Mitochondrial Mas70p Signal Anchor Sequence

MUTATIONS IN THE TRANSMEMBRANE DOMAIN THAT DISRUPT DIMERIZATION BUT NOT TARGETING OR MEMBRANE INSERTION*

(Received for publication, December 13, 1993, and in revised form, January 14, 1994;
Douglas G. Millar and Gordon C. Shore
From the Department of Biochemistry, McIntyre Medical Sciences Building, McGill University,
Montreal H3G 1Y6, Canada)

Mas70p is an integral membrane protein in Saccharomyces cerevisiae that is targeted and inserted into the mitochondrial outer membrane in an N-termina C-termina orientation by its NH2-terminal 29-amino acid signal anchor sequence. Recently, we demonstrated that the signal anchor was capable of mediating homo-oligomerization of a fusion protein, pOMD29, in the outer membrane in vitro (Millar, D. G., and Shore, G. C. (1992) J. Biol. Chem. 268, 18403–18406). Consistent with this finding, we show here that a synthetic peptide corresponding to the Mas70p signal anchor is capable of independent membrane insertion and dimerization with pOMD29. To further map the oligomerization domain in the signal anchor sequence, a deletion mutant of pOMD29 that lacks amino acids 2–10 was constructed. This protein, pOMD29Δ2–10, efficiently participated in dimer formation following import, indicating that dimerization was mediated by the putative membrane spanning segment (amino acids 11–29). This segment is predicted to form an α-helix that has an alanine-rich face and contains multiple copies of a pentapeptide dimerization motif that is widespread among members of the receptor tyrosine kinase family. Substitution of the alanine residues in one of these copies with isoleucine, producing a potentially bulkier contact surface, resulted in a protein which was targeted and inserted into the outer membrane but failed to assemble into dimers. Taken together, these results identify a structural feature of the signal anchor transmembrane domain that is important for oligomerization but is not required for targeting and membrane insertion.

Transmembrane domains of integral membrane proteins, in addition to passively anchoring soluble domains to a particular membrane, often contain important structural features that contribute to a variety of functional properties. This includes information for targeting and insertion of proteins into the endoplasmic reticulum (1–3) and mitochondrial outer membrane (4–6), protein retention in the inner nuclear membrane (7, 8) and Golgi membrane compartments (9, 10), growth factor receptor signal transduction (11), interaction with chaperones (12), polytopic protein folding (13), and assembly of homo- and hetero-oligomeric complexes (14–18). Elucidation of the specific structural features of transmembrane segments that encode this information, and how they are recognized, is a major goal of membrane protein research.

* This study was supported in part by operating grants from the Medical Research Council and National Cancer Institute of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
†Recipient of a Studentship from the Medical Research Council.

To study some of these structure-function aspects of transmembrane domains as they relate to the mitochondrial outer membrane, we have concentrated on a bitopic protein in Saccharomyces cerevisiae, Mas70p, that functions as a receptor component of the mitochondrial protein import apparatus (19–22). Mas70p is specifically targeted to mitochondria and inserted into the outer membrane via its NH2-terminal 29 amino acids (19, 23–25). This sequence comprises a 10-amino acid stretch rich in basic and hydroxylation residues followed by a 19-amino acid uncharged, hydrophobic region predicted to anchor the protein in the outer membrane. The sequence functions as a signal anchor (4) analogous to that of type II and III integral membrane proteins of the endoplasmic reticulum (1, 2). The 19-amino acid predicted transmembrane domain by itself contains sufficient information for targeting and stable integration of a passenger protein into the lipid bilayer of the outer membrane (4). Although the transmembrane domain of Mas70p has not yet been directly related to the function of the protein as an import receptor, the fact that its homolog in Neurospora crassa, MOM72 (26, 27), exists as multiple copies within the import complex (28) suggests that homo- and/or hetero-oligomeric interactions might be important.

Previously, we demonstrated the assembly of a chimeric protein containing the Mas70p signal anchor sequence fused to dihydrofolate reductase, termed pOMD29, into homodimers (18). An interesting feature of the signal anchor sequence is the clustering of alanine residues on one face of the predicted transmembrane α-helix, and the existence of residues on this face that exactly match a consensus motif found in the transmembrane domains of receptor tyrosine kinases that dimerize in response to signal transducing ligands (14). Small residue interfaces between interacting transmembrane α-helices have been suggested to be important for the dimerization of glycoprotein A (29), members of the tyrosine kinase family of receptors (14, 30), and the α and β chains of the class II major histocompatibility complex proteins (15). Here we have localized the dimer-mediating sequence of pOMD29 to within the transmembrane domain of the signal anchor. Substitution mutations introduced into the alanine-rich face of the predicted transmembrane α-helix are tolerated by the signal anchor sequence, which retains its outer membrane targeting, insertion and anchoring functions. However, we show that substitutions with residues of increased side chain volume, which produce a potentially bulkier contact surface in the α-helix face, disrupt dimer formation. The implications of these findings for oligomeric assembly, receptor function, and outer membrane sorting of Mas70p are discussed.

MATERIALS AND METHODS

General—Previous articles (Ref. 18 and references therein) describe the routine procedures used in this study. These include in vitro transcription of pSP64-derived plasmids, translation of the resulting mRNA
in rabbit reticulocyte lysate in the presence of \(^{35}\text{S}\)methionine, purification of mitochondria from rat heart, protein import of pOMD29 in vitro, alkali extraction of membranes with 0.1 M NaCO\(_3\), pH 11.5, chemical cross-linking with BMH, and analysis of import products by SDS-PAGE and fluorography. Additional details are provided in the figure legends.

**Peptide—pSP(pOMD29) and pSP(pOMD29Δ2–10)**—have been described previously (4). A mutation (T15 to A15) originally introduced into pOMD29 during DNA manipulations (4) was corrected and the wild-type sequence (T15) was used throughout this study. pSP(pOMD29) was modified by oligonucleotide-directed mutagenesis to create pSP(pOMD29-34G). The changes were confirmed by DNA sequencing.

**RESULTS AND DISCUSSION**

Our earlier observation that the Mas70p signal anchor mediated dimerization in the outer mitochondrial membrane in the context of a chimeric protein containing monomeric, cytosolic dihydrofolate reductase, termed pOMD29 (18), has led us to investigate the structural and functional properties of the signal anchor on its own, and to more precisely localize regions within the sequence involved in dimerization. As described in the earlier study, homodimers of pOMD29 in the outer membrane were detected by chemical cross-linking of adjacent polypeptides following import, in vitro, via a unique cysteine residue in pOMD29 (residue 34) which is located near the signal anchor on the cytosolic side of the membrane. Cross-linking was performed with the thiol-specific homobifunctional reagent, BMH, and the major cross-linked product shown to be a dimer of pOMD29 rather than a heterodimer containing pOMD29 and a resident outer membrane protein of similar size. As predicted, co-import of \(^{35}\text{S}\)pOMD29 obtained by \(^{35}\text{S}\)pOMD29 in a concentration-dependent manner (Fig. 1, lanes 3 and 5). The extent of cross-linking of \(^{35}\text{S}\)pOMD29 with peptide was similar to that observed with unlabeled bacterial-expressed pOMD29 (Fig. 1). To determine if dimer formation with \(^{35}\text{S}\)pOMD29, decreasing the amount of \(^{35}\text{S}\)pOMD29 homodimers below control levels (data not shown). Insertion and dimer assembly of the peptide was independent of the mitochondrial transmembrane electrochemical potential (\(\Delta \Psi\)), yielded alkali-resistant product, and used the bona fide saturated import pathway followed by pOMD29. These results confirm that the signal anchor is an independent functional domain containing the necessary structural information for mitochondrial targeting, outer membrane insertion, and dimer assembly.

**Role of the Transmembrane Segment**—It was shown previously that the 19-amino acid hydrophobic core (amino acids 11–29) of the signal anchor alone can target the deletion mutant pOMD29Δ2–10 to mitochondria and lead to its stable integration into the outer membrane in vitro (4). To determine if the transmembrane domain alone was sufficient for dimer formation, we performed import and cross-linking with the pOMD29Δ2–10 mutant (Fig. 2). pOMD29Δ2–10 participated in dimer formation with unlabeled pOMD29 with an efficiency approaching that of pOMD29 containing the entire 29-amino acid signal anchor (Fig. 2, compare lanes 3 and 6). pOMD29Δ2–10 also formed a heterodimer with pOMD1–34 peptide (data not shown). These results strongly suggest that the structure mediating dimerization of the signal anchor is contained in the 19-amino acid membrane spanning segment. Similarly, a synthetic peptide of the glycoporphin A transmembrane domain contained all the information for dimer assembly in membranes (31).

As noted previously (18), the hydrophobic core of the Mas70p signal anchor contains multiple copies of the pentapeptide consensus motif that has been implicated in the formation of dimers among members of the receptor tyrosine kinase family (14). One of these copies, comprising amino acids 14–18, contains multiple alanosines at positions that fall on one-half of the predicted transmembrane \(\alpha\)-helix. The contribution of these residues to the sidedness of this domain is evident in a helical wheel projection of the hydrophobic core (amino acids 11–28) of the signal anchor (Fig. 3). To address the possibility that this region is involved in a closely packed helix-helix dimer interface, 4 residues at positions 14, 15, 17, and 18 in the signal

\[\text{BMH} \quad \text{unlabeled bact. pOMD29 (\mu M)} \quad \text{pOMD(1–34) peptide (\mu M)}\]

\[\begin{array}{cccc}
- & + & + & + \\
- & .5 & 1.0 & - \\
- & - & 0.1 & 0.2 \\
\end{array}\]

\[\text{49.5 kDa} \quad 32.5 \text{kDa} \quad 27.5 \text{kDa}\]

**FIG. 1. Dimerization of \(^{35}\text{S}\)pOMD29 and synthetic pOMD1–34 peptide.** In vitro translated \(^{35}\text{S}\)pOMD29 was co-imported into purified mitochondria with unlabeled pOMD1–34 or purified pOMD29 from overexpressing bacteria, at the indicated concentrations under standard import conditions (see "Materials and Methods"). Following isolation of mitochondria, samples were treated with 10 mM BMH (lanes 2–5) or untreated (lane 1), exactly as described previously (18). After 60 min on ice, the reaction was stopped by the addition of 100 mM dithiothreitol and the samples processed for SDS-PAGE and analyzed by fluorography. The positions of pOMD29, the pOMD29 dimer, and the pOMD29–pOMD1–34 heterodimer (asterisk) are indicated. The positions of molecular mass markers are shown (dashes).

1 The abbreviations used are: BMH, bis-maleimidohexane; PAGE, polyacrylamide gel electrophoresis.

anchor, were mutated to isoleucine (Fig. 3). Isoleucine is often found in membrane spanning domains and its side chain conformations is compatible with an α-helical secondary structure (32, 33). However, substituting isoleucine for alanine increases the volume and contact surface area at each position and thus may disrupt helical interactions involving that face.

pOMD29-ile4—The isoleucine substitution mutant, designated pOMD29-ile4, was analyzed for outer mitochondrial membrane targeting, insertion, and assembly (Fig. 4). The mutant was imported into intact mitochondria and was stably inserted into the outer membrane as judged by temperature-sensitive acquisition of resistance to extraction with alkali (Fig. 4, panel A, compare lanes 3 and 4). Also, it assumed the same Nα-Cα orientation as pOMD29 as demonstrated by sensitivity of the bulk of the protein to exogenous trypsin (Fig. 4, panel A, lane 5). Like pOMD29, pOMD29-ile4 required protease-sensitive mitochondrial membrane surface components and ATP but did not depend on Δψ for import (data not shown). The efficiency of temperature-dependent membrane insertion of pOMD29-ile4 was comparable to that of wild-type pOMD29 when normalized to the 4°C background control (Fig. 4, panel A, compare lanes 3 and 4). This indicates that the signal anchor sequence can tolerate 4 amino acid substitutions in the transmembrane domain without compromising the mitochondrial targeting or outer membrane insertion functions. In contrast to the cross-linking of pOMD29 dimers, however, membrane-inserted pOMD29-ile4 did not exhibit detectable cross-linking, even in the presence of unlabeled pOMD29 (Fig. 4, panel B, compare lanes 2 and 3, with lanes 5 and 6).

The cross-linking reagent, BMH, that was used in Fig. 4, panel B, has a linear length of 16.1 Å. However, the dimer of pOMD29 could also be detected using the shorter cross-linking reagents, 1,4-phenylene diamidine and 1,3-phenylene diamidine, which have lengths of ~12 Å and ~10 Å, respectively (Fig. 4, panel C). This indicates close proximity as well as considerable flexibility of the cysteine-containing region of pOMD29 immediately outside the membrane. The fact that neither a pOMD29-ile4 homodimer nor a pOMD29-ile4-pOMD29 heterodimer could be detected, even with the longest cross-linking reagent used (BMH, 16.1 Å) makes it unlikely that the bulkier isoleucine face was simply separating a dimer...
out of the reach of the cross-linking reagent in the region of the cysteine residues.

Conclusions—Our results provide evidence that the ability of the Mas70p signal anchor to assemble into homo-oligomers relates to a structural feature within its predicted transmembrane domain, in which alanine residues form a structure compatible with dimerization between the transmembrane domains. Substitutions with isoleucine residues disrupt this structure. One interpretation of this result is that the alanine face is at the helix-helix dimer interface and is required for close helix-helix packing. Such packing of hydrophobic or amphiphilic α-helices has been observed in several membrane as well as soluble proteins. Packing can involve a “ridges into grooves” fitting of side chains from two interacting α-helical chains that frequently includes small hydrophobic residues (34, 35). Such a transmembrane helix packing has been observed in the bacterial photosynthetic reaction center (36) and the pea membrane domain, in which alanine residues form a structure compatible with dimerization between the transmembrane do-

REFERENCES

Mitochondrial Mas70p Signal Anchor