (50 µl) were removed and layered over 500 µl 0.25 M sucrose, 10 mM Hepes, pH 7.5, 1.0 mM dithiothreitol, and mitochondria were collected by centrifugation for 2 min at 12,000 g.

**Alkali Extraction**

After import, mitochondria were recovered by centrifugation, suspended in freshly prepared 0.1 M Na2CO3, pH 11.5, to a final protein concentration of 0.25 mg/ml, and incubated on ice for 30 min, with periodic vortexing. Membranes were collected by centrifugation at 30 psi. for 10 min at 4°C in a Beckman Airfuge (Beckman Instruments, Carlsbad, CA).

**Results and Discussion**

A hybrid protein, pOMD29, was created by fusing amino acids 1-29 of yeast OMM70 (Hase et al., 1984) through a glycine (residue 30) to amino acids 4-186 of dihydrofolate reductase (DHFR). The NH2-terminal sequence of pOMD29 and its mutant derivatives are shown in Fig. 1. As documented elsewhere (Li and Shore, 1992a), pOMD29 was imported into the outer membrane of intact rat heart mitochondria in vitro by a process dependent on ATP and ATPase-sensitive mitochondrial surface components, and in which the orientation of the native OMM70 protein was retained (Nw-Cys). In common with all other outer membrane proteins examined to date, insertion of pOMD29 did not require the mitochondrial electrochemical potential and the protein was not proteolytically processed (Li and Shore, 1992a).

Insertion of pOMD29 into the lipid bilayer of the mitochondrial outer membrane was assayed by its acquisition of resistance to extraction at pH 11.5, a property that is common to integral membrane proteins (Fujiki et al., 1982). Fig. 2 A demonstrates that recovery of the alkali-resistant form of pOMD29 was dependent on the presence of mitochondria during import (compare lanes 2 and 4). However, appearance of the alkali-resistant form of pOMD29 occurred after binding of the protein to mitochondria at 30°C (Fig. 2 A, compare lanes 3 and 4) but much less so at 4°C (Fig. 2 A, compare lanes 5 and 6), suggesting that alkali extraction can distinguish between pOMD29 that is merely bound to the surface of the organelle (4°C, Fig. 2 A) and that which is inserted into the bilayer (30°C, Fig. 2 A). As expected, after import of a hybrid protein containing the matrix-targeting signal of preornithine carbamyl transferase fused to DHFR (i.e., pO-DHFR, Skerjanc et al., 1990), both the full-length precursor that was recovered with the organelle and the processed product, previously shown to be located exclusively in the matrix compartment (Skerjanc et al., 1990), were completely extracted at pH 11.5 (Fig. 3 A, top). As shown in Fig. 2 B, insertion of pOMD29 into the outer membrane and uptake of pO-DHFR to the matrix were both significantly reduced by pretreatment of the intact mitochondria with trypsin (see also Li and Shore, 1992a).

**Targeting and Membrane-Anchor Domains of pOMD29**

Deletions were introduced into pOMD29 to remove either

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1. Abbreviation used in this paper: DHFR, dihydrofolate reductase.

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the hydrophilic, positively charged NH$_2$-terminus of the protein (amino acids 2-10, Fig. 1) or a large portion of the predicted (Hase et al., 1984; Glick and Schatz, 1991) transmembrane segment (amino acids 16-29, Fig. 1). As shown in Fig. 3 A, pOMD29A2-10, which contains only the transmembrane segment at its NH$_2$-terminus, was capable of binding to intact mitochondria in vitro (compare lanes 2 and 3) and, of the bound fraction, a significant amount was alkali-insoluble (lane 4). Like pOMD29, pOMD29A2-10 required ATP for import (not shown) and was inserted in the N$_\text{t}$-C$_\text{cyt}$ orientation, i.e., the bulk of the protein was accessible to externally-added trypsin (Fig. 3 B). Also, neither protein was imported into or across pancreatic ER microsomal membranes when mitochondria were replaced with these membranes in the import assay (Fig. 3 C), as judged by resistance to external trypsin (lane 4) or resistance to extraction by alkali (lane 5). These microsomes efficiently translocated and processed a major histocompatibility class 1 protein, HLA-2A; the predicted 3 kD cytoplasmic COOH-terminal tail of the polypeptide (Ennis et al., 1990) was accessible to external protease whereas the bulk of the polypeptide in the lumen was protected (Fig. 4, lane 2), except in the presence of detergent (Fig. 4, lane 3). In contrast to pOMD29A2-10, removal of a large portion of the transmembrane segment of pOMD29 resulted in a protein (pOMD29A16-19, Fig. 1) whose import was below the levels of detection in the heterologous system described here (mitochondria from rat heart) (data not shown). Taken together, therefore, these findings suggest that the transmembrane segment of pOMD29 makes an important contribution to targeting, as well as to membrane insertion.

**Role of the Positively-Charged NH$_2$ Terminus of pOMD29**

Despite the fact that amino acids 1-15 of pOMD29 (OMM70) on their own cannot support import of DHFR into rat heart mitochondria in vitro, the possibility that the hydrophilic NH$_2$-terminus of pOMD29 cooperates with the membrane-anchor segment to give optimal import was examined. A helical wheel projection of the NH$_2$ terminus of pOMD29 predicts that such a helix would be amphipathic, with the basic residues at positions 2, 7, and 9 clustered on the hydrophilic face (Fig. 5). As a further consideration, therefore, the lysine residues at positions 2 and 9 and the arginine at position 7 were mutated to glutamine (Figs. 1 and 5). Like lysine and arginine, glutamine is compatible with an $\alpha$-helix (Chou and Fasman, 1974), but its side chain amide is uncharged. The mutant was designated pOMD29KR2,7,9Q and was found to be competent for import (Fig. 3 A, bottom).

In Fig. 6, the rates of import and acquisition of alkali-insolubility for pOMD29, pOMD29A2-10, and pOMD29KR2,7,9Q were analyzed by SDS-PAGE, and the results quantified by determining the radioactive content of gel slices containing the [$^{35}$S]-labeled proteins. The data show that the NH$_2$ terminus of pOMD29 makes a significant contribution to import, to the extent that pOMD29A2-10 was imported at a rate that was approximately five times slower than the rate of import of pOMD29. pOMD29KR2,7,9Q, on the other hand, exhibited a rate of insertion into the outer membrane that was approximately threefold lower than that of pOMD29 (Fig. 6). Thus, the positive charges at positions 2, 7, and 9 of pOMD29 contribute significantly, though not completely, to the optimal rate of import that is conferred by the hydrophilic NH$_2$ terminus of the protein. Like pOMD29 and pOMD29A2-10, pOMD29KR2,7,9Q was inserted into the outer membrane in the N$_\text{t}$-C$_\text{cyt}$ orientation (Fig. 3 B).

**Effects of a Bacterial-expressed Mitochondrial Matrix Precursor Protein on Import and Insertion of pOMD29 into the Outer Membrane**

As reported earlier (Sheffield et al., 1990), the hybrid protein pO-DHFR (Fig. 3 A, top) has been expressed in bacteria and purified in a form that is efficiently imported into the matrix compartment of mitochondria in vitro. When the purified bacterial expression product was added to reticulocyte lysate containing [$^{35}$S]pO-DHFR produced by in vitro transcription-translation of the recombinant plasmid, pSP (pODHFR) (Skerjanc et al., 1990; Fig. 3 A), import of the radioactive precursor was effectively competed by bacterial pO-DHFR at concentrations of 1-6 $\mu$M (Fig. 7). This concentration range is very similar to the amount of a synthetic
Figure 3. Import of pO-DHFR and normal and mutant forms of pOMD29. (A) Translation products containing [35S]pO-DHFR (amino acids 1-36 of preornithine carbamyl transferase fused to DHFR, Skerjanc et al., 1990), [35S]pOMD29, [35S]pOMD29Δ2-10, and [35S]pOMD29KR2,7,9Q were incubated with (lanes 3 and 4) or without (lane 2) mitochondria (M/TO) under standard import conditions. Recovery of mitochondria, extraction with 0.1 M Na2CO3, pH 11.5 (lane 4) (ALKALI), and analysis by SDS-PAGE and fluorography were as described in Fig. 2 and Materials and Methods. (Lane 1) 10% of input [35S]precursor protein; (lane 2) [35S]precursor protein that sedimented in the absence of mitochondria. (B) As in A, except that, after import, reaction mixtures were treated either with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) trypsin (POST-TRYPSIN) (Li and Shore, 1992a). Mitochondria were recovered and analyzed by SDS-PAGE and fluorography. (C) As in A except that mitochondria were substituted with dog pancreas microsomes (Walter and Blobel, 1981) in import reactions containing pOMD29 and pOMD29Δ2-10 and the reaction mixtures subsequently subjected to trypsin treatment as in (A) (lane 4) or extraction with alkali (Materials and Methods) (lane 5). The amount of ER membrane protein and mitochondrial outer membrane protein in A and C were the same (40 μg/ml).

Concluding Remarks

Our results indicate that the positively-charged NH2 terminus of pOMD29 (OMM70) cooperates with the transmembrane segment to create the requisite topogenic domain for insertion of pOMD29 into the outer membrane. By analogy to the topogenic sequences of type II and type III proteins (von Heijne, 1988) inserted into the endoplasmic reticulum (Wickner and Lodish, 1985), we term this domain a signal-anchor sequence, in which the targeting domain is coinci-
Figure 4. Insertion of HLA-2A into microsomal membrane. Human HLA-A2 cDNA (Ennis et al., 1990) was transcribed and translated in the presence of [35S]methionine and dog pancreas stripped microsomes (Walter and Blobel, 1981). Microsomes were recovered, treated with (lanes 2 and 3) or without (lane 1) 100 μg proteinase K per ml in the presence (lane 3) or absence (lanes 1 and 2) of 1% (w/v) Triton X-100, and subjected to immunoprecipitation with W32 antibody (Barnstable et al., 1978). Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

Figure 5. Helical wheel projection of amino acids 1-12 of pOMD29 (OMM70). Asterisks denote residues mutated to glutamine in pOMD29KR2,7,9Q. Hydrophobic amino acids are circled. Amino acids are designated by the single letter code.

Figure 6. Rates of import and membrane insertion of pOMD29, pOMD29A2-10, and pOMD29KR2,7,9Q. Import reactions containing the various [35S]-labeled precursor proteins were carried out for 4, 8, 16, 20, 25, 30, and 40 min., at which times mitochondria were recovered by centrifugation through a sucrose cushion, and alkali-insoluble protein obtained and subjected to SDS-PAGE and fluorography as described in Fig. 2 and Materials and Methods. Radioactive precursor proteins were located on the dried gel by aligning with an exposed x-ray film, and the bands were excised, dissolved in H2O2 and 0.7 M NH4OH, and radioactivity determined by scintillation counting. The input amounts of the three [35S]precursor proteins were normalized, and the results for each time point expressed as a percentage of the maximal import that was observed for pOMD29 (set at 100).
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