Signal Anchor Sequence Insertion into the Outer Mitochondrial Membrane

COMPARISON WITH PORIN AND THE MATRIX PROTEIN TARGETING PATHWAY*

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We have addressed the question of overlap between the pathways for protein insertion into the outer mitochondrial membrane and import to the matrix compartment, using competition studies in vitro. A synthetic peptide corresponding to the matrix-targeting signal of pre-ornithine carbamyl transferase competed for outer membrane insertion of porin but did not compete for membrane insertion of outer membrane signal anchor-containing proteins. Conversely, however, a synthetic peptide corresponding to the signal anchor sequence of Tom70 competed for import of all proteins examined. Both peptides competed for a step beyond receptor binding. Import of all precursors examined was inhibited by antibodies raised against the import receptor Tom20. Following binding to the surface of the organelle, outer membrane integration of porin was sensitive to depletion of nucleoside triphosphates by apyrase, whereas signal anchor protein insertion was not. The results demonstrate that outer membrane signal anchor insertion overlaps with a general insertion pathway. However, it exhibits both properties and steps that differ from the pathway followed by porin and matrix-targeted protein.

All integral membrane proteins of the outer mitochondrial membrane (OM) identified to date are encoded in the nucleus and posttranslationally targeted to the organelle. Unlike the majority of matrix-targeted proteins that contain a cleavable N-terminal matrix-targeting sequence (MTS), the signal that targets OM proteins resides within the mature protein sequence. A well characterized signal mediating OM protein targeting is the signal anchor sequence (SAS) (for recent reviews, see Refs. 1 and 2). Research on this targeting signal has revealed that the transmembrane domain is necessary and sufficient for proper sorting of passenger proteins and that it can be located at either the N terminus, as in the protein Tom70 (3), or the C terminus, as in Bcl-2 (4). In addition, the transmembrane segment of the SAS of yeast TOM70 contains a motif that mediates assembly into homo-oligomers in the OM (5, 6), and regions flanking the transmembrane domain influence the translocation or retention of the N terminus, thus specifying transmembrane topology (7, 8). The mechanisms employed by the mitochondrial import apparatus to recognize, insert, and laterally release an SAS into the OM remain enigmatic except for a few observations. In the case of Tom70 and Bcl-2, it is known that OM insertion requires cytosolic ATP, mitochondrial surface-exposed proteins, and physiological temperatures, is independent of the inner membrane electrochemical potential, ΔΨ, and is kinetically inhibited by high concentrations of the Tom70 SAS (3, 4, 9). However, the sequential steps forming the import pathway of OM proteins targeted by an SAS have so far been poorly characterized.

Extensive investigation of protein import into mitochondria in yeast and Neurospora has elucidated a multistep pathway followed by the majority of proteins examined (reviewed in Refs. 10–12). Several distinct steps at the OM were found to mediate intermediate stages of the import pathways of the OM protein porin and the inner membrane ADP/ATP carrier protein (AAC). Competition studies employing purified, denatured porin revealed a common step at the OM involved in porin insertion and translocation of AAC to an intermediate import stage at a site inaccessible to external protease and extractable by alkali (13, 14). The site, termed the general insertion pore (GIP), was found to be common to a variety of intermembrane space, inner membrane, and matrix-targeted proteins (15). Cross-linking of GIP site intermediates led to the identification of a protein of approximately 40 kDa apparent molecular mass in proximity to the translocating polypeptide and the proposal that this protein may line an aqueous pore (i.e., the GIP) (16–18). Additional steps in the import pathway that have been examined in detail include precursor protein interaction with cytosolic chaperones (19–21), binding of precursors to receptor proteins (reviewed in Ref. 22), release of cytosolic chaperones (23, 24), and transfer to the GIP site (25). The emerging picture of the protein import apparatus of the OM includes a protein receptor complex comprised of the proteins Tom70, Tom37, Tom22, and Tom20 mediating initial precursor binding steps and the membrane-embedded proteins Tom40, Tom7, Tom6, and Tom5 making up the GIP site (for recent reviews, see Refs. 26–28).

It was previously found that a hybrid protein, containing the SAS of yeast Tom70 fused to DHFR, followed a pathway that was not competitively inhibited by an MTS-containing protein that saturated matrix protein import (3). This suggested that the step competed by the MTS protein might be distal to the OM insertion step or that SAS insertion might occur independently of an MTS import pathway localized at contact sites between the outer and inner membranes (29).

We have examined the OM insertion pathway of SAS-con YES collects traffic and usage data for this website using cookies. By using this website, you will be providing your consent to the use of cookies. For more information about how we use cookies, please see the privacy policy.
taining proteins with respect to porin and MTS-containing proteins. An effective approach has been the use of synthetic signal sequence peptides in competition studies to saturate rate-limiting components during import. This strategy has been widely used to demonstrate participation of distinct steps in the import of signal peptides on the pathway followed by bona fide precursor proteins and to identify overlapping steps in the import pathways of different precursors (30–38). Here we have combined this approach with the analysis of import intermediates, involvement of specific OM components, and consecutive precursor transfer steps and propose a model for the SAS import pathway. Our results demonstrate a common import pathway followed by SAS proteins, OM porin, as well as MTS proteins. Interestingly, the import pathways of porin and MTS proteins appear to contain an additional step that, when competitively blocked by peptide, does not block SAS protein insertion.

MATERIALS AND METHODS

General—Previous articles (Ref. 39 and references therein) describe the routine procedures used in this study. These include in vitro transcription of recombinant plasmids, translation of the resulting mRNA in rabbit reticulocyte lysate in the presence of [35S]methionine, purification of mitochondria from rat heart, protein import in vitro, recovery of mitochondria from import reactions, and analysis of the import products by SDS-PAGE and fluorography. Additional details are provided in the figure legends.

Plasmids—cDNA encoding the eukaryotic porin, human voltage-dependent anion channel isoform-1 (hVDAC1, kindly provided by Michael Forte), was subcloned into the BamH1 and SmaI sites of pSP64 (Promega) using standard recombinant DNA methodology to produce pSP(hVDAC1). Plasmids containing the cDNA of all the other precursor proteins used in this study have been described previously (3, 4, 40).

Synthetic Peptides—The synthetic peptides pOCT-(1–27) and pOMD-(1–34) have been described previously (6, 30).

Antibodies against human TOM20—Production of antihuman TOM20 antibodies has been described previously (39).

RESULTS AND DISCUSSION

Matrix-targeting Signal Peptide Competes for Porin but Not Signal Anchor Protein Insertion into the Outer Membrane—To investigate the import pathway of SAS proteins with respect to the pathways followed by porin and MTS proteins, we examined the effect of a synthetic peptide comprising the MTS of pre-ornithine carbamyltransferase (pOCT), termed pOCT-(1–27). This peptide was shown to block protein import at a step common to matrix- and inner membrane-targeted proteins (30). The peptide efficiently competed for import of an in vitro translated 35S-labeled hybrid protein, pO-DHFR (40), which

FIG. 1. Import competition by the matrix-targeting signal peptide, pOCT-(1–27). Increasing amounts of peptide (in 7 M urea, 10 mM dithiothreitol, 10 mM Hepes, pH 7.4) were diluted 25-fold into standard import reactions at 0 °C containing 25 μg of purified rat heart mitochondria in a total volume of 50 μl. 35S-Labeled in vitro translated precursor protein was added to each reaction, and import was initiated by shifting the temperature to 30 °C. Following 10 min of incubation, samples containing Tom70-DHFR and Bcl-2 were extracted with 7 M urea as described (39), and samples containing porin and pO-DHFR were treated with trypsin as described (9). Mitochondrial pellets were recovered by centrifugation, and imported protein was analyzed by SDS-PAGE and fluorography. The insets show imported protein recovered with the indicated final concentrations of peptide. 10% of precursor added to each reaction and background import at 4 °C are shown. The position of the mature protein is indicated by an arrow. Densitometric analysis was performed on the scanned fluorograms using NIH-Image version 1.57 software. The amount of imported protein in the absence of competitor peptide was set to 100%.
contains the MTS of pOCT fused to DHFR (Fig. 1). The peptide had no effect on the transmembrane electrochemical potential, $\Delta \Psi$, at the concentrations used (30) (data not shown). Likewise, OM insertion of porin, as judged by acquisition of a membrane-embedded and protease-inaccessible form (39), was also competed by pOCT-(1–27) in a concentration-dependent manner similar to pO-DHFR (Fig. 1). In contrast, however, OM insertion of Tom70-DHFR and Bcl-2, as judged by resistance to extraction from the membrane with urea (39), was unaffected by similar concentrations of pOCT-(1–27) under the same conditions that blocked import of pO-DHFR and porin (Fig. 1). Competition was performed during the initial phase of import, which was linear for all the precursors examined (data not shown). The inhibitory effect of the peptide was reversible and could be overcome by increasing the amount of precursor added to the reaction (30) (data not shown). This result is consistent with the previous demonstration that overexpressed pO-DHFR purified from bacteria saturated the MTS pathway but did not affect the OM insertion of Tom70-DHFR (3). This observation suggests that the step that is competed by pOCT-(1–27) on the MTS import pathway overlaps with the porin insertion pathway but is not a limiting step for SAS protein insertion into the OM.

Signal Anchor Peptide Competes for Import of Porin and Matrix-targeted Protein—To examine the participation of a saturable binding step in the SAS insertion pathway, we employed a synthetic peptide containing the SAS of yeast TOM70, termed pOMD-(1–34). The peptide was previously shown to be inserted into the OM where it assembled into dimers with membrane-inserted Tom70-DHFR, demonstrating its ability to function as an OM-targeting and anchor signal (6). Also, this peptide was able to kinetically compete for OM insertion of Bcl-2, indicating that the peptide followed a saturable import pathway mediating OM SAS protein insertion (4). OM insertion of in vitro translated $^{35}$S-labeled Tom70-DHFR was efficiently competed by pOMD-(1–34) (Fig. 2). Insertion of Bcl-2 was also competed by pOMD-(1–34) in a concentration-dependent manner similar to Tom70-DHFR (Fig. 2). In contrast to the pathway differences suggested by the pOCT-(1–27) competition experiments in Fig. 1, pOMD-(1–34) efficiently competed for import of pO-DHFR to the matrix as well as for porin insertion into the OM (Fig. 2). The peptide had no effect on $\Delta \Psi$ at the concentrations used as determined by accumulation of the potential sensitive probe methyltriphenylphosphonium iodide (data not shown). This observation suggests the step that is competed by pOCT-(1–27) on the OM insertion pathway of SAS proteins is also a common limiting step involved in the insertion of porin and the OM translocation of MTS proteins.

**Peptide Competition Occurs Downstream of Receptor Binding**—To address the possibility that different receptors might be involved in different import pathways and that this might account for the different competition results, we examined the
involvement of OM surface-exposed proteins for precursor binding. Pretreatment of mitochondria with low concentrations of trypsin reduced the association of porin and pOCT with the organelle by 70–80% and reduced binding of Tom70-DHFR by approximately 50% (Fig. 3). It is possible the slight difference observed in surface protein-dependent binding between porin/pOCT and Tom70-DHFR could reflect components with different protease sensitivity mediating the binding step. In any case, binding of the precursors was unaffected by signal peptides in the binding reaction at concentrations that maximally competed the binding step. The characteristics of competition by pOMD-(1–34), involving a postreceptor site common to both OM insertion and translocation, are reminiscent of those of the GIP. The characteristics of the pOCT-(1–27)-competed step suggest additional limiting components on the porin and MTS import pathways.

**Antihuman TOM20 Antibodies Inhibit Import of Porin, Matrix-targeted, and Signal Anchor Proteins**—Since the requirement for different components of the import machinery in yeast and Neurospora could be identified by selective import inhibition of certain precursors by antibodies to OM receptors (41–44), we tested whether antibodies against the mammalian homolog of Tom20 affected OM insertion and translocation. Tom20 is found in a protein complex containing the putative pore-lining proteins Tom40, Tom7, and Tom5 (45, 46). The sequence of mammalian Tom20 is highly conserved from rat to humans, and antibody raised against the cytosolic domain of human Tom20 cross-reacts with the rat mitochondrial protein (39). Preincubation of mitochondria with antihuman TOM20 efficiently inhibited import of pO-DHFR, porin, Tom70-DHFR, and Bcl-2 while preimmune immunoglobulin had little effect (Fig. 4), consistent with the proposed role of Tom20 as a protein import receptor and its presence in the import complex (39, 47, 48). The inhibitory effect of the antibody on the import pathways examined in this study indicates a general requirement for this receptor, or for a protein complex containing this receptor, for OM insertion of the SAS proteins, as well as for porin insertion and translocation of pO-DHFR across the OM. A sterically on precursor access to other components of the import apparatus or other inhibitory effects on import activity cannot be ruled out. However, the interpretation that all of the import pathways converge at a step blocked by Tom20 antibodies is consistent with either mechanism.

**Postreceptor ATP Requirements**—Tom70-DHFR and Bcl-2 had previously been shown to require cytosolic ATP for insertion into the OM (4, 9). Such a requirement is thought to be due to the activity of the ATP-dependent cytosolic chaperones that prevent aggregation and maintain the precursors in an import-competent conformation (19, 49). A cytosolic ATP requirement for import of porin, as well as for AAC, has also been well established (50, 51). This requirement seems to be related to the maintenance of an unfolded state of the precursor or to maintaining its ability to be unfolded, since acid/base-denatured porin could assemble into the OM in an ATP-independent manner (52). Furthermore, passage of porin and AAC from the receptor-bound and protease-accessible site to the protease-inaccessible GIP site was demonstrated to require elevated levels of ATP (49). To address whether ATP was required at a similar postreceptor binding step on the SAS import pathway, we examined the effect of ATF depletion on the ability of prebound precursors to be chased into the OM. Following a standard binding reaction, samples were treated with apyrase to hydrolyze free nucleoside triphosphates, and subsequent import of bound protein was assessed (Fig. 5). Porin, which had been allowed to bind to mitochondria at 4°C, was subsequently sensitive to degradation by externally added trypsin (Fig. 5, lane 1), whereas the protein acquired a membrane-assembled protease-resistant structure after incubation at 30°C (Fig. 5, lane 2). Depletion of ATP with apyrase prevented the OM insertion of porin (Fig. 5, compare lanes 2 and 3). In contrast, however, insertion of Tom70-DHFR and Bcl-2 into the OM occurred independently of ATP when depletion was performed following the binding step (Fig. 5, compare lanes 5 and 8 with lanes 6 and 9). This suggests that the SAS proteins are able to insert into the OM from the receptor-bound form in the absence of external ATP while porin requires an additional ATP-dependent step for its OM insertion.

**Conclusions**—The results presented here, taken together with previous findings, suggest the following model (Fig. 6). Assembly of Tom70-DHFR (Fig. 6A) follows an import pathway in mammalian mitochondria mediated first by ATP-dependent chaperones in the cytosol. Step 2 involves receptor-mediated binding to the mitochondrial surface and sensitivity to protease digestion of the organelle. Step 3 involves a GIP-like site, saturable by pOMD-(1–34) (Fig. 6B). The last step overlaps with the TOM and MTS protein import pathways and may contain Tom20 as part of the receptor-pore complex. The OM assembly pathway of porin (Fig. 6D) differs from the SAS insertion pathway in the requirement for elevated ATP following receptor-mediated binding for passage to the membrane-embedded form. A GIP-like pOMD-(1–34)-competed step (Fig. 6E) is also involved in the porin insertion pathway as well as in MTS protein import. The ability of pOCT-(1–27) to compete for porin insertion and MTS protein import suggests two possibl-
Fig. 4. Import inhibition by antibodies to Tom20. Increasing amounts of antibody raised against human TOM20 (△) or preimmune antibody (●) were added to standard import reactions and left on ice for 30 min. Import and analysis were performed exactly as in Fig. 1. 10% of precursor added to each reaction and background import at 4 °C are shown. The position of mature proteins is indicated by an arrow.

Fig. 5. Effect of apyrase treatment on membrane insertion following precursor binding to mitochondria. 35S-Labeled precursors were bound to mitochondria for 10 min as in Fig. 3. Samples in lanes 3, 6, and 9 were then treated with 0.1 unit/μl apyrase (Sigma) for 30 min on ice. Import was initiated by shifting the temperature to 30 °C. Following incubation for 10 min, samples were extracted and analyzed as in Fig. 1. The amount of imported protein in untreated samples (lanes 2, 5, and 8) was set to 100%. Lanes 1, 4, and 7, background import at 4 °C.
Insertion Pathways of Outer Mitochondrial Membrane Proteins

The postreceptor requirement of ATP observed for porin at the OM suggests an additional unfolding or chaperone-mediated step on its insertion pathway. Recently, import pathways of precursors exhibiting different cytosolic ATP requirements were shown to be determined by interaction with the cytosolic chaperones of the mitochondrial import stimulation factor or Hsp70 (24). Release of mitochondrial import stimulation factor from precursors required ATP and occurred at the step involving binding to a receptor complex containing Tom70 and a 37-kDa component. Proteins without a strong import requirement for ATP were bound to the receptor Tom20 and released from Hsp70 independently of ATP hydrolysis. Interestingly, porin was bound to mitochondrial import stimulation factor, and the complex was dissociated at the mitochondrial surface in an ATP-dependent reaction (24). Tom70, however, was reported to interact with Hsp70 (56). Such a component- and precursor-specific step involved in mediating chaperone release might represent the pOCT-(1–27)-competed step and could account for the pathway difference observed for SAS proteins.

Our study demonstrates that a limiting kinetic step on the import pathways of porin and MTS proteins is not limiting for SAS protein insertion into the OM. Otherwise, however, the pathways of OM insertion and translocation overlap at a common OM GIP-like site. Further work is required to identify the distinct components mediating recognition of different signal sequences and to elucidate the mechanisms determining protein integration into and translocation across the OM.

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