PROTEIN IMPORT INTO AND ACROSS THE MITOCHONDRIAL OUTER MEMBRANE.

by

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Short title: Mitochondrial protein import.

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Abstract

Protein import into the mitochondria is a result of a series of sequential binding interactions between a mitochondrial targeting signal and the translocation machinery of both mitochondrial membranes. The targeting signals contained within protein of the outer membrane are distinct from those which target proteins to other subcompartments. The transmembrane domain of the yeast outer membrane receptor protein yTom70 is capable of both targeting and inserting the protein into the outer membrane. The efficiency of this process is increased by the addition of a positively-charged region preceding the transmembrane region. These two structural domains co-operate to form a signal-anchor sequence selective for the outer membrane, since this is the first membrane encountered by the targeting signal.

Consistent with this model, the signal-anchor sequence of the outer membrane protein yTom70 is also capable of importing into the inner membrane of mitochondria when the outer membrane is selectively removed. Import into the inner membrane requires the presence of an electrochemical potential across the lipid bilayer. Import proceeds in the absence of $\Delta\psi$ only when constructs are used which lack the positively-charged amino terminal region of the signal-anchor sequence. These results suggest that the positively-charged presequence leads the transmembrane domain into the import machinery and that $\Delta\psi$ is required to clear this region in order that the distal transmembrane region can enter the translocation pathway.

The charged N-terminal 10 residues of yTom70 are incapable of directing import into intact mammalian mitochondria, however, are able to efficiently direct import into the matrix of yeast mitochondria or mammalian mitoplasts. This potentially cryptic signal is excluded from intact mammalian mitochondria due to the presence of the receptor protein Tom20, since replacement of yeast Tom20 with mammalian Tom20 confers the mammalian phenotype onto yeast. These results suggest that receptor proteins may also have the ability to screen potentially cryptic signals from distal components of the outer and inner membrane translocation machinery.

Résumé

L'importation de protéines dans la mitochondrie est un méchanisme résultant d'une série séquentielle d'intéractions entre le signal mitochondrien ciblé et l'appareil de translocation des deux membrane mitochondriales.

Le signal ciblé des protéines de la membrane mitochondrienne externe sont distinct de ceux qui dirigent les protéines vers d'autres compartiments mitochondriales. Chez la levure, le domaine transmembranaire de la protéine yTom70, une protéine-récepteur qui se retrouve que dans la membrane mitochondrienne externe, est capable de diriger et d'insérer cette dernière dans la membrane externe de la mitochondrie. L'éfficacité avec laquelle ce domaine transmembranaire remplie ses functions est augmentée par l'addition d'une région riche en charges positives précédant ce dernier. Ces deux structures coopèrent, formant ainsi un signal ancré sélectif à la première membrane mitochondrienne rencontrée, I. e. la membrane externe.

Ce signal ancré est aussi capable de diriger et de négocier l'insertion de la protéine yTom70 dans la membrane mitochondrienne interne lorsque la membrane externe a été préalablement détruite. L'importation de protéines dans cette membrane interne est dépendante de la présence d'un potentiel électrochimique ($\Delta\Psi$). La disruption de ce potentiel électrochimique prévient l'importation de protéines seulement si celles-ci ont toujours le domaine de charges-positives précedant le domaine transmembranaire.

Ces résultats suggèrent que le domaine de charges positives amène le domaine transmembranaire vers l'appareil de translocation et que le potentiel électrochimique ($\Delta\Psi$) est nécessaire pour détruire la nuisance que le domaine de charges positives crée envers l'insertion du domaine transmembranaire dans l'appareil de translocation.

Les dix premières acides aminées de la protéine yTom70 sont chargées positivement et sont incapables de diriger l'importation dans des mitochondries mammifères. Néanmoins, ces mêmes acides aminées sont capables de diriger l'importation dans des matrices de mitochondries de levures ou dans des mitoplasmes mammifères. Ce signal potentiellement cryptique et spécifique à la levure est exclus du system mammifère du à la présence de la protéine-récepteur Tom20. Ainsi, l'intégration de la protéine mammifère Tom20 chez la levure bloque l'importation de yTom70. Ces résultats suggèrent que des protéines-récepteurs tels que Tom20, ont aussi l'abilité de cribler les signaux potentiellement cryptique des autres composantes de l'appareil mitochondrien de translocation.

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And to Rob, for sharing his life and love with me.

Preface

In accordance with the regulations described in item 3 of the Guidelines concerning thesis preparation of McGill University Faculty of Graduate Studies and Research, as cited in full below, and as approved by the Department of Biochemistry, three manuscripts published or to be published have been incorporated into this thesis.

> "Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.
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> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
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> In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent**. Supervisors must attest to the accuracy of such statements at

the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**"

Chapter 2: McBride, H. M., Millar, D. G., Li, J. M., and Shore, G. C. (1992) *J. Cell Biol.* **119**, 1451-1457.

Chapter 3: McBride, H. M., Silvius, J. R., and Shore, G. C. (1995) *Biochim. Biophys. Acta.* **1237**, 162-168.

Chapter 4: McBride, H. M., Goping, I. S., and Shore, G. C. (1996) *J. Cell Biol.* **134**, 1-7.

In chapter 2, figure 2b and 7 were performed by D. G. Millar and figure 4 was performed by Dr. Weija Ou. Dr. John Silvius supplied me with the liposomes used in chapter 3 and Ing Swie Goping supplied me with the hTom20 complemented yeast strain used in figure 6 of chapter 4. Other than these exceptions, all work described in these three manuscripts is entirely my own.

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List of Abbreviations

AAC	ATP/ADP carrier
UCP	uncoupling protein
GIP	general insertion pore
kDa	kilodaltons
ER	endoplasmic reticulum
SRP	signal recognition particle
tRNA	transfer ribonucleic acid
NEM	N-ethyl-maleimide
DHFR	dihydrofolate reductase
Tom	translocase of the outer membrane of mitochondria
Tim	translocase of the inner membrane of mitochondria
pOCT	pre-ornithine carbamyl transferase
Bcl-2	the second protein identified with a B-cell lymphoma
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SBTI	soybean trypsin inhibitor
TPMP	triphenylmethylphosphorium
RO-mitochondria	mitochondria with a ruptured outer membrane
NCBR	NADH cytochrome b_5 reductase

Chapter 1: Introduction

1. Introduction.

1.1. A new understanding of an old organelle

It is generally accepted that mitochondria once existed as an autonomous eubacteria and has since entered into a symbiotic relationship with the eukaryotic cell. In order to maintain this relationship, the eukaryotic cell trapped much of the mitochondria's genome within its own nucleus. The successful acquisition of the eubacteria granted the eukaryote the special ability to recycle its energy in a process called oxidative phosphorylation. Mitochondria are now left with an intron-less, circular DNA encoding 13 proteins, as well as two classes of structural RNA's, the large and small ribosomal RNA's and 23 tRNA's (Wallace 1986, 1994). The remaining approximately 1000 mitochondrial proteins are encoded in the nucleus and they must be translated on free polyribosomes before subsequent translocation into mitochondria. The symbiotic existence of mitochondria within the eukaryotic cell has resulted in the ability to evolve multi-cellular organisms and mitochondria have since become an essential component of every functional eukaryotic cell.

An enormous amount of research in last decade has increased our understanding of this essential organelle far beyond its previously ascribed function as the "energy powerhouse" of the cell. The tissue specific expression of proteins has allowed the development of mitochondria as an essential component in many unique cell functions. In the liver, for example, mitochondria house important enzymes responsible for steps along the tricarboxylic acid cycle. The fact that mitochondria store a large amount of cellular calcium intricately links this organelle with the regulation of cell signaling (Gunter *et al.*, 1994). Brown adipose tissue of hibernating animals

(and human infants) expresses the uncoupling protein (UCP) responsible for releasing the potential energy stored in the mitochondria (in the form of a proton electrical gradient) as heat in a process called non-shivering thermogenesis (Ricquier *et al.*, 1991). Other unique functions include the synthesis of heme, amino acids, lipids, steroids, the β-oxidation of fatty acids and the list is growing. The localization of the anti-apoptotic protein Bcl-2 to the outer membrane of mitochondria has implicated the involvement of this organelle in programmed cell death (Nguyen *et al.*, 1993, 1994). This, coupled with the fact that the mitochondrion is the primary producer of oxygen free radical species (also thought to play a role in initiating programmed cell death), is another reason for this new splash of excitement in the mitochondrial field (Korsmeyer *et al.*, 1995).

These new insights have also had an impact in the clinical perspective of mitochondria. Since the mitochondrion plays such an important role in every cell, clinicians assumed that mitochondrial deficiencies could not support life and therefore did not consider this organelle as a direct cause of any diseases. Unfortunately, clinicians have now identified a number of mitochondrially inherited diseases and genetic myopathies whose phenotypes and time of onset vary greatly (Luft 1994, Beal 1995). Constant exposure of mitochondria to free radicals produced by the organelle results in time-dependent degeneration and the onset of a condition commonly referred to as "growing old". Mitochondria are also important for researchers in the archeological fields who routinely sequence mitochondrial DNA in order to determine the phylogenetic origins of human existence as well as the migratory patterns responsible for the development of races and cultures around the world.

However, there are many basic questions which need to be answered in order to fully understand how this organelle functions within the cell. For

example, it remains a mystery how mitochondrial division is initiated and the mechanism by which division occurs. Also, although it is known that mitochondria are responsible for calcium homeostasis, no molecules have been found which are responsible for signaling between the nucleus and mitochondria. Now that the true importance of this exceptional organelle is beginning to be recognized, the emphasis in the future will undoubtedly involve the identification of signaling molecules which allow the mitochondria to communicate with the cellular environment.

1.2. An essential cellular function, mitochondrial protein import.

One of the most obvious mechanisms by which the nucleus could communicate with mitochondria is by regulation of protein synthesis of mitochondrial precursors. Interestingly, all of the mitochondrial proteins essential for cell viability are also involved in the import of nuclear-encoded precursors into the organelle (Baker and Schatz, 1991, Lithgow *et al*, 1994). The essential nature of components of the import machinery have been identified by screening yeast mutants with growth defects. Alternatively, much of the import machinery has been identified both in yeast *Saccharomyces cerivisiae* and *Neurospora crassa* by arresting precursors during import and then cross-linking the precursor to neighboring proteins of the translocation machinery (Sollner *et al.*, 1992, Kubrich *et al.*, 1995). The genetic approach coupled with the biochemical identification of import machinery has allowed great advances in understanding the components of the import machinery. However, since the mitochondrion contains two lipid bilayers, an outer and an inner membrane, the mechanism of protein import becomes intricately linked with the problems of protein sorting. As most precursors are destined for only one final destination, the import machinery must be able to recognize

precursors as mitochondrial, and import them to either the outer membrane, intermembrane space, inner membrane or matrix.

The study of protein translocation across any lipid bilayer begins by addressing the following questions: a) what are the mechanisms by which proteins are directed to the surface of the organelle, b) what constitutes the translocation competence of the polypeptide, c) what is the identity and general properties of the translocation channel or pore, d) what are the energetics required for both initial and complete polypeptide translocation across the lipid bilayer and e) what are the requirements for proper final assembly of the protein. As these questions apply also to the mitochondria, this introduction will take the reader through each of these steps of protein translocation. The first section explains the nature of mitochondrial targeting signals, followed by a discussion of the factors which assist in maintaining polypeptides in an import competent state. Both outer and inner membrane translocation machineries are described with a discussion of the transfer of polypeptides from the cis to trans side of each bilayer. The final section describes sorting pathways taken by proteins destined to the different mitochondrial subcompartments. Figure 1 illustrates the various stages of mitochondrial protein import (page 10a).

2. Sorting signals

2.1 General features of a sorting signal.

An fundamental criterion for protein targeting to any organelle or targeting system is that the protein contain sorting information within either the primary amino acid sequence or in some structural feature of the protein. Therefore, each targeting system has evolved unique signals for proper sorting of all proteins within the cell. Targeting signals are characterized by their ability to direct the import of a cytosolic reporter protein to a given organelle.

Recombinant studies employing such strategies have allowed generalizations to be made which help identify signals within many proteins, however there remain many whose signals are not yet identified.

Proteins destined for the ER, chloroplasts, mitochondria and bacterial export employ structural signals rather than primary amino acid sequences (von Heijne, 1988). A common structural feature found in most targeting signals is the propensity to form an alpha-helix. The usefulness of this structure is highlighted when one considers the plethora of protein-protein interaction throughout the cell which are mediated through the faces of each helix. As the amino acids wind into a coil, the physical properties of each residue combine with those of its neighbours which results in an additive hydrophobicity or charge (Chothia *et al.*, 1981). These properties are capable of directing specific protein-protein interactions without a dependence on primary sequence. Targeting sequences are generally amino-terminal extensions from the mature protein which are responsible for targeting and, once translocation is initiated, are cleaved by proteases within the various organelles.

Most proteins of the ER contain a 15-30 amino acid sequence located at the amino terminus which contains a hydrophobic region (with the propensity to form alpha helices) flanked by positive charges (Rapoport, 1991). The hydrophobic region contains the requisite information to target the protein to the ER and effect lateral release into the lipid bilayer (termed a signal-anchor sequence). For soluble lumenal ER proteins, the hydrophobic signal-anchor is cleaved during transit by signal peptidases which releases the mature domain into the lumen.

Bacterial proteins destined for the inner membrane or for export also contain an amino terminus targeting region 15-30 amino acids in length (the leader sequence) which is a composite of three features, a positively charged

amino terminus region followed by a hydrophobic sequence and a hydrophilic carboxyl terminal (Arkowitz and Bassiliana, 1994). The carboxyl terminal region also may contain a cleavage consensus for essential peptidases in the periplasmic space responsible for the final release of soluble precursors. Chloroplasts and peroxisomal type 2 (PTS2) signal sequences contain cleavable transit sequences which resemble mitochondrial matrix targeting signals (Douwe de Boer and Weisbeek, 1991, Purdue *et al.*, 1991).

Unlike most organelle signals, some peroxisomal and nuclear proteins contain targeting signals encoded at the primary sequence level. The peroxisomal type 1 signal sequences or PTS1 generally consist of a three amino acid consensus sequence "SKL", located at the extreme carboxyl terminal (Subramani, 1993, Rachubinski and Subramani, 1995). Nuclear localization signals (NLS) are located at the amino terminus of the protein and contain a loose consensus sequence "PKKKRKV" (Silver, 1991), which was originally observed as the polyoma Large-T antigen NLS.

2.2. Matrix signals.

Mitochondrial matrix presequences are generally described as positively-charged amino terminus regions with an increased number of hydroxylated residues and are predicted to form amphipathic alpha helices (Attardi and Schatz, 1990, Pfanner and Neupert, 1990). They contain no negatively charged amino acids and most have consensus cleavage sites for essential matrix peptidases. Some chloroplast proteins can be imported into mitochondrial matrix compartment and *visa versa*, which demonstrates structural similarities between these signals (Brink *et al.*, 1994). In the case of the peroxisomal enzyme alanine:glyoxylate aminotransferase, a single amino acid substitution in the PTS2 signal results in mis-sorting to the mitochondria

(Purdue *et al.,* 1991, Danpure, 1993), which demonstrates the similarity in these types of signals.

2.3 Outer membrane signals.

Proteins destined for the outer membrane do not contain cleavable pre-sequences and are of two types: those with transmembrane segments which form alpha-helixes, and beta-barrel proteins. Signals residing within a beta-barrel protein such as porin have not been identified to date. The alpha-helical transmembrane domains of a bitopic receptor protein Tom70 has been characterized in detail and is the subject of Chapter 2 of this thesis. It has been shown that, like proteins of the ER, insertion into the outer membrane of mitochondria is directed by the hydrophobic transmembrane region. The overall efficiency of import is greatly enhanced by the presence of a positively-charged amino-terminal helix (McBride *et al*, 1992). Since this is highly homologous to signal-anchor sequences found in the ER., the terminology has been maintained and signal-anchor sequences are now accepted as *bone fide* mitochondrial outer membrane signals.

2.4. Inner membrane signals.

In contrast to signal-anchor sequences of the outer membrane, some bitopic inner membrane proteins contain a cleavable matrix targeting signal followed by a hydrophobic stretch which functions as a stop-transfer to arrest translocation at the level of the inner membrane (Nguyen and Shore, 1987, Glaser *et al.,* 1990). It is predicted that the downstream stop-transfer region does not insert into the outer membrane because the matrix targeting signal is engaged in the machinery of the inner membrane (Nguyen *et al.,* 1988). Polytopic inner membrane proteins such as the uncoupling protein (UCP) and

the ATP/ADP carrier (AAC) span the inner membrane 6 times, with the transmembrane regions forming paired domains. UCP and AAC do not contain cleavable presequences and the first transmembrane segment is at the immediate N-terminal. The targeting signals located within such polytopic proteins are predicted to be either within the amino acids between the paired transmembrane domain or perhaps the paired domains alone represent import signals. The mechanism by which such hydrophobic regions escape insertion into the outer membrane is unknown and the signals directing inner membrane biogenesis remain the subject of current investigation.

2.5. Intermembrane space signals.

Signals directing proteins to the intermembrane space are believed to be a combination of positively-charged amphipathic alpha helices and hydrophobic stop-transfer regions. Some intermembrane space proteins also contain an apolar sorting signal responsible for arresting translocation at the level of the inner membrane. This pause in transport allows signal peptidases of the inner membrane and matrix to cleave the polypeptide on either side of the membrane resulting in a released, soluble protein (Glick *et al.*, 1992b).

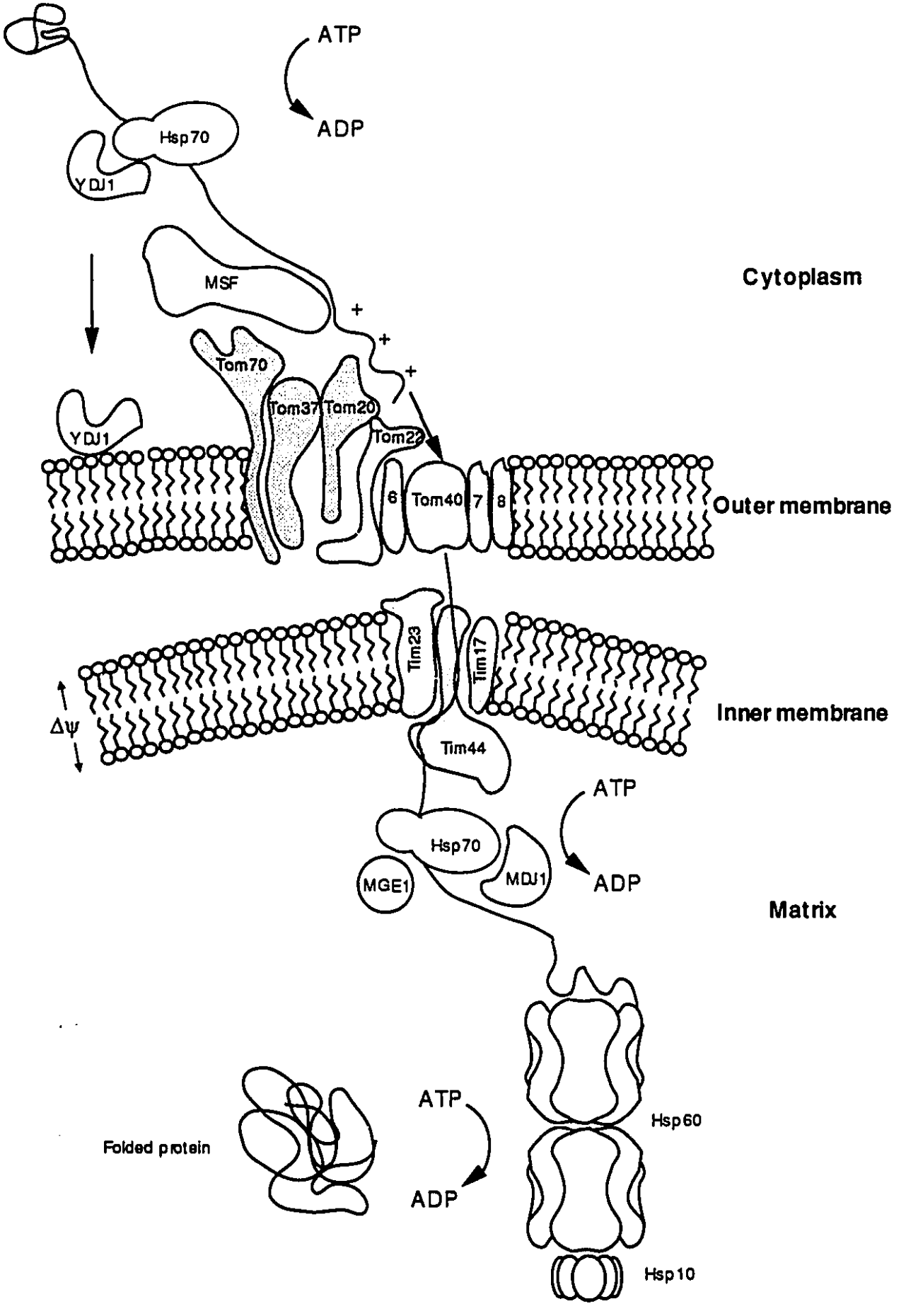
3. Translocation competence - the role of cytosolic chaperones

Protein import into mitochondria is known to occur primarily after translation on free polyribosomes in the cytosol. A number of early studies demonstrated the requirement for cytosolic factors of various masses which could stimulate mitochondrial protein import (Ohta and Schatz, 1984, Argan and Shore, 1985). The first of these factors was identified as a member of the heat-shock protein family, hsp70 (Deshaies *et al*, 1988, Murakami *et al.*, 1988,).

Early experiments also indicated the involvement of an NEM-sensitive cytosolic factor which has since been cloned and termed the mitochondrial import stimulating factor (MSF) (Hachiya *et al.*, 1993). The present understanding of cytosolic factors in mitochondrial protein import is that their role is three-fold: a) to assist in bringing the protein directly to the proper organelle and present the signal sequence for interaction with the surface, b) prevent hydrophobic regions of the protein from aggregating or misfolding before it has been properly sorted to the final destination and c) retain the polypeptide in an unfolded state in order that it be capable of translocating across the lipid bilayer in an extended polypeptide chain (Hachiya *et al.*, 1993, Lithgow *et al.*, 1993, Hohfeld and Hartl, 1994, Hachiya *et al.*, 1994, Lithgow *et al.*, 1995, Mihara and Omura, 1996).

Ssa1p and Ssa2p are the major hsp70 heat shock proteins of yeast involved in maintaining translocation competence (Craig *et al.*, 1993, Ellis and van der Vies, 1991). Hsp70 is also involved in many cellular functions including the ATP-dependent uncoating of clathrin-coated vesicles (Chappell *et al.*, 1986, Prasad *et al.*, 1994) and the complex formation with the cytosolic chaperone Hsp90 is responsible for numerous cellular functions (Pratt, 1993). Members of the hsp70 family generally require the presence of co-factors, which have been most extensively characterized in bacterial homologues. Hsp70 is a homologue of the bacterial DnaK protein which functions with the aid of two co-chaperones. The bacterial DnaJ protein stimulates the ATP hydrolyzing activity of DnaK, thereby triggering the release of bound polypeptides. Precursor release is facilitated by another co-factor, GrpE (Hartl *et al.*, 1994). The yeast DnaJ homologue, Ydj1, has been identified and functions as a chaperone and regulator of the hsp70 (Caplan and Douglas, 1991, Atencio and Yaffe, 1992, Hartl *et al.*, 1994). Ydj1 is post-translationally modified through the addition of a

Figure 1. Mitochondrial protein import. Presequence interaction with cytosolic chaperones Hsp70/Ydj1 and the mitochondrial import stimulating factor (MSF) are shown. These chaperones facilitate interactions between the presequence and receptor components of the outer membrane Tom70/Tom37 and Tom20/Tom22. Tom22 hands off the presequence to the general insertion pore (GIP), which is comprised of Tom40, Tom6, Tom7 and Tom8. Passage through the inner membrane is mediated by the inner membrane machinery, Tim44,Tim23 and Tim17, and depends on the electrochemical potential ($\Delta\psi$). Tim44 and mtHsp70 function together as an "import motor" and drive unidirectional transport of the presequence into the matrix in a manner dependent on ATP. Mge1 and Mdj1 function as co-factors of mtHsp70. Hsp60/Hsp10 complexes facilitate proper ATP-dependent folding and oligomerization of incoming proteins.


ATP
ADP
Hsp70
YDJ1
MSF
Cytoplasm
+
+
+
Tom70
Tom37
Tom20
Tom22
YDJ1
6
Tom40
7
8
Outer membrane
Tim23
Tim17
Δψ
Inner membrane
Tim44
ATP
Hsp70
MGE1
MDJ1
ADP
Matrix
Folded protein
ATP
ADP
Hsp60
Hsp10

carboxyl terminal farnesyl group which has been proposed as the mechanism by which it interacts with the mitochondrial surface (Caplan et al, 1992). A eukaryotic homologue of GrpE in the cytosol has not yet been identified.

Although the involvement of cytosolic chaperones in mediating protein import into mitochondria was well established, there was confusion concerning the mechanism by which chaperone involvement resulted in specific targeting to the proper organelle. However, the identification of chaperones which bind specifically to mitochondrial precursors, such as MSF (mitochondrial import stimulating factor) (Hachiya *et al.*, 1993) and PBF (mitochondrial presequence binding factor) (Murakami *et al.*, 1992), has resulted in new speculations that chaperones can function to direct precursors specifically to a single organelle (Lithgow *et al.*, 1993). Much progress has been made in recent years on the precise mechanism by which cytosolic chaperone proteins specifically mediate precursor interactions with the mitochondrial surface. It was demonstrated that MSF can bind directly to the amino-terminal presequence and actively disperse mitochondrial protein aggregates in an ATP-dependent reaction (Hachiya *et al.*, 1993, 1994, Komiya *et al.*, 1996) thereby rendering the precursor competent for import. Hsp70 on the other hand, binds to mature regions of polypeptides and probably co-operates with MSF in the ATP-dependent maintenance of import competence (Fourie *et al.*, 1994, Komiya *et al.*, 1994).

The next important function of the chaperones is the presentation of the presequence to the mitochondrial surface. Mitochondrial-specific MSF was shown to bind to the surface of mitochondria only when complexed with presequence. This binding could be competed for by the addition of specific antibodies against individual components of the outer membrane receptor complex (discussed below). In contrast, the non-specific chaperone, hsp70, continued to facilitate presequence binding to mitochondria even in the

presence of the same receptor antibodies (Hachiya *et al.*, 1995). Furthermore, it was shown that the receptor complex to which the MSF-presequence bound was distinct from that which the hsp70-presequence bound, suggesting the possible existence of an MSF specific receptor on the surface of the mitochondria. Although interactions between the presequence and specific outer membrane receptor molecules has been documented through binding studies, a direct interaction between the chaperones and a receptor component has not been shown. However, the proposed existence of such a docking receptor similar to that observed in SRP-mediated sorting to the endoplasmic reticulum is tantalizing. The mechanisms and specificity through which chaperone molecules bind and release precursors is an active area of research in which advances will benefit the understanding of protein folding in general.

4. The outer membrane.

4.1 Outer membrane import machinery

Early experiments demonstrated that import into the matrix compartment was inhibited upon pre-treating the mitochondria with protease, which indicated the requirement for proteinaceous components on the surface of the organelle required for import (Argan *et al.*, 1983, Zwizinski *et al.*, 1984, Pfaller *et al.*, 1988). Furthermore, Fab fragments purified against a 45 kDa outer membrane protein inhibited import of various precursors (Ohba and Schatz, 1987a, Vestweber *et al.*, 1989). Since that time, a number of experiments using both yeast genetics and chemical cross-linking to incoming polypeptides have identified a number of proteins which make up the translocation apparatus of the outer membrane.

We now know that the recognition of precursors at the surface of the mitochondrion is the responsibility of receptor proteins whose bulk is exposed

to the cytosol and is accessible to externally added proteases and antibodies. To date, 4 receptor proteins have been identified in *Saccharomyces cerivisiae*, *Neurospora crassa* and one mammalian homologue, and are universally called Tom's (translocase of the outer mitochondrial membrane (Pfanner *et al.*, 1996, Lill and Neupert, 1996)). These receptor proteins are, Tom70 (Hines *et al.*, 1990, Sollner *et al.*, 1990), Tom37 (Gratzer et al, 1995), Tom20 (Ramage *et al.*, 1993, Moczko *et al.*, 1994, Goping *et al.*, 1995) and Tom22 (Kiebler *et al*, 1993, Lithgow *et al.*, 1994, Nakai and Endo, 1995a). Tom22 is the only receptor molecule which is essential for yeast cell viability (Lithgow *et al.*, 1994, Honlinger et al, 1995). Components which comprise the translocation pore itself, also referred to as the general insertion pore (GIP), have been identified in yeast and *Neurospora crassa* and are termed Tom40 (Vestweber *et al.*, 1989, Baker *et al.*, 1990, Kiebler *et al.*, 1990), Tom6 (Kassenbrock *et al.*, 1993), Tom7 and Tom8 (Sollner *et al.*, 1992)

4.2 Structure and assembly of outer membrane proteins

The receptor components of the outer membrane import machinery are oriented in the membrane such that the bulk of the polypeptides are exposed to the cytosol where they are capable of facilitating productive interactions with incoming polypeptides. Tom70, Tom20 and Tom37 are inserted into the membrane in an Nin-Cout orientation, whereas Tom22 is inserted in a Cin-Nout orientation with approximately 45 residues with an overall negative charge exposed to the intermembrane space. The negatively charged intermembrane space region of Tom22 was shown to be required for efficient import of some precursors (Bolliger *et al.*, 1995) but not all (Nakai *et al.*, 1995b). Structural information on the cytosolic portion of these molecules is limited However Tom70, Tom20 and Tom37 all contain a number of tetratricopeptide repeats

(TPR) known to mediate protein-protein interactions between a number of cellular proteins including that of actin and spectrin (Goebl and Yanagida, 1991, Lamb et al, 1995). The function of TPR domains within the mitochondrial receptor molecules has not yet been investigated. For example, it is unknown whether these domains are responsible for receptor-receptor, receptor-chaperone or for receptor-precursor interactions.

A picture is emerging based on cross-linking experiments and receptor requirements that most likely Tom70/Tom37 and Tom20/Tom22 form heterodimers, (Gratzer *et al.*, 1995, Hachiya *et al.*, 1995, Mayer *et al.*, 1995c). In a reconstituted yeast import system, a Tom70/Tom37 heterodimer could bind precursors via the liver specific cytosolic chaperone, MSF. In such a model, following hydrolysis of ATP by MSF, the precursor would be released from Tom70/Tom37 and transferred to Tom20/Tom22 (Hachiya *et al.*, 1995). It was also shown that not all precursors required an initial interaction with Tom70/Tom37 and are instead capable of direct interaction with Tom20/Tom22 (Ryan and Jensen, 1995). The latter complex is responsible for the transfer of the polypeptide to the translocation machinery itself (Kiebler *et al.*, 1993, Mayer *et al.*, 1995c).

It has also been demonstrated that Tom70 is capable of forming homodimers through a proposed alanine face of the alpha-helical transmembrane region following insertion into the lipid bilayer (Millar and Shore, 1993, 1994). This finding does not rule out potential interactions with other receptor molecules through the cytosolic domains. There is evidence that both the mechanism of assembly as well as the partners within the import complex may be somewhat species dependent. For example, in yeast, the correct assembly of yTom70 into the receptor complex was shown to require the presence of 37 kDa protein (probably yTom37) whereas in *Neurospora crassa*,

nTom70 assembly did not require the 37 kDa protein and was instead found in proximity to nTom22 (Schlossmann and Neupert, 1995).

The components of the GIP do not contain large portions exposed to either the cytosol or the intermembrane space region and are mostly embedded in the lipid bilayer. Tom40 is predicted to form a multi-membrane spanning beta-barrel structure with protease accessible residues exposed to the intermembrane space (Court *et al.*, 1995). Tom6 was identified as a high-copy suppresser of the Tom40 yeast mutant and is now known to functionally interact with Tom40 and together constitute the translocation channel with a single transmembrane spanning region and a short amino terminus region exposed to the cytosol (Kassenbrock *et al.*, 1993, Cao and Douglas, 1995, Alconada *et al.*, 1995). Two other components of the translocation pore, Tom7 and Tom8, have been crosslinked biochemically to incoming polypeptides in *Neurospora crassa* and have not yet been cloned therefore structural information is unknown (Sollner *et al.*, 1992). Thus, although many of the components of the import machinery have been identified, the mechanism by which they assemble in the bilayer and interact with each other remains a subject of further investigation.

4.3. Topogenic determinants of outer membrane proteins.

An important consideration in the biogenesis of membrane proteins is the determinants of polypeptide orientation. The amino terminus signal anchor sequence of Tom70 is known to insert the protein in an Nin-Cout orientation (McBride *et al.*, 1992, Li and Shore, 1992b). The orientation was reversed by replacing the amino terminus 10 hydrophilic residues of the signal-anchor with a highly charged matrix targeting signal (Li and Shore, 1992b). These studies have led to a model whereby the matrix targeting signal is functioning as a retention signal to hold the amino terminus at the cis side of the machinery.

The hydrophobic signal anchor would then engage the translocation machinery prior to insertion of the remainder of the molecule into the intermembrane space. The mechanism by which the matrix-targeting signal is retained is uncertain. However protease treatment of the mitochondrial surface had no effect on the orientation, which indicates that the determination of protein orientation may be a result of lipid interaction with the matrix signal rather than a specific receptor binding event (Steenaart *et al.*, 1996). Consistent with this, protein-free liposomes were shown to bind the matrix presequence, but did not bind the 11 amino acid N-terminal region of the Tom70 signal anchor (Steenaart *et al.*, 1996).

The topogenic features of beta-barrel proteins such as porin, Tom40 and monamine oxidase are far less understood, if at all. Recent experiments using porin indicate that the insertion into the outer membrane may be due to conserved tracts of lysine residues at the C-terminal of the protein (Smith *et al.*, 1995), however a complete understanding of the biogenesis of such proteins is awaited.

4.4 Energetics of translocation across the outer membrane

Import experiments using isolated outer membrane vesicles have demonstrated that outer membrane proteins can be imported in the absence of the inner membrane machinery (Mayer *et al.*, 1993, 1995a, Iwahashi *et al.*, 1994). Moreover, matrix signals can be transiently inserted into the translocation machinery, but not completely translocated . These experiments demonstrated that the outer membrane translocation machinery can function independently in the recognition, insertion and translocation of some mitochondrial proteins. The mechanism of precursor translocation from the cis to trans side of the outer membrane is proposed to be the result of a series of

binding and release steps based primarily on proposed electrostatic interactions between the positively charged signal sequence and "acid bristles" located on the cytosolic domain of receptor proteins (Sollner *et al.*, 1989, Honlinger *et al.*, 1995, Bolliger *et al.*, 1995). However, whether a productive binding event is determined by the length of time such an electrostatic interaction is maintained or whether a direct "lock-and-key" correct match is required for productive binding is unknown. The model of sequential binding through the receptor complex on the surface of the outer membrane accounts for the mechanism by which the precursor reaches the GIP, but not how the presequence travels across the GIP. Recent data indicates that the acidic intermembrane space domain of Tom22 is capable of binding to positively charged presequences *en route* across the outer membrane (Bollinger *et al.*, 1995). This suggests that the binding of incoming presequences to regions on the intermembrane space side of the outer membrane may be a driving force for translocation across the GIP. The presence of such a trans binding site was predicted previously when outer membrane vesicles prepared with the matrix-processing peptidase (MPP) enclosed in the lumen to monitor the appearance of the signal on the trans side of the vesicle (Mayer *et al.*, 1995a). Binding to a trans site accommodated limited unfolding on the cis side however the exact mechanism of this unfolding remains speculative (Mayer *et al.*, 1995a).

Cytochrome c heme lyases in the intermembrane space have been shown to be imported through the GIP in a manner independent of the electrochemical potential ($\Delta\psi$) across the inner membrane or ATP (Lill *et al.*, 1992, Steiner *et al.*, 1995). A folded domain of this protein was found to occur at the same time as import, and also did not require ATP or any chaperones of the peptidylprolyl cis-trans isomerases (Steiner *et al*, 1995). The authors suggest that the machinery of the GIP such as Tom40, rather than Tom22, may

function not only in translocating the polypeptide across the lipid bilayer, but also may hold regions of the protein in order to facilitate proper folding during import. Therefore, a driving force of the import of cytochrome c heme lyase may be intricately associated with binding to a site on the trans side of the translocation machinery which facilitates folding. This again supports the notion that protein translocation across the outer membrane is a result of a series of binding and release interactions initiated by cytosolic chaperones which lead to interactions with the receptor components on the surface and passage through the GIP. In the case of molecules like cytochrome c heme lyase, such a trans site on the membrane may facilitate folding which would constitute the driving force for further unidirectional translocation of the molecule across the lipid bilayer. In other cases, interaction of the precursor with components of the inner membrane translocation machinery would initiate downstream interactions (discussed later) resulting in complete translocation across the outer membrane. The nature of binding interactions with various components along the import pathway and the requirements for unidirectional transport remains the subject of further investigation.

The unique example of apocytochrome c demonstrates that perhaps not all proteins require the import machinery and instead can spontaneously and reversibly insert across the lipid bilayer (Mayer *et al.*, 1995b). Upon initial appearance of the apocytochrome c at the trans-side of the outer membrane, it interacts with its partner molecule, cytochrome c heme-lyase, and the formation of an oligomeric complex drives complete, unidirectional translocation. The formation of oligomeric complexes may be a unidirectional driving force for many other proteins as well.

Although ATP is required for release of precursors from cytosolic chaperones (Hohfeld and Hartl, 1994), it has been difficult to determine whether

or not energy in the form of hydrolyzable nucleotide triphosphates (NTP) is required for the transfer of the polypeptide from site to site within the translocation machinery. The importance of GTP as an energy and regulator of import in protein transport has been aptly demonstrated in a number of import pathways. For example, protein traffic through the Golgi and endosomes (Balch, 1990) requires a number of proteins in the "ras" family of GDP/GTP exchange factors. In chloroplasts, at least two components of the machinery has been shown to bind GTP (Kessler *et al.*, 1994) and GTP hydrolysis is required for protein import (Kessler *et al.*, 1994, Schnell *et al*, 1994). However, to date, there has been no evidence of a GTP requirement in mitochondrial protein import. Another important potential energy source (other than ATP or GTP) possibly affecting passage of proteins through the outer membrane machinery is the thermodynamic "pull" into the lipid bilayer due to the hydrophobicity of a transmembrane region (Shore *et al.*, 1995). Whether or not these domains physically encounter lipids while within the pore is unknown, although the recent discovery that incoming precursors could be directly cross-linked to lipid molecules within the translocation pore of the endoplasmic reticulum lends credence to such a hypothesis (Martoglio *et al.*, 1995).

4.5. Do Receptors screen out cryptic signals?

The receptor components of the import machinery are responsible not only for recognizing mitochondrial signals, but also to screen out non-mitochondrial proteins (McBride *et al.*, submitted). Early studies demonstrated a rather loose signal specificity *in vitro* of yeast mitochondria, which resulted in yeast mitochondria misinterpreting false or cryptic signals as matrix targeting signals when placed at the amino terminus of passenger proteins (Allison and Schatz, 1986, Baker and Schatz, 1987, Hurt and Schatz, 1987, Roise *et al.*,

1988). Data presented in Chapter 4 demonstrate that for some cryptic matrix-targeting signals, such promiscuous behavior is limited in the mammalian systems. The human receptor Tom20 can functionally complement a yeast mutant (Goping *et al.*, 1995), demonstrating that the mechanism of import is more similar than it is different between species. However, interestingly, the greatest divergence in sequence between receptors of different species is observed within the cytosolic domain (Goping *et al.*, 1995). With the increased complexity of a mammalian cell relative to yeast, it is tempting to speculate that the import machinery may have evolved a more stringent mechanism for screening out potentially harmful foreign proteins. In humans, there are examples of mis-sorting aberrations in which, most commonly, peroxisomal proteins become sorted to the mitochondria (Danpure 1993). In the case of alanine:glyoxylate aminotransferase, a single amino acid substitution generates an efficient matrix-targeting signal and the protein is thereby re-routed to the mitochondria (Purdue *et al.*, 1991). This single amino acid substitution is predicted not to alter the proposed helical structure of the sequence. Thus the wild type peroxisomal precursor contains a targeting signal that differs only slightly from a mitochondrial signal and is, perhaps suprisingly, not targeted to the mitochondria. The mechanisms by which yeast and mammalian mitochondria receptor molecules exclude cryptic proteins is unknown (McBride *et al.*, submitted). One could envision a model whereby the precursors bind to a region of the cytosolic domain of receptor molecules distinct from the binding sites for *bone fide* signals. This would result in a dead-end interaction whereby each receptor could not hand off the precursor protein to the next component along the import pathway.

5. The contact site.

Translocation of proteins into the matrix or inner membrane requires that the polypeptide engage the machinery of the inner membrane. By arresting a polypeptide "in transit" across the mitochondrial membrane, researchers were able to determine that a) incoming polypeptides were translocated in a highly unfolded state and b) that translocation could occur through both membranes simultaneously (Schleyer *et al.*, 1985, Schwaiger *et al.*, 1987, Pfanner *et al.*, 1990, Glick and Schatz, 1991). This suggests that there is a site of contact between the two membranes and/or membrane machineries. Whether or not contact sites were stable or dynamic structures was a question of debate. However, upon inactivation of the yeast outer membrane machinery by protease treatment, import could be restored by selectively rupturing the outer membrane with osmotic shock (Ohba and Schatz, 1987b, Hwang *et al.*, 1989). Furthermore, it was subsequently shown that a fusion product of the matrix targeting signal of the F_1-ATPase b-subunit followed by cytochrome c heme lyase could be efficiently imported into the intermembrane space of mitochondria whose electrochemical potential was uncoupled. This intermembrane space intermediate could then be chased into the matrix compartment by re-energizing the mitochondria. This demonstrated that the inner membrane machinery is capable of functioning independently of the outer membrane, moreover, that the formation of a contact site was not required for translocation across the inner membrane (Segui-Real *et al.*, 1993). The functional independence of the inner membrane machinery was further proven in rat heart, rat liver and *Neurospora crassa* mitochondria in a number of experimental systems (Hwang *et al.*, 1991, Rassow and Pfanner, 1991, Li and Shore, 1992a, McBride *et al.*, 1995). Experiments using yeast mitochondria have since shown that components of the inner membrane import machinery can not be co-immunoprecipitated using an outer membrane antibody against

Tom40. However, upon the addition of precursor protein and initiation of import, components of the inner membrane translocation machinery (see below) can then be immunoprecipitated using the anti-Tom40 antibody (Horst *et al*, 1995). This experiment suggests that the inner membrane is not in direct contact with the outer membrane until the appearance of the incoming polypeptide at the trans side of the outer membrane. It is now generally accepted that contact sites are dynamic in nature and are formed upon appearance of a precursor at the trans side of the outer membrane. However, it is unknown whether the components of the translocation machineries interact directly or if the translocating polypeptide is the only component that bridges the two membranes.

6. The inner membrane.

6.1 Inner membrane translocation machinery

The inner membrane has evolved unique machinery in order to translocate proteins across the lipid bilayer (Pfanner *et al.*, 1994). The fact that this apparatus is capable of functioning in the absence of the outer membrane indicates that the inner membrane machinery contains the requisite components in order to recognize specific signals and translocate proteins without the outer membrane.

To date, three proteins of the inner membrane translocation machinery have been identified genetically and are essential in yeast (Pfanner *et al*, 1994, Kubrich *et al.*, 1995). At least two others have been identified biochemically in cross-linking experiments using incoming precursors (Berthold *et al.*, 1995). Tim44 (translocase of the inner mitochondrial membrane of 44 kDa) was the first genetically identified protein in yeast (Maarse *et al.*, 1992, Emtage and Jensen, 1993, Blom *et al.*, 1993, Horst *et al.*, 1993, Pfanner *et al.*, 1996, Ryan

and Jensen, 1993, Rassow *et al.*, 1994, Berthold *et al.*, 1995). Tim44 is responsible for transferring the incoming presequence by directly interacting with the matrix hsp70 chaperone protein (Rassow *et al.*, 1994, Schneider *et al*, 1994, Berthold *et al.*, 1995). The two molecules together act as a translocation motor to pull the precursor into the matrix compartment. The translocation pore itself is known to contain 2 cloned proteins identified as Tim23 and Tim17 (Kubrich *et al.*, 1994), and other cross-linked products called Tim14 and Tim33 (Berthold *et al.*, 1995). Tim23 is thought to span the lipid bilayer twice and contains a large, acidic region exposed to the intermembrane space (Dekker *et al.*, 1993). Tim17 is thought to be mostly membrane-embedded, and is also an essential component of the pore (Maarse *et al.*, 1994). Until the cross-linked proteins Tim14 and Tim33, are conclusively shown to be components of the translocation pore and not neighboring members of the respiration complex, their involvement in protein import will remain unknown. The mechanism by which the channel proteins recognize precursors and actively transport them through the pore is unknown. However, it has been shown that once the presequence has been presented to the matrix, the proteins of the channel itself apparently do not to interact with the incoming precursor and functions as a passageway (Ungermann *et al.*, 1994). Although the import channel may not directly interact with the incoming protein, it must retain a very tight seal such that the tight ionic barrier of the inner membrane not be compromised during import.

6.2 Energy for transport: The electrochemical potential .

Cellular ADP must enter into the matrix of the mitochondria in order to be regenerated into a useful energy form, ATP. This "recycling" process occurs at the inner membrane and relies on oxidation events resulting from oxidative

phosphorylation (reviewed in Hatefi, 1985). Energy resulting from the transfer of electrons (accepted by oxygen) through the electron transport chain allows protons to be pumped out of the matrix. This creates an energy potential in the forms of chemical and electrical gradients. The electrochemical potential ($\Delta\psi$) is a gradient of about 220mV, inside negative, and is drawn upon by ATP synthase which pumps a proton back into the matrix to release phosphorylated ADP (ATP) from the enzyme complex. The ATP/ADP carrier (AAC) then exchanges a new ATP from the matrix for an ADP from the intermembrane space in a new round of recycling. Not only is the electrochemical potential essential for the phosphorylation of ADP, it is also essential for protein import into or across the inner membrane (Martin *et al.,* 1991). More specifically, it has been demonstrated that $\Delta\psi$ is required to initiate translocation across the inner membrane but can then can be uncoupled and translocation will proceed (Schleyer and Neupert, 1985, Pfanner and Neupert, 1987, Hwang *et al.,* 1991). This is in contrast with the requirement for the proton-motive force across the inner membrane of bacteria in the initiation of translocation and throughout translocation (Driessen, 1992). The reason for this requirement in import across the inner, but not outer membrane of mitochondria is unknown. There are two models to account for this difference: a) that $\Delta\psi$ is required to open/close what would be voltage-gated import machinery, or b) that the positively-charged presequences require $\Delta\psi$ to exert an electrophoretic pull in order to cross the lipid bi-layer. In Chapter 3 of this thesis I address this problem using fusion constructs to study the dependence for $\Delta\psi$ during import into mitoplasts. It is shown that $\Delta\psi$ is required only in the presence of a presequence which contains positive charges, which suggests that the negative-inside electrochemical gradient exerts an electrophoretic pull in order to clear

positively-charged presequences from the import machinery (McBride *et al.*, 1995).

6.3 Energy for transport: Matrix Chaperones and ATP.

Complete translocation of a polypeptide across the inner membrane is known to require ATP hydrolyzing activity of the matrix chaperone, mtHsp70 (Beasley *et al.*, 1992, Gambill *et al.*, 1993, Voos *et al.*, 1993, Stuart *et al.*, 1994). mtHsp70 has a dual role in protein import in which it both facilitates complete unfolding of the cytosolic portion of the polypeptide chain for translocation and drives the actual protein movement across the membrane. mtHsp70 is a member of the Hsp70 family which includes bacterial DnaK and lumenal Bip in the ER (Sanders and Schekman, 1992) and, like these family members, is bound to a co-chaperone homologous to bacterial GrpE, termed Mge1p or Yge1 (Voos *et al*, 1994, Ikeda *et al*, 1994) and the DnaJ homologue Mdj1p (Rowley *et al.*, 1994). mtHsp70 is now considered a genuine component of the translocation machinery since it was found in direct interactions with the inner membrane protein Tim44. mtHsp70 and Tim44 function together as an ATP-dependent "import motor" which pulls the precursor into the matrix in multiple binding and release cycles (Rassow *et al.*, 1994, Schneider *et al*, 1994, Berthold *et al.*, 1995).

Once mtHsp70 has pulled in the imported protein, the chaperone complex acts in concert with the oligomeric mitochondrial matrix chaperonin hsp60, a member of a family of chaperones including the bacterial GroEL and chloroplast RUBISCO chaperones (Reading *et al.*, 1989, Cheng *et al.*, 1989, Hartl *et al.*, 1994). This second class of molecular chaperones is essential in order to facilitate oligomeric assembly and refolding of monomeric proteins (Cheng *et al.*, 1989, Ostermann *et al.*, 1989, Manning-Krieg *et al.*, 1991, Stuart

et al., 1994). All members of this family consist of two stacked rings containing 7 subunits of identical 60 kDa proteins which form a toroid structure with a large central cavity (Hutchinson *et al.*, 1989, Braig *et al.*, 1994). Like bacterial GroEL, mitochondrial hsp60 also requires the regulatory function of a co-chaperone homologue of GroES, called hsp10 (Rospert *et al.*, 1993a, 1993b). Together, hsp60 and hsp10 form a molecular scaffold which assists in ATP dependent folding and sorting of incoming polypeptides whose precise mechanism, like that of all chaperones, remains unclear.

Although hsp70 and hsp60 chaperone involvement in the matrix is well established, there are other chaperone-like proteins which have also been identified in the matrix whose functions may be more specific. For example, a Clp homologue, hsp78, has been identified in yeast and can partially substitute for mt-hsp70 function (Schmitt *et al.*, 1995). Matrix cyclophilin Cpr3p in both yeast and *Neurospora crassa* is also involved in folding probably as a proline isomerase (Matouschek *et al.*, 1995, Rassow *et al.*, 1995).

7. Sorting Pathways.

7.1. Intermembrane space sorting.

To transport nuclear encoded proteins to the matrix, a signal sequence is required which directs transport across both lipid bilayers. Intermembrane space proteins can be sorted in one of two ways: either directly across the outer membrane to the intermembrane space ("unidirectional", Glick *et al.*, 1992a), or a longer route through the matrix via a matrix signals which becomes cleaved upon entrance into the matrix thereby revealing a "conserved" signal which drives export via conserved eubacterial export machinery (the "conservative sorting pathway", Hartl and Neupert, 1990). It was considered that the unidirectional pathway would facilitate newly evolved mitochondrial precursors

and the conservative sorting pathway was reserved for evolutionarily conserved proteins (Hartl and Neupert, 1990).

The first example of conservative sorting to the intermembrane space was the Reiske iron-sulfur protein (FeS) (Hartl *et al.*, 1986). Import of the FeS protein was shown to require hydrolysis of matrix ATP by mt-hsp70 and hsp60 and could be stalled as an intermediate in the matrix compartment (Hartl *et al.*, 1986, Hartl and Neupert, 1990, Kang *et al.*, 1990). There are two other examples, however, in which it is not clear if the precursors are traveling a conservative or non-conservative route. Cytochrome c_1 and cytochrome b_2 contain a bi-partite signal sequence consisting of a matrix-targeting signal followed by an apolar region postulated to comprise the intermembrane space export signal (Sadler *et al.*, 1984, Guiard, 1985, van Loon and Schatz, 1987). It has also been suggested that these proteins follow a unidirectional pathway whereby the apolar region arrests translocation at the level of the inner membrane (stop transfer model, Blobel, 1980, Glick *et al.*, 1992a.). Once arrested (but not inserted into) the inner membrane, signal peptidases on both sides of the membrane cleave the signals resulting in a soluble intermembrane space enzyme (Glick *et al.*, 1992b). Although the conservative sorting pathway would require mt-hsp70 for translocation into the matrix, a requirement for mtHsp70 in cytochrome b2 import seemed to depend on the construct used. Fortunately, this discrepancy has recently ended with a unifying model (Gartner *et al.*, 1995). In this study, a construct whereby the apolar region was deleted, resulted in complete translocation into the matrix. However, if the heme-binding domain was placed at the C-term of the protein, mtHsp70 was unable to unfold it and the protein was incompletely translocated. It was reasoned that the apolar sorting signal arrests translocation at the inner membrane resulting in an early divergence from the matrix sorting pathway. According to their model, the

tightly folded heme-binding domain located on the carboxyl terminal of the apolar region required the co-operation of mtHsp70 to unfold this region in the cytosol.

A critical element missing in the conservative pathway story has been the identification of the conserved protein export machinery. Fusing an *Neurospora crassa* Su9 matrix targeting signal to the cytosolic reporter protein DHFR targets it to the matrix of yeast mitochondria where it is subsequently re-exported (Rojo *et al.*, 1995). In this study, the authors were able to stall the precursor in the matrix prior to export and demonstrated that export required the proton gradient component of the electrochemical potential and ATP (Rojo *et al.*, 1995). Subsequently, it was demonstrated that the export machinery is capable of translocating mitochondrial encoded cytochrome oxidase II fused to passenger proteins such as DHFR (Herrmann *et al.*, 1995). Therefore it can potentially export many nuclear-encoded precursors. The nature of the mitochondrial export machinery will certainly be of great interest to the field, especially since one would predict a system evolutionarily conserved from bacterial export machinery.

7.2. Inner membrane sorting.

An ongoing problem with membrane sorting in the mitochondria concerns the ability of inner membrane proteins to escape detection by the outer membrane such that transmembrane domains pass freely across this lipid bilayer. An obvious difference between inner and outer membrane insertion is the requirement for $\Delta\psi$ as a form of energy, however, it is uncertain what creates the driving force for lateral release into the lipid bilayer. There are two types of proteins destined for insertion into the inner membrane, single-spanning proteins such as the small subunit of cytochrome c-oxidase, COX Va, and

polytopic proteins such as the uncoupling protein, UCP. Proteins such as COX Va are imported via a cleavable N-terminal matrix targeting signal (Glaser *et al.*, 1990) and are unidirectionally inserted into the inner membrane via a stop-transfer domain of high hydrophobicity located in the C-terminal third of the molecule (Miller and Cumsky, 1993). It is generally considered that this hydrophobic region escapes detection by the outer membrane since its N-terminus has already engaged the inner membrane machinery. This machinery may require ATP hydrolysis, however this has not yet been addressed. Polytopic protein import into the inner membrane is poorly understood, as is the energetics of insertion.

8. Perspectives.

The last 5 years have been witness to an explosion in knowledge concerning the mechanisms of protein translocation across the mitochondrial lipid bilayers. The identity of the translocation machineries of both membranes has advanced the field, and the search for mammalian homologues continues. We have learned much about mitochondrial import pathways and, as divergent as they can be, common themes are emerging as their signals become decoded. Yet to be understood in the mitochondrial and other translocation systems is the nature of the driving force for protein unfolding and early steps which commit the polypeptide to unidirectional translocation across the lipid bilayers. The work presented in this thesis addresses these questions in three ways. First, the identity of a transmembrane domain which can act as a signal to direct both initiation of translocation across the lipid bilayer, as well as the abrogation of translocation resulting in the lateral release into the lipid bilayer, suggests that not all productive binding interactions are based on the presence of positively charged helical N-terminal regions. Furthermore, the lateral

release of such a signal-anchor sequence into the lipid bilayer may present a strong driving force for directional import. Secondly, the precise mechanism by which the electrochemical potential across the inner membrane drives initial insertion to the trans side of the inner membrane is investigated, and found to be a function of an electrophoretic effect responsible for clearing positively charged signals from the translocation machinery. Finally, data presented in Chapter 4 suggest that the sequential binding interactions responsible for initiating translocation at the level of outer membrane receptor molecules are also required to discriminate against any productive binding of potentially cryptic mitochondrial signals. This finding emphasizes early binding events as a requirement for directional import.

Note: References included in the Introduction and General discussion (Chapter 5) are combined at the end of the thesis, beginning on page 65.

Chapter 2: A signal-anchor sequence selective for the mitochondrial outer membrane.

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_{in}-C_{cyto} 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), transcription of pSP64 constructs (Nguyen and Shore, 1987), translation of pSP64-derived mRNA in a rabbit reticulocyte cell-free system in the presence of [^{35}S]methionine, and isolation of mitochondria from rat heart (Argan et al., 1983). Additional details are provided in the figure legends.

Recombinant Proteins

A 650-bp *TaqI-BglII* fragment was excised from pSV$_2$DHFR 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'-AGCTATGAAGAGCTTCATTACAAGGAACAAGACAGCCATTTTGGCTGCA and 3'-TACTTCTCGAAGTAATGTTCCTTGTTCTGTCGGTAAAACCG; adaptor II: 5'-GTTGCTGCTACAGGTACTGCCATCGGTGCCTACTATTATTACGCTGCA and 3'-ACGTCAACGACGATGTCCATGACGGTAGCCACGGATGATAATAATGCG). 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)[1], and in which amino acid 15 was changed from threonine to alanine. The plasmid pSP(pOMD29Δ16-29) was created by employing only adaptor I in the manipulations described above. pSP(pOMD29Δ2-10) was formed by deleting adaptor I from pSP(pOMD29) and replacing it with an adaptor (5'-AGCTATGGCCATTTTGGCTGCA and 3'-TACCGGTAAAACCG) that encodes amino acids 1 and 11–15 of pOMD29. Finally, adaptor I of pSP(pOMD29) was replaced with the adaptor, 5-AGCTTATGCAGAGCTTCATTCACAGAACCAGACAGCCATTTTGGCTGCA and 3'-ATACGTCTCGAAGTAATGTGTCTTGGTCTGTCGGTAAAACCG, to yield pSP(pOMD29KR2,7,9Q), in which amino acids 2, 7, and 9 of pOMD29 were replaced by glutamine.

Mitochondrial Import

Reaction mixtures contained 10% (v/v) rabbit reticulocyte lysate transcription-translation products labeled with [^{35}S]methionine, mitochondria (0.5 mg protein/ml), 0.125 M sucrose, 40 mM KCl, 1.0 mM $MgAc_2$, 10.0 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 mM 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 Na_2CO_3, 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 NH_2 terminal sequence of pOMD29 and its mutant derivatives are shown in Fig. 1. As documented elsewhere (Li and Shore, 1992*a*), pOMD29 was imported into the outer membrane of intact rat heart mitochondria in vitro by a process dependent on ATP and protease-sensitive mitochondrial surface components, and in which the orientation of the native OMM70 protein was retained (N_m-C_{cyt}). 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, 1992*a*).

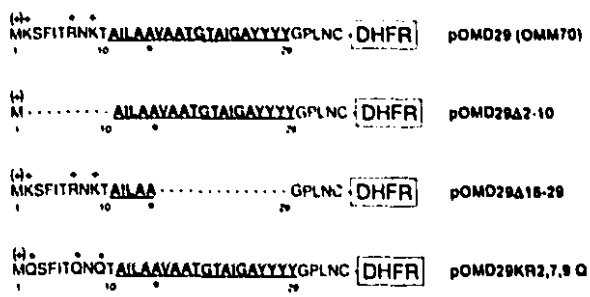

Figure 1. Recombinant proteins. The NH_2-terminal sequences of the proteins encoded by the various recombinant plasmids described in Materials and Methods are shown, using the single-letter code for amino acids. The predicted transmembrane segment (amino acids 11–29, Hase et al., 1984; Nakai et al., 1989) is underlined; •, alanine substitution for threonine at residue 15 of OMM70; (*dashes*) deletions; (*asterisks*) substitutions of lysine and arginine by glutamine; (*DHFR*) murine dihydrofolate reductase (see Materials and Methods).

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° (Fig. 2 *A*, compare lanes *3* and *4*) but much less so at 4° (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°, Fig. 2 *A*) and that which is inserted into the bilayer (30°, 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, 1992*a*).

Targeting and Membrane-Anchor Domains of pOMD29

Deletions were introduced into pOMD29 to remove either

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

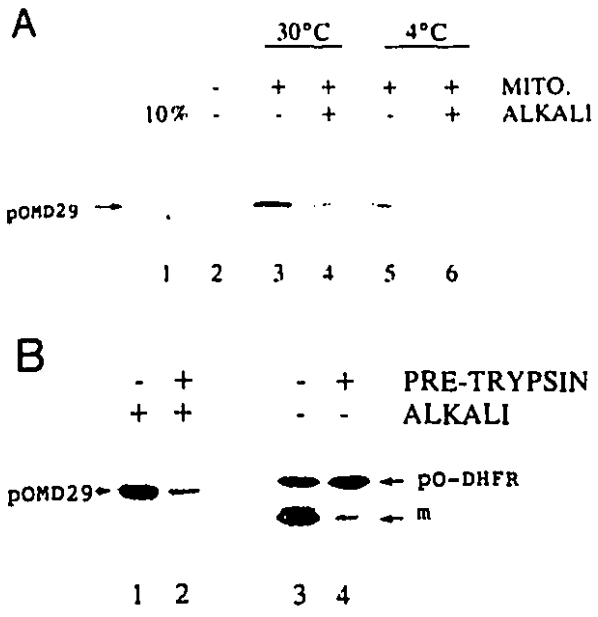

Figure 2. Temperature-dependent and protease-sensitive insertion of pOMD29. (*A*) pSP(pOMD29) was transcribed and translated in the presence of [^{35}S]methionine, and import of pOMD29 into purified rat heart mitochondria (MITO) was carried out at either 30 or 4°C. Aliquots from the reaction mixtures were removed and layered over a cushion of sucrose and centrifuged to recover mitochondria. Pellets were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) either directly (lanes *2*, *3*, and *5*) or after extraction with 0.1 M Na_2CO_3, pH 11.5 (*ALKALI*) (lanes *4* and *6*), and the products visualized by fluorography. (Lane *1*) 10% of input [^{35}S]pOMD29; (lane *2*) [^{35}S]-pOMD29 that sedimented in the absence of mitochondria. (*B*) Before import, mitochondria were treated with trypsin (*PRE-TRYPSIN*) in the presence (lanes *1* and *3*) or absence (lanes *2* and *4*) of excess soybean trypsin inhibitor, exactly as described (Li and Shore, 1992*a*). Import was carried out for [^{35}S]pOMD29 and [^{35}S]pO-DHFR as described in *A* and Fig. 3 *A*, respectively, and mitochondria were recovered and analyzed either directly (lanes *3* and *4*) or after extraction with alkali (lanes *1* and *2*) (*ALKALI*). The positions of pOMD29 and of the precursor and mature (*m*) forms of pO-DHFR are indicated.

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*, pOMD29Δ2-10, 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, pOMD29Δ2-10 required ATP for import (not shown) and was inserted in the N_{in}-C_{cyto} 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 pOMD29Δ2-10, removal of a large portion of the transmembrane segment of pOMD29 resulted in a protein (pOMD29Δ16-19, 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 amphiphilic, 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 α-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, pOMD29Δ2-10, and pOMD29-KR2,7,9Q 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 pOMD29Δ2-10 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 pOMD29Δ2-10, pOMD29KR2,7,9Q was inserted into the outer membrane in the N_{in}-C_{cyto} 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 μM (Fig. 7). This concentration range is very similar to the amount of a synthetic

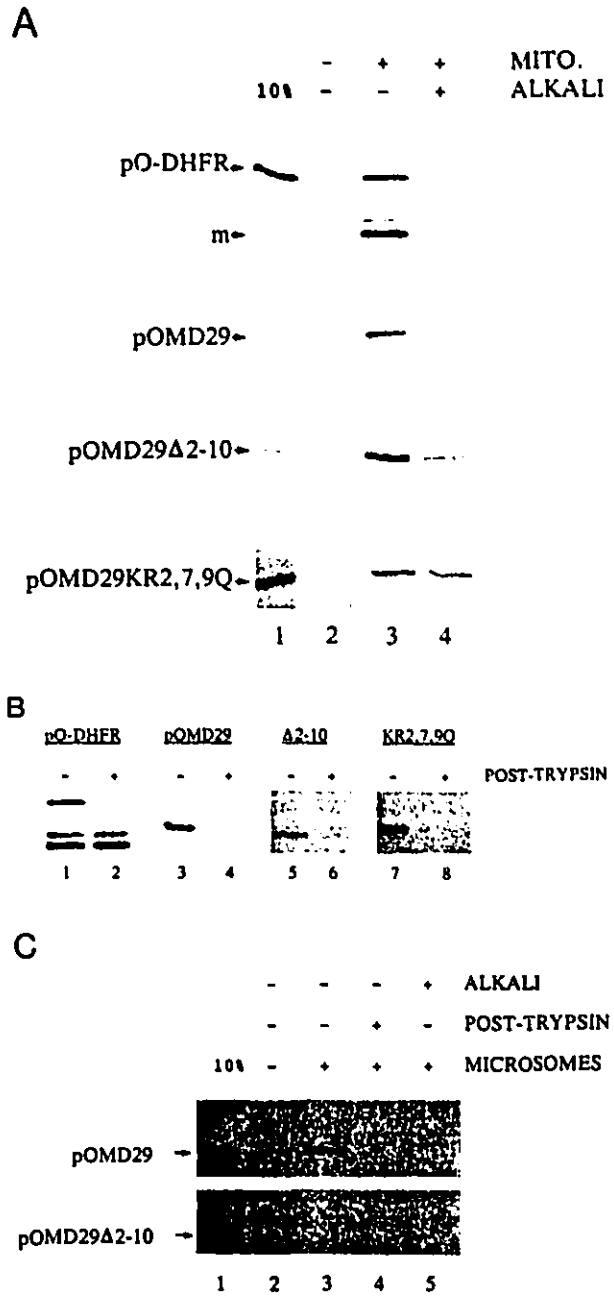

Figure 3. Import of pO-DHFR and normal and mutant forms of pOMD29. (*A*) Translation products containing [^{35}S]pO-DHFR (amino acids 1-36 of preornithine carbamyl transferase fused to DHFR, Skerjanc et al., 1990), [^{35}S]pOMD29, [^{35}S]pOMD29Δ2-10, and [^{35}S]pOMD29KR2,7,9Q were incubated with (lanes *3* and *4*) or without (lane *2*) mitochondria (*MITO*) under standard import conditions. Recovery of mitochondria, extraction with 0.1 M Na_2CO_3, 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 [^{35}S]precursor protein; (lane *2*) [^{35}S]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, 1992*a*). 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).

peptide corresponding to the preornithine carbamyl transferase signal sequence of pO-DHFR that is required to block import of proteins to the matrix under similar conditions (Gillespie et al., 1985). Control experiments (data not shown) revealed that mitochondrial $\Delta\Psi$ was unaffected by the bacterial precursor, as judged by the $\Delta\Psi$-dependent uptake of [^{3}H]triphenylmethylphosphonium (iodine salt) (Bakker, 1978) into mitochondria in the import reaction. In contrast to the competition of import of pO-DHFR by itself, import and insertion of pOMD29 into the outer membrane was relatively unaffected by concentrations of bacterial pO-DHFR up to 6 μM, the highest concentration tested (Fig. 7). This was the case for import at 30° (Fig. 7), and as well for import after combining the two proteins with mitochondria at 4° for 10 min, followed by a chase at 30° (not shown). At higher levels, pO-DHFR tended to aggregate and, therefore, was not examined for its ability to compete for import of pOMD29.

Because the two proteins, pO-DHFR and pOMD29, differ only in their NH_2-terminal topogenic sequence (either 36 amino acids from pre-ornithine carbamyl transferase or 29 amino acids from OMM70 fused to DHFR, respectively), it is presumably these topogenic sequences alone that account for the differences in the competition profiles seen in Fig. 7. It is important to note, however, that the *Neurospora* homolog of OMM70 (MOM72) may employ the same master import receptor on the surface of mitochondria as do proteins that are imported to the matrix (Söllner et al., 1990). We conclude, therefore, that bacterial pO-DHFR at the concentrations examined competes for import of itself but not for pOMD29 because the rate-limiting step for import of pO-DHFR is at a point on the import pathway that is distal to translocation across the outer membrane. Such a distal rate-limiting step may explain why translocation intermediates in transit to the matrix can be detected that simultaneously span both the outer and inner mitochondrial membranes at translocation contact sites (Schleyer and Neupert 1985; Vestweber and Schatz, 1988). pOMD29, on the other hand, may insert into the outer membrane without penetrating into contact sites, a view that is compatible with the recent evidence that the translocation machineries of the two membranes are not permanently coupled (Glick et al., 1991; Pfanner et al., 1992).

Concluding Remarks

Our results indicate that the positively-charged NH_2 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-

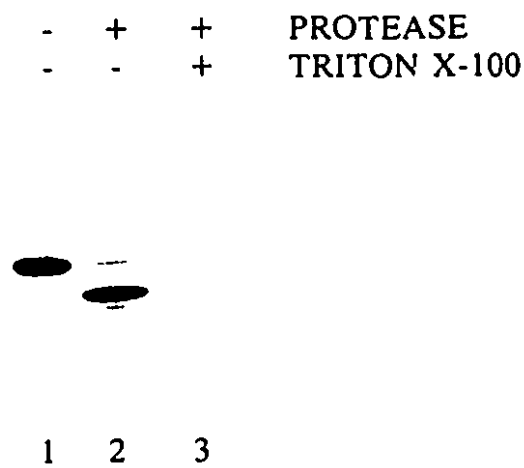

Figure 4. Insertion of HLA-2A into microsomal membrane. Human HLA-A2 cDNA (Ennis et al., 1990) was transcribed and translated in the presence of [^{35}S]methionine and dog pancreas stripped microsomes (Walter and Blobel, 1981). Microsomes were recovered, treated with (lanes *2* and *3*) or without (lane *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.

dent with, or overlaps, the membrane anchor domain. An important consequence of a signal-anchor function is that the domain that specifies targeting and initial translocation across the membrane is also the domain that abrogates this

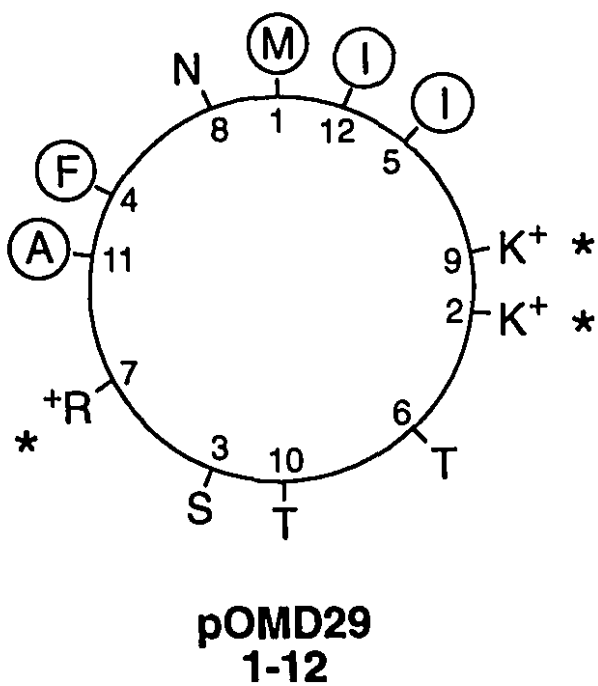

Figure 5. Helical wheel projection of amino acids 1–12 of pOMD29 (OMM70). Asterisks denote residues mutated to glutamine in pOMD29KR2,7,9Q. Hydrophobic amino acids are circled. Amino acids are designated by the single letter code.

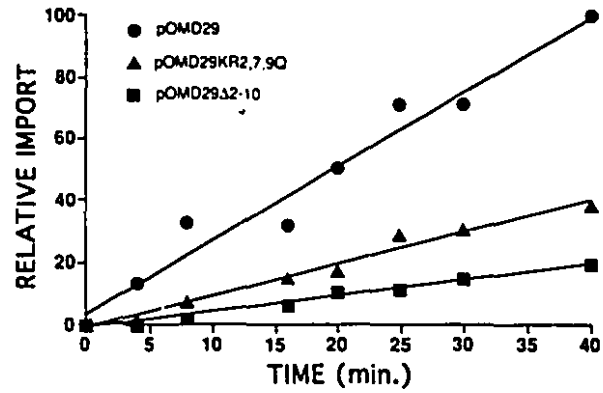

Figure 6. Rates of import and membrane insertion of pOMD29, pOMD29Δ2-10, and pOMD29KR2,7,9Q. Import reactions containing the various [^{35}S]-labeled precursor proteins were carried out for 4, 8, 16, 20, 25, 30, and 40 min., at which times mitochondria were recovered by centrifugation through a sucrose 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 H_2O_2 and 0.7 M NH_4OH, and radioactivity determined by scintillation counting. The input amounts of the three [^{35}S]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).

process and results in release of the segment into the surrounding lipid bilayer (Blobel, 1980; Singer, 1990). If these principles extend to mitochondrial membranes, the presence of a signal-anchor sequence may result in selection of the mitochondrial outer membrane for insertion, simply because this is the first membrane encountered by the incoming precursor protein. Similarly, the combination of a matrix-targeting signal followed immediately by a stop-transfer domain may also select the outer membrane for insertion if, again, the stop-transfer segment enters the outer membrane translocation machinery and abrogates translocation before the protein is committed for import into the interior of the organelle (Nguyen et al., 1988; Singer and Yaffe, 1990). It is interesting in this regard that the *Neurospora* homolog of OMM70, MOM72, may employ the same import receptor as do matrix-destined proteins (Söllner et al., 1990). What we have demonstrated, however, is that the transmembrane segment of pOMD29 specifies targeting and insertion, whereas the positively-charged NH_2-terminus affects only the efficiency of this process. When amino acids 1–10 of pOMD29 were replaced with a strong matrix-targeting signal (from preornithine carbamyl transferase), the protein was inserted into the outer membrane, but in an inverted orientation compared to pOMD29 (Li and Shore, 1992*b*). Both MOM72 (Steger et al., 1990) and MOM19 (Schneider et al., 1991), which are inserted into the outer membrane of *Neurospora* mitochondria with the same topology as pOMD29 (OMM70), lack a basic region upstream of the predicted transmembrane segment. It remains to be determined if the predicted transmembrane segments in these proteins, which are located at (MOM19) or toward (MOM72) the NH_2 terminus, function as signal-anchor sequences. Finally, certain proteins destined for the mitochondrial inter-

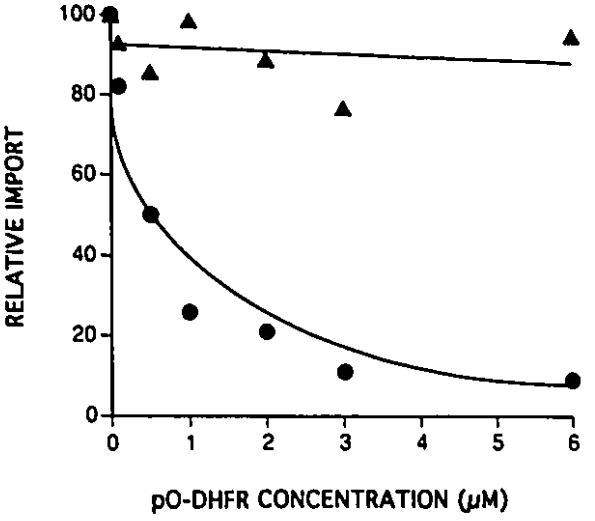

Figure 7. Insertion of pOMD29 into the mitochondrial outer membrane in the presence of bacterial-expressed pO-DHFR. pO-DHFR was expressed in bacteria, purified, and kept in 10 mM Hepes, pH 7.4, 7 M urea, and 1.0 mM dithiothreitol until use (Sheffield et al., 1990). Various concentrations of pO-DHFR in this mixture were rapidly diluted 50-fold into standard import reactions containing [^{35}S]pO-DHFR or [^{35}S]pOMD29 obtained by transcription-translation in reticulocyte lysate, and mitochondria were added to initiate import. After 10 min., reactions containing [^{35}S]pOMD29 were subjected to alkali extraction (Materials and Methods) and those containing [^{35}S]pO-DHFR were treated with trypsin (Li and Shore, 1992*a*). The products were recovered, analyzed by SDS-PAGE and fluorography, and the relative amounts of alkali-resistant pOMD29 and trypsin-resistant, processed pO-DHFR were quantified by laser densitometry (LKB2202 UltroScan) of bands on exposed x-ray film. Values obtained in the absence of bacterial pO-DHFP were arbitrarily set at 100. ●, [^{35}S]pO-DHFR; ▲, [^{35}S]pOMD29.

membrane space contain an apolar segment located toward the NH_2-terminus, immediately downstream of a matrix-targeting signal. However, whereas these sequences contribute to sorting, they probably do not function as membrane anchor sequences, i.e., these proteins do not appear to become embedded, even transiently, into the lipid bilayer (Glick et al., 1992; Koll et al., 1992). Characteristics other than simple hydrophobicity alone, therefore, contribute to the signals that specify sorting to the outer membrane and intermembrane space.

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Chapter 3: Insertion of an uncharged polypeptide into the mitochondrial inner membrane does not require a trans-bilayer electrochemical potential: effects of positive charges.

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Insertion of an uncharged polypeptide into the mitochondrial inner membrane does not require a trans-bilayer electrochemical potential: effects of positive charges

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Abstract

Mitochondria with a ruptured outer membrane exhibited impaired import into this membrane of an outer membrane fusion protein containing the signal-anchor sequence of Mas70p. However, the Mas70p signal-anchor efficiently targeted and inserted the protein directly into exposed regions of the inner membrane. Import into the inner membrane was dependent on $\Delta\psi$ and this dependence was due to the presence of the positively-charged amino acids located at positions 2, 7, and 9 of the signal-anchor. In contrast to wild-type signal-anchor, mutants lacking the positively-charged residues mediated import into the inner membrane in both the presence and absence of $\Delta\psi$. The results suggest two conclusions: (1) $\Delta\psi$-dependent import of the signal-anchor sequence was due exclusively to an effect of $\Delta\psi$ on the positively-charged domain of the signal-anchor, rather than to an effect of $\Delta\psi$ on a property of the inner membrane import machinery; (2) in the absence of $\Delta\psi$, the positively-charged domain of the signal-anchor *prevented* the otherwise import-competent signal-anchor from inserting into the membrane. This suggests that the positively-charged domain leads import across the inner membrane, and that $\Delta\psi$ is required to vectorially clear this domain in order to allow the distal region of the signal-anchor to enter the translocation pathway. The implications of these findings on the mechanism of import into the mitochondrial inner membrane and matrix are discussed.

Keywords: Polypeptide; Import mechanism; Mitochondrial inner membrane; Electrochemical potential, transbilayer

1. Introduction

The step on the mitochondrial protein import pathway that involves translocation of the polypeptide chain into or across the inner membrane has been shown to require two primary sources of energy: matrix ATP [1–3] and the transmembrane electrochemical potential [4–6]. ATP is employed by mtHsp70 in the matrix to fuel a binding and release cycle that enables the chaperone to drive import of the incoming polypeptide from the *trans* side of the membrane [7–9]. The electrochemical potential, on the other hand, is required to initiate translocation of the polypeptide, but is no longer essential once the polypeptide establishes interaction with mtHsp70 [6,7]. Several studies have demonstrated that it is the positively-charged matrix-targeting signal of the precursor protein that first moves across the inner membrane [6,10,11], and that it does so by responding to the electrical component, $\Delta\psi$ (inside negative), of the total transmembrane proton motive force [12,13]. An obvious role for $\Delta\psi$, therefore, is to impose a voltage-driven electrophoretic motive force on the positively-charged signal sequence, thereby driving vectorial translocation of this domain across the membrane to the next step of the import pathway, which is the binding of the precursor polypeptide to mtHsp70. Consistent with this 'electrophoresis model' are the findings that different presequences exhibit different thresholds for inhibition of translocation by the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), i.e., $\Delta\psi$ did not have an all or nothing effect which might have been expected, for example, if $\Delta\psi$ controlled a voltage-gated import channel inde-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; pOCT, pre-ornithine carbamyl transferase; DHFR, dihydrofolate reductase; PAGE, polyacrylamide gel electrophoresis; RO-mitochondria, mitochondria with a ruptured outer membrane; TPMP, triphenylmethylphosphonium.

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pendently of its electrophoretic effect on the signal sequence [13]. These two possibilities, that $\Delta\psi$ acts either on the substrate or on the membrane machinery, however, are not mutually exclusive since $\Delta\psi$ could contribute to the function of one or more components of the inner membrane import machinery, but at a threshold which is below the level of $\Delta\psi$ that drives the movement of the signal sequence.

To further address the role of $\Delta\psi$ in protein import, we have taken advantage of the earlier findings in yeast that exposed regions of the inner membrane in mitoplasts are capable of direct and efficient import of proteins to the matrix [14,15]. This has also been observed in mammalian mitochondria, where it has been extended as well to direct insertion of integral proteins into the inner membrane [16]. Partial disruption of the outer mitochondrial membrane, therefore, allows for delivery to the inner membrane of a precursor protein which otherwise would not encounter the inner membrane during normal import into the intact organelle. One such protein is a fusion protein in which DHFR is linked to the signal-anchor sequence of the yeast outer mitochondrial membrane protein Mas70p. This topogenic domain is normally responsible for targeting and inserting Mas70p into the outer membrane in the N_{in}-C_{cyto} orientation, leaving the bulk of the polypeptide facing the cytosol [16,18]. The three positively-charged amino acids which are located in the hydrophilic NH_2-terminal region of the signal-anchor are not required for import, but rather they cooperate with the transmembrane portion (amino acids 11–29) of the signal-anchor to enhance the overall rate of import [19]. Here, we demonstrate that mitochondria with a ruptured outer membrane lose the ability to integrate the Mas70p signal-anchor into the outer membrane. Rather, the outer membrane is bypassed and this domain efficiently inserts into the inner membrane. Insertion is dependent on $\Delta\psi$ and this dependence is due to the positively-charged amino acids within the NH_2-terminal hydrophilic domain of the signal-anchor, rather than to an effect of $\Delta\psi$ on the inner membrane import machinery. We present evidence that the positively-charged residues prevent the *$\Delta\psi$-independent* insertion of the transmembrane domain, suggesting that the positively-charged residues must otherwise be vectorially cleared from the import machinery by $\Delta\psi$ to allow subsequent translocation of the distal portion of the signal-anchor.

2. Experimental procedures

2.1. General

Previous articles describe the standard procedures used in this study. These include in vitro transcription of recombinant plasmids [20], translation of the resulting mRNA in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine [20], import of radiolabelled recombinant proteins into mitochondria [21], and analysis of import products by SDS-PAGE and fluorography [21]. Additional details are provided in the figure legends.

2.2. Mitochondria

For isolation of intact mitochondria, livers from 200–250 g male Sprague-Dawley rats were minced and washed in ice-cold HIM (220 mM mannitol, 70 mM sucrose, 10 mM Hepes, pH 7.5, 1 mM ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, and 2 mg/ml de-lipidated bovine serum albumin). The minced liver (3 g wet weight) was gently homogenized in 9 ml HIM in a Wheaten Dounce glass homogenizer using two complete up and down cycles of a glass 'B'-type pestle. The homogenate was diluted 2-fold with HIM and centrifuged at 4°C for 10 min at $600 \times g$ in a Sorval SS34 rotor. The supernatant was recovered, centrifuged at $7000 \times g$ for 15 min, and the pellet resuspended to the original homogenate volume in HIM minus albumin. After centrifuging at $600 \times g$, mitochondria were recovered from the supernatant by centrifuging at $7000 \times g$ for 15 min. The mitochondria were uniformly suspended in 0.5 ml MRM (250 mM sucrose, 10 mM Hepes, pH 7.4, 1 mM ATP, 5 mM Na succinate, 0.08 mM ADP, 1 mM dithiothreitol, and 2 mM K_2HPO_4, pH 7.4). Alternatively, mitochondria with a ruptured outer membrane were prepared as above, except that albumin was excluded at all stages and liver tissue was homogenized in a motorized Potter Elvehjem homogenizer using four up and down cycles with a tight-fitting teflon pestle operating at 400 rpm.

2.3. Liposomes

Liposomes were prepared by extrusion [38,39] from 40:45:12.5:2 (molar proportions, based on phosphorus) bovine heart phosphatidycholine, phosphatidylethanolamine and cardiolipin plus *N*-(biotinylaminocaproyl)phosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL). For import, liposomes were diluted 1000-fold into MRM (10 μM phospholipid final concentration) and added to a standard import reaction, after which the liposomes were aggregated by addition of avidin (0.20 nmol/nmol biotinyl groups) and incubation for 30 min on ice. The reaction mixture was diluted 8-fold in 80 mM KCl, 10 mM Hepes, pH 7.4, and 2 mM Mg acetate and the cross-linked liposomes recovered by centrifugation at $12000 \times g$ for 10 min.

3. Results

As described in Experimental procedures, mitochondria from rat liver were isolated either with an intact outer membrane or with an outer membrane that was ruptured. The latter were designated RO-mitochondria (mitochondria

with a *R*uptured *O*uter membrane). In contrast to mitoplasts, in which the outer membrane has largely been stripped from the organelle [23], RO-mitochondria retained 60–80% of the outer membrane, as judged by immunoblot analysis employing an antibody against the outer membrane marker, monoamine oxidase A (not shown). Electron microscopy revealed that the remaining outer membrane was similar in morphology to that of intact mitochondria (i.e., a right-side out sheet), but contained gaps that left regions of the inner membrane exposed to the exterior of the organelle (not shown).

When analyzed for import of a matrix precursor protein, little difference was observed between intact mitochondria and RO-mitochondria. If anything, the latter were more effective than the intact organelle (Fig. 1). The precursor protein that was used in this analysis, pODHFR, contained the matrix-targeting signal of pre-ornithine carbamyl transferase (pOCT) fused to DHFR (Fig. 1). For both intact and RO-mitochondria, uptake and processing of pODHFR (Fig. 1, lanes 3 and 9) was abolished by CCCP (lanes 4 and 10) due to the dependence of this matrix precursor protein on $\Delta\psi$ for import across the inner membrane. In contrast to the full-length precursor outside the organelle, the processed product was protected against exogenous proteinase (not shown) (see Ref. [19]). Import of pODHFR was largely prevented by lowering the temperature of the import reaction to 4°C (lanes 5 and 11) or by pretreating the organelle with trypsin prior to import (lanes 6 and 12). Trypsinization of intact and RO-mitochondria had no effect on the electrochemical potential, as judged by the uptake of [^{3}H]TPMP (not shown, Ref. [24,25]). Although the route of import of pODHFR into RO-mitochondria was not examined in this study, earlier studies employing rat heart mitochondria revealed that pOCT was capable of being imported directly via exposed regions of the inner membrane [16].

In contrast to the results obtained for pODHFR, major differences between intact and RO-mitochondria were observed when examined for import of an integral protein of the outer membrane. This protein was constructed by

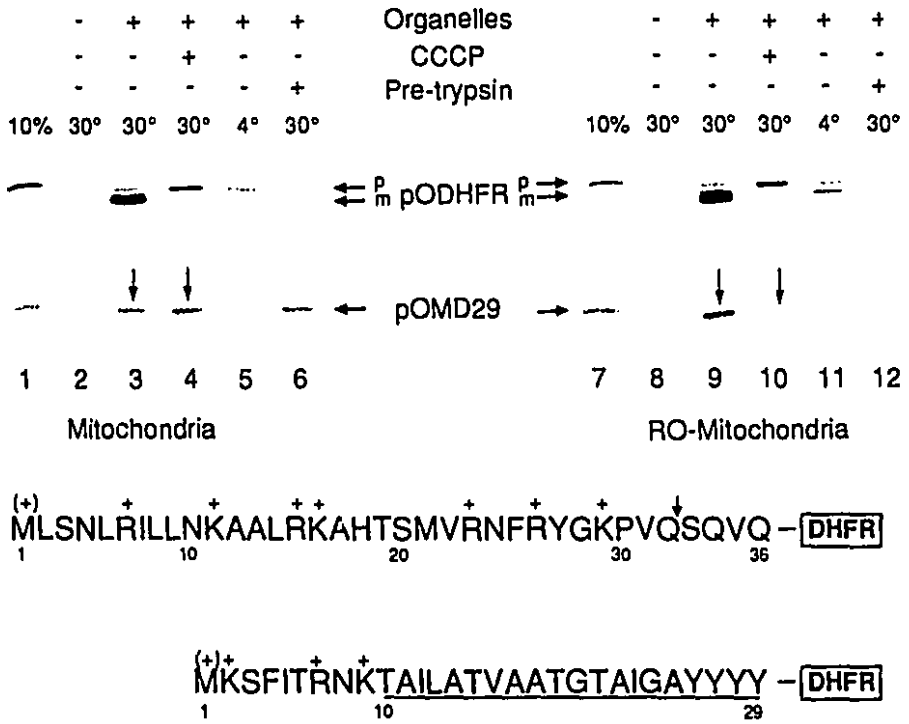

Fig. 1. Insertion of the outer membrane protein, pOMD29, into the inner membrane is dependent on $\Delta\psi$. Import reactions included either [^{35}S]pODHFR (upper panels) or [^{35}S]pOMD29 (lower panels) and were carried out in the presence of intact mitochondria (lanes 2–6) or mitochondria containing a ruptured outer membrane (RO-mitochondria) (lane 8–12) at a concentration of 0.5 mg organellar protein/ml, for 30 min at 4°C or 30°C, in the presence (lanes 4 and 10) or absence (lanes 2, 3, 5, 6 and lanes 8, 9, 11, 12) of 1 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). In certain cases, the organelles were first treated with 0.125 mg/ml trypsin for 20 min, on ice, at which time 1.25 mg/ml soybean trypsin inhibitor [19] was added, and import performed (Pre-trypsin, lanes 6 and 12). After import, reaction mixtures (50 μl) were layered over 0.5 ml 250 mM sucrose in a 1.5 ml eppendorf tube, the organelles recovered by centrifugation and analyzed by SDS-PAGE and fluorography, either directly (pODHFR) or following extraction in 0.1 M Na_2CO_3, pH 11.5 [16,19] (pOMD29). Lanes 1 and 7, 10% of input radiolabelled protein. Arrows denote pOMD29 and the precursor (*p*) and processed (*m*) forms of pODHFR. At the bottom of the figure are shown the sequences (single letter amino acid code) of the NH_2-terminal topogenic domains of pODHFR (upper) and pOMD29 (lower), fused to amino acids 4–186 of mouse dihydrofolate reductase (DHFR). Arrow, processing site of pODHFR; underline, predicted transmembrane segment of pOMD29; plus signs, positively-charged amino acids.

replacing the pOCT matrix-targeting signal of pODHFR with the NH_2-terminal 29 amino acid signal-anchor sequence of yeast Mas70p (Fig. 1). Targeting and insertion of the resulting fusion protein, pOMD29 [16] into the outer membrane of intact mitochondria in vitro has been extensively characterized [16,19,26–28]. Like Mas70p [17,29], pOMD29 is inserted into the outer membrane in the N_{in}-C_{cyto} orientation via the predicted transmembrane domain of the signal-anchor (amino acids 11–29, Fig. 1), leaving the bulk of the protein exposed to the cytosol. Import of pOMD29 was assayed by the acquisition of resistance to extraction of the protein from the organelle at alkaline pH [16,19]. For both intact and RO-mitochondria, resistance to alkaline extraction was dependent on the presence of the organelle in the import reaction (Fig. 1, compare lanes 3 and 2, and lanes 9 and 8) and was temperature-sensitive (lanes 5 and 11). However, whereas import of pOMD29 into the outer membrane of intact mitochondria was, as expected, unaffected by the collapse of $\Delta\psi$ by CCCP (compare lanes 3 and 4, see also Refs. [16,19,26]), import into RO-mitochondria was reduced by CCCP to background levels (lanes 8–10, see also Ref. [16]). Moreover, pretreatment of RO-mitochondria with trypsin severely inhibited subsequent import of pOMD29 (lanes 9 and 12), whereas treatment of intact mitochondria at this concentration of trypsin had very little effect (lanes 4 and 6). This suggests that different receptors, exhibiting different availabilities to exogenous proteinase, were recognized by pOMD29 at the outer and inner membrane, respectively. Like import of pOMD29 into the outer membrane of intact mitochondria, however, pOMD29 was inserted into RO-mitochondria in the N_{in}-C_{cyto} orientation (not shown).

Based on the finding that CCCP prevented import of pOMD29 into RO-mitochondria, we conclude that the outer membrane was largely by-passed during import and that pOMD29 was inserted into the inner membrane. Import into the inner membrane is defined here operationally as $\Delta\psi$-dependent acquisition of resistance to extraction by alkali, since all proteins examined to date that enter or cross the inner membrane require $\Delta\psi$ for import whereas $\Delta\psi$ is not required for protein insertion into the outer membrane [30,31]. The fact that RO-mitochondria contain areas in which the outer membrane is intact, yet is largely incapable of importing pOMD29 into this membrane, suggests that the import capacity of the outer membrane has been severely compromised in RO-mitochondria. Similar observations have been made for impaired import of porin into ruptured outer membrane of yeast mitochondria. Failure to import into the ruptured outer membrane may have been due to loss of intermembrane space proteins during isolation of the organelle [32].

3.1. Import competition of pOMD29

The $\Delta\psi$-dependent import of pOMD29 into the inner membrane of RO-mitochondria was examined for competition by a synthetic peptide corresponding to amino acids 1–27 of the pOCT matrix-targeting signal [25] (Fig. 2). This peptide functions in a manner very similar to bacterially-expressed pODHFR in competing for import of both pOCT and an integral protein of the inner membrane, UCP, in intact mitochondria [33]. The rate-limiting step of import that is competed by matrix-targeting signal peptides, however, likely resides at the level of the inner membrane [34]. As shown in Fig. 2, the profile of competition of pOMD29 into RO-mitochondria was very similar to that exhibited by UCP and, therefore, the two proteins shared a common or overlapping pathway into the inner membrane. Competition for import of pODHFR was less than that observed for pOMD29 and UCP at relatively low concentrations of the synthetic pOCT signal peptide (Fig. 2), perhaps reflecting different translocation efficiencies for the different imported proteins. The electrochemical potential in RO-mitochondria was unaffected over the concentration range of peptide that was employed (0.2–2.0 μM).

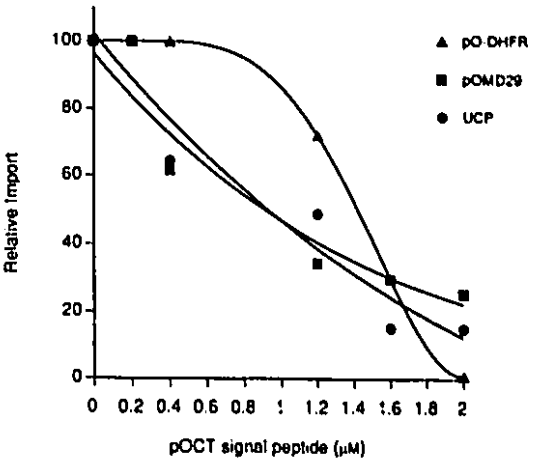

Fig. 2. Competition for pOMD29 import into the mitochondrial inner membrane by a synthetic matrix-targeting signal. Solution containing 7 M urea, 10 mM Hepes, pH 7.4, and various concentrations of the synthetic peptide, pO(1–27)cys [38] were flash diluted 25-fold into standard import reactions containing RO-mitochondria (0.5 mg protein/ml), and [^{35}S]pODHFR (▲), [^{35}S]pOMD29 (■), or [^{35}S]UCP (●). The final concentration of peptide (0–2.0 μM) is indicated. After 15 min at 30°C, RO-mitochondria were recovered and analyzed by SDS-PAGE and fluorography, either directly (pODHFR) or following alkali-extraction (pOMD29 and UCP) (see Fig. 1). Bands corresponding to alkali-resistant pOMD29 and UCP and to the processed form of pODHFR were quantified using a FUJI BAS 2000 bioimager, employing as baseline the product obtained for import in the presence of CCCP. Results obtained in the absence of peptide were arbitrarily set at 100.

3.2. Mutants of pOMD29

In Fig. 3, the effect of modifying charged amino acids in the pOMD29 signal-anchor sequence on $\Delta\psi$-dependent import of the protein into RO-mitochondria was examined. The charged residues were removed either by deleting

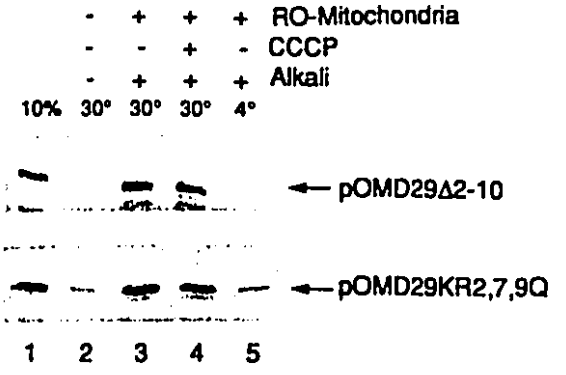

Fig. 3. Import of pOMD29Δ2–10 and pOMD29KR2,7,9Q into the inner membrane does not require $\Delta\psi$. Translation mixtures containing [^{35}S]pOMD29Δ2–10 (arrow, upper panel) or [^{35}S]pOMD29KR2,7,9Q (arrow, lower panel) were incubated with (lanes 3–5) or without (lane 2) RO-mitochondria, in the presence (lane 4) or absence (lanes 2, 3, 5) of CCCP, under standard import conditions for 30 min at the indicated temperature. Analysis was as described in Fig. 1. Lane 1, 10% of input radiolabelled protein.

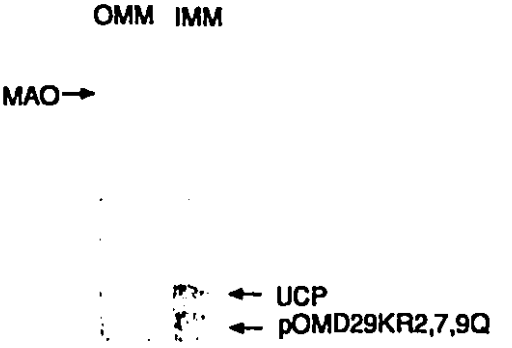

Fig. 4. Sucrose density gradient sedimentation. Reticulocyte lysate containing both [^{35}S]UCP and [^{35}S]pOMD29KR2,7,9Q was combined with RO-mitochondria, and a standard import reaction was conducted. The RO-mitochondria were recovered, subjected to sonication in hypo-osmotic medium, and outer (OMM) and inner (IMM) fractions obtained following separation in a sucrose density gradient, exactly as described in Ref. [16]. Aliquots of the fractions were subjected to SDS-PAGE and the gels analyzed either by immunoblotting with monospecific antibody against monoamine oxidase B (upper panels), or by fluorography (lower panels). OMM and IMM containing fractions are shown, and positions of monoamine oxidase A (MAO), UCP, and pOMD29KR2,7,9Q are indicated by arrows.

amino acids 2–10 (pOMD29Δ2–10) or by converting residues at positions 2, 7, and 9 to glutamine (pOMD29KR2,7,9Q) [19] (see Fig. 1). Earlier studies using intact mitochondria revealed that the positively-charged residues in the signal-anchor are not essential for protein targeting and insertion into the outer membrane, but rather that they cooperate with the transmembrane domain to enhance the overall rate of import [19]. For RO-mitochondria, import of pOMD29Δ2–10 and pOMD29-KR2,7,9Q was dependent on the presence of the organelle (Fig. 3, lanes 2 and 3) and was temperature-sensitive (lanes 4 and 5). However, in contrast to the wild-type signal-anchor (Fig. 1), the mutants forms of pOMD29 exhibited targeting and insertion that was no longer dependent on the electrochemical potential (Fig. 3, lanes 3 and 4). After import into RO-mitochondria, both mutant proteins were accessible to exogenous proteinase following import into RO-mitochondria (not shown), indicating that they had been inserted in the N_{in}-C_{cyto} orientation (not shown).

To examine the membrane location(s) of pOMD29KR2,7,9Q in RO-mitochondria, fractions enriched in outer or inner membranes were obtained by sonication of RO-mitochondria, and resolution by sucrose density gradient centrifugation [16]. As shown in Fig. 4, pOMD29KR2,7,9Q was recovered predominantly in the inner membrane fraction, together with the inner membrane marker, UCP. Conversely, pOMD29KR2,7,9Q and

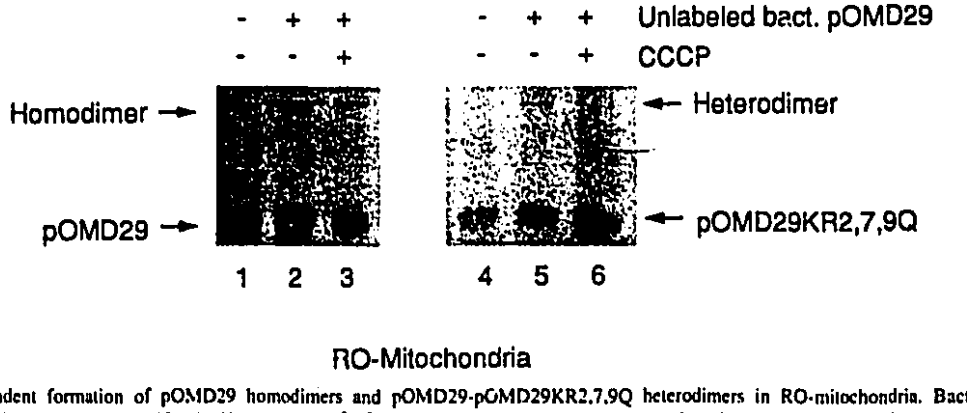

Fig. 5. $\Delta\psi$-dependent formation of pOMD29 homodimers and pOMD29-pOMD29KR2,7,9Q heterodimers in RO-mitochondria. Bacterial-expressed pOMD29 was purified in 7 M urea, 10 mM Hepes, pH 7.4 [33] and diluted 25-fold into import reactions (lanes 2, 3, 5, 6; final concentration = 0.5 μg protein/ml) containing RO-mitochondria and either [^{35}S]pOMD29 (arrow, lanes 1–3) or [^{35}S]pOMD29KR2,7,9Q (arrow, lanes 4–6). Lanes 1 and 4, urea solution minus bacterial pOMD29; lanes 3 and 6, import in the presence of CCCP. After 30 min at 30°C, RO-mitochondria were recovered, suspended and subjected to chemical cross-linking with bismaleimidohexane, as described in Ref. [27]. The products were analyzed as described in Fig. 1. Dimers are indicated by arrows.

UCP were barely detectable in the outer membrane fraction, whereas the outer membrane marker, monoamine oxidase, was enriched here (Fig. 4). The major location of pOMD29KR2,7,9Q in RO-mitochondria, therefore, was the inner membrane.

Earlier studies showed that pOMD29 is capable of forming homodimers following insertion into the outer membrane, mediated by specific helical packing between transmembrane segments [27,28]. When redirected to the inner membrane [16], pOMD29 is also capable of forming homodimers. Since insertion of wild-type pOMD29 into the inner membrane of RO-mitochondria depends on $\Delta\psi$ (Fig. 1), it follows that the formation of heterodimers between pOMD29 and pOMD29KR2,7,9Q in the inner membrane will also depend on $\Delta\psi$, despite the fact that insertion of the mutant is $\Delta\psi$-independent. To examine this possibility, [^{35}S]pOMD29KR2,7,9Q was co-imported into RO-mitochondria with non-radioactive pOMD29 purified from bacteria, at concentrations of the latter that stimulate the formation of dimers but are below the level required to compete for import (for details, see Refs. [27,28]). The formation of pOMD29 and [^{35}S]pOMD29 homodimers was sensitive to CCCP (Fig. 5, lanes 2 and 3), as was the formation of pOMD29 and [^{35}S]pOMD29KR2,7,9Q heterodimers (lanes 5 and 6). This suggested that pOMD29 and pOMD29KR2,7,9Q resided within the same $\Delta\psi$-dependent membrane compartment of RO-mitochondria. Similar results were observed for pOMD29Δ2–10 (not shown). Together with the results from sucrose gradient sedimentation (Fig. 4), these findings demonstrated that the mutant proteins were imported into the inner membrane, even though they lacked a requirement for $\Delta\psi$.

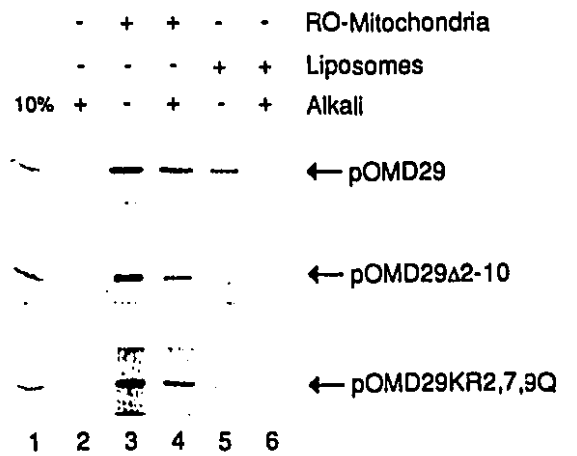

Fig. 6. Import into RO-mitochondria versus liposomes. Translation products containing [^{35}S]pOMD29 (top panel), [^{35}S]pOMD29Δ2–10 (middle panel), or [^{35}S]pOMD29KR2,7,9Q (bottom panel) were incubated without membranes (lane 2), with RO-mitochondria (0.5 mg/ml protein and 0.17 μM phospholipid) (lanes 3 and 4), or with Liposomes (2.5 μM phospholipid) (lanes 5 and 6), under standard import conditions for 30 min at 30°C. RO-mitochondria and liposomes were recovered and analyzed either directly (lanes 3 and 5) or following alkali extraction (lanes 4 and 6), as described in Fig. 1 and experimental procedures. Lane 1, 10% of input radiolabelled protein. Arrows denote the precursor proteins.

To rule out the possibility that the observed import of pOMD29Δ2–10 or pOMD29KR2,7,9Q into the inner membrane of RO-mitochondria was the result of non-specific insertion of the polypeptides directly into the lipid bilayer, the association of these mutant proteins was examined with liposomes whose phospholipid composition was similar to that of the inner membrane [35]. As documented previously, the liposomes contained biotinylated phosphatidylethanolamine which facilitated quantitative recovery of the liposomes from import reactions by cross-linking with avidin [22]. Liposomes were included in standard import reactions at a concentration of lipid that was about 15-times greater than the lipid content of the RO-mitochondria used in these assays. Compared to RO-mitochondria, the liposomes exhibited reduced binding of both the wild-type and mutant pOMD29 protein (Fig. 6, lanes 3 and 5). Moreover, virtually all of the protein that was recovered with the liposomes was extracted with alkali (Fig. 6, lanes 4 and 6), indicating that it had been associated peripherally with the liposome surface rather than integrating into the bilayer.

4. Discussion

We have found that a ruptured outer membrane severely compromised insertion of the Mas70p signal-anchor into the regions of this membrane that remained associated with the organelle and appeared otherwise normal morphologically. One explanation for this is that exposed regions of the inner membrane were far more efficient at importing pOMD29 than was the available outer membrane. A more likely possibility, however, may be that the integrity of the outer membrane import machinery was lost in RO-mitochondria, perhaps due to loss of a critical component during the isolation of the RO-mitochondria [11]. This interpretation is consistent with the findings that isolated outer membrane vesicles are relatively inefficient in integrating outer membrane proteins compared to the intact organelle (Refs. [36,37], and unpublished data). Nevertheless, properties of the Mas70p signal-anchor that relate both to its transmembrane segment and its positively-charged hydrophilic domain allowed the signal-anchor to by-pass the outer membrane of RO-mitochondria, and effectively target the inner membrane.

Insertion of the Mas70p signal-anchor into the inner membrane of RO-mitochondria followed the same import pathway as proteins native to the inner membrane and matrix, indicating that this otherwise outer membrane signal-anchor is recognized by the inner membrane translocation machinery, with a dependence on $\Delta\psi$ (inside negative). The charge-deficient mutants of the Mas70p signal-anchor were also integrated into the inner membrane, but

without a dependence on $\Delta\psi$. The requirement for $\Delta\psi$, therefore, was a property of the positively-charged domain of the signal-anchor sequence rather than an effect of $\Delta\psi$ on, for example, a component of the translocation machinery. This finding is consistent with the suggestion that $\Delta\psi$ exerts its effect by imposing an electrophoretic motive force on positively charged amino acids, resulting in vectorial conductance of the domain through the predicted translocation pore [13]. Once the translocating polypeptide chain establishes contact with mthsp70 on the *trans* side of the inner membrane, subsequent dependence on $\Delta\psi$ is overcome [7].

Despite the fact that the positive charge deficient mutants of the Mas70p signal-anchor were capable of insertion into the inner membrane in the absence of $\Delta\psi$, the wild-type signal-anchor was not. Thus, the positively-charged residues at positions 2, 7, and 9 of the signal-anchor *prevent* $\Delta\psi$-independent import, and this inhibition overrides the ability of the rest of the signal-anchor to independently insert into the membrane. One interpretation of this finding is that positively-charged residues within the signal-anchor sequence precede the transmembrane segment into the predicted translocation pore of the inner membrane, and that $\Delta\psi$ is required to vectorially clear these residues to the matrix, thereby allowing the adjacent transmembrane portion of the signal-anchor to enter the translocation pathway. This is consistent with the suggestions that an essential component of the inner membrane translocation machinery, MIM44, recognizes positively-charged targeting signals ([40], reviewed in [41]). $\Delta\psi$-dependent release of positively-charged residues from interactions with proteins such as MIM44 within the translocation pore might constitute a rate-limiting step during normal import into or across the inner membrane.

Acknowledgements

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Chapter 4: The human mitochondrial import receptor, hTom20p, prevents a cryptic matrix targeting sequence from gaining access to the protein translocation machinery.

Abstract

Yeast Mas70p and NADH cytochrome b_5 reductase are bitopic integral proteins of the mitochondrial outer membrane, and are inserted into the lipid-bilayer in an N_{in}-C_{cyto} orientation via an NH_2-terminal signal-anchor sequence. The signal-anchor of both proteins is comprised of a short positively-charged domain followed by the predicted transmembrane segment. The positively-charged domain is capable of functioning independently as a matrix-targeting signal in yeast mitochondria in vitro but does not support import into mammalian mitochondria (rat or human). Rather, this domain represents a cryptic signal that can direct import into mammalian mitochondria only if proximal components of the outer membrane import machinery are removed. This can be accomplished either by treating the surface of the intact mitochondria with trypsin or by generating mitoplasts. The import receptor Tom20p (Mas20p/MOM19) is responsible for excluding the cryptic matrix-targeting signal from mammalian mitochondria since replacement of yeast Tom20p with the human receptor confers this property to the yeast organelle, while at the same time maintaining import of other proteins. In addition to contributing to positive recognition of precursor proteins, therefore, the results suggest that hTom20p may also have the ability to screen potential matrix-targeting sequences, and exclude certain proteins that would otherwise be recognized and imported by distal components of the outer and inner membrane protein translocation machinery. These findings also indicate, however, that cryptic signals, if they exist within otherwise native precursor proteins, may remain topogenically silent until the precursor successfully clears hTom20p, at which time the activity of the cryptic signal is manifested and can contribute to subsequent translocation and sorting of the polypeptide.

Introduction

Biochemical and genetic studies in yeast and Neurospora have identified a number of proteins within the mitochondrial outer membrane that are responsible for recognizing and translocating cytosolic precursor proteins into the organelle. Together, they form an import machine comprised of a dynamic complex of receptor molecules that feed the incoming precursor protein to an associated translocation pore (Lithgow *et al.*, 1995, Kubrich *et al.*, 1995, Ryan and Jensen, 1995, Lill and Neupert, 1996). Subunits of the receptor complex that have been identified to date include Tom20p (Ramage *et al.*, 1993, Moczko *et al.*, 1994), Tom22p (Keibler *et al.*, 1993, Lithgow *et al.*, 1994), Tom37p (Gratzer *et al.*, 1995), and Tom70p (Hines *et al.*, 1990, Sollner *et al.*, 1990), whereas Tom40p (Vestweber *et al.*, 1989, Baker *et al.*, 1990, Keibler *et al.*, 1990) and associated proteins (Tom6p, Kassenbrock *et al.*, 1993; Tom7p and Tom8p, Sollner *et al.*, 1992) likely cooperate to form the translocation pore (for current nomenclature, see Pfanner et al., 1996). The existence of multiple receptor subunits may reflect an exceptionally diverse array of cytosolic precursor proteins whose delivery to the organelle is mediated by different types of signal sequences, with quite different properties. These include positively-charged matrix-targeting signals (Attardi and Schatz, 1988, Hartl and Neupert, 1990) and hydrophobic outer membrane signal-anchor sequences (McBride *et al.*, 1992, Schlossmann and Neupert, 1995, Shore *et al.*, 1995), both of which are usually located at the NH_2-termini of precursor proteins, as well as a number of internal signals within polytopic proteins of the mitochondrial outer and inner membranes whose character remains to be determined (e.g., porin, Smith *et al.*, 1995; uncoupling protein, Liu *et al.*, 1988). Additionally, the cytosolic signal recognition factor, MSF (Hachiya *et al.*, 1993), contributes to the recognition of at least certain precursor proteins by the

mitochondrion, in part via direct interactions of MSF with components of the receptor complex (Hachiya *et al.*, 1995). Not surprisingly, therefore, different precursor proteins may exhibit differential dependencies on individual subunits of the receptor complex for recognition by the import machinery.

Recent studies suggest that, following recognition by the receptor, subsequent vectorial movement of precursor proteins from the proximal to distal side of the outer membrane translocon may be mediated, in part, by a series of binding reactions involving the signal sequence (Hachiya *et al.*, 1995, Mayer *et al.*, 1995). Such findings raise important questions. For example, what is the specificity of these distal binding reactions relative to the interaction with the receptor complex? Also, with what degree of efficiency and specificity can an individual precursor protein by-pass the receptor complex and still execute productive downstream interactions and, therefore, translocation into the organelle? Certainly, enforced protein import via a receptor by-pass mechanism has been well documented in lower eukaryotes (Pfanner *et al.*, 1988). Moreover, genetic studies have revealed that numerous genomic open reading frames have the potential to encode sequences that can function as promiscuous matrix-targeting signals when located at the NH_2-terminus of a passenger protein (Allison and Schatz, 1986, Baker and Schatz, 1987, Hurt and Schatz, 1987, Roise *et al.*, 1988). One mechanism to prevent unscheduled import of proteins into mitochondria, therefore, might be a receptor that on the one hand contributes to the positive recognition of a *bona fide* precursor protein, but on the other excludes foreign proteins that bear a cryptic signal sequence from gaining access to downstream binding sites within the translocation machinery, an event that might allow the protein to be functionally imported.

Here, we have gained insight into this problem as the result of finding that a domain located at the NH_2-termini of certain precursor proteins in yeast can function independently as a matrix-targeting signal in yeast mitochondria but not in mammalian mitochondria. Under normal conditions, mammalian Tom20p (Goping et al., 1995, Seki *et al.*, 1995, Hansen et al., 1996) denies this domain access to the protein translocation machinery, but once access is achieved by physically removing the Tom20p barrier, this sequence is capable of mediating efficient import of attached proteins across both the outer and inner membranes. The physiological significance of these findings, and the possible mechanisms whereby Tom20p prevents cryptic targeting sequences from gaining access to the import machinery, are discussed.

Materials and Methods

General

Previous articles (McBride *et al.*, 1992, Li and Shore, 1992a, McBride *et al.*, 1995 and references therein) describe the routine procedures employed for recombinant DNA manipulations, transcription of pSP64 constructs, translation of the resulting mRNA in rabbit reticulocyte lysate in the presence of [^{35}S]methionine, and import of radiolabelled precursor proteins into rat heart or liver mitochondria and mitoplasts. Purification and import into yeast mitochondria was performed according to Glick *et al.*, 1992. Additional information is provided in the figure legends.

Recombinant Plasmids

pSP(pOMD29D16-29) (McBride *et al.*, 1992) encodes amino acids 1-15 of Mas70p fused to amino acids 4-186 of dihydrofolate reductase (DHFR) [1]. Following digestion with PstI and HindIII to remove the *MAS70* sequence, adapters were introduced that encode the following changes to wild-type Mas70p. (1) Lysine at position 9 was mutated to an isoleucine and isoleucine at position 12 was mutated to a lysine, and the protein designated Mas70p(1-15)K9:I12DHFR. The adapters were 5'-AGCTTATGAAGAGCTTCATTACAAGGAACATTACAGCCAAGTT GGCTGCA-3' and 5' -GCCAACTTGGCTGTAATGTTCCTTGTAATGAAGCTCTTCATA-3'. (2) The positively-charged amino acids at positions 2, 7, and 9 of wild-type Mas70p were replaced with glutamine, employing the adapters 5'-AGCTTATGCAGAGCTTCATTACACAGAAC CAGACAGCCATTTTGGCTGCA-3' and 5'-GCCAAAATGGCTGTCTGGTTCTGTGTAAT GAAGCTCTGCATA-3'. This new protein was designated Mas70p(1-15)KR2,7,9QDHFR. (3) Amino acids 1-15 of Mas70p were replaced with NCBR amino acids 1-12 using the adaptors

5'-GCTTTTGAGTGAGATCTGGATAATCTGGAAAACATA-3' and 5'-AGCTTATGTTTTCCA GATTATCCAGATCTCACTCAAAAGCTGCA-3'. This new protein was termed NCBR(1-12)DHFR. Finally, the DHFR sequence in pSP(pOMD29D16-29) was replaced with DNA encoding amino acids 62-355 of rat pOCT by replacing a PstI-EcoRI fragment (Nguyen and Shore, 1987) with the corresponding restriction fragment generated from pSPO19 (Nguyen et al., 1986), yielding the new protein Mas70p(1-15)OCT. All changes were verified by nucleotide sequencing.

Results and Discussion

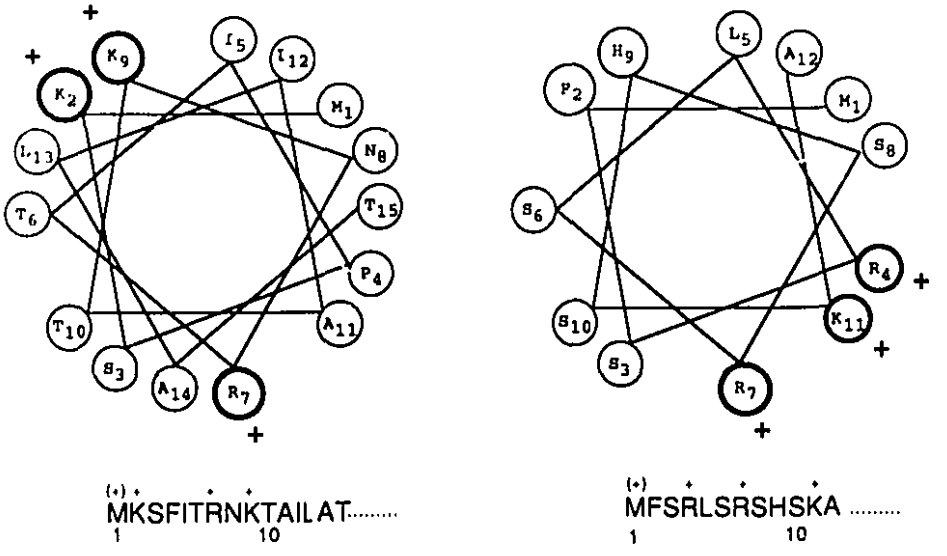
Mas70(1-15)DHFR and NCBR(1-12)DHFR are Imported into Yeast but not Rat Heart Mitochondria in vitro

The NH_2-terminal signal-anchor sequences of yeast Mas70p and NADH cytochrome b_5 reductase (NCBR)[1] are responsible for targeting and inserting these proteins into the mitochondrial outer membrane in the N_{in}-C_{cyto} orientation , leaving the bulk of the protein facing the cytosol (Hase *et al.*, 1984, McBride *et al.*, 1992, Hahne *et al.*, 1994). Interestingly, the signal-anchor of NCBR is bi-functional and delivers a portion of the NCBR molecules to the inter-membrane space in yeast mitochondria (Hahne *et al.*, 1994). The signal-anchor of both proteins is comprised of a short amphiphilic, positively-charged region at the extreme NH_2-terminus followed by the predicted transmembrane segment (Hase *et al.*, 1984, Hahne *et al.*, 1994). The NH_2-terminal domain of Mas70p can support matrix targeting of a fused passenger protein into mitochondria from yeast (Hurt *et al.*, 1985), but not mammals (rat heart) (McBride *et al.*, 1992). Fig.1 documents this fact and shows as well that this disparity between mammalian and yeast mitochondria extends to NCBR. Import of porin, on the other hand, was observed for mitochondria isolated from either source.

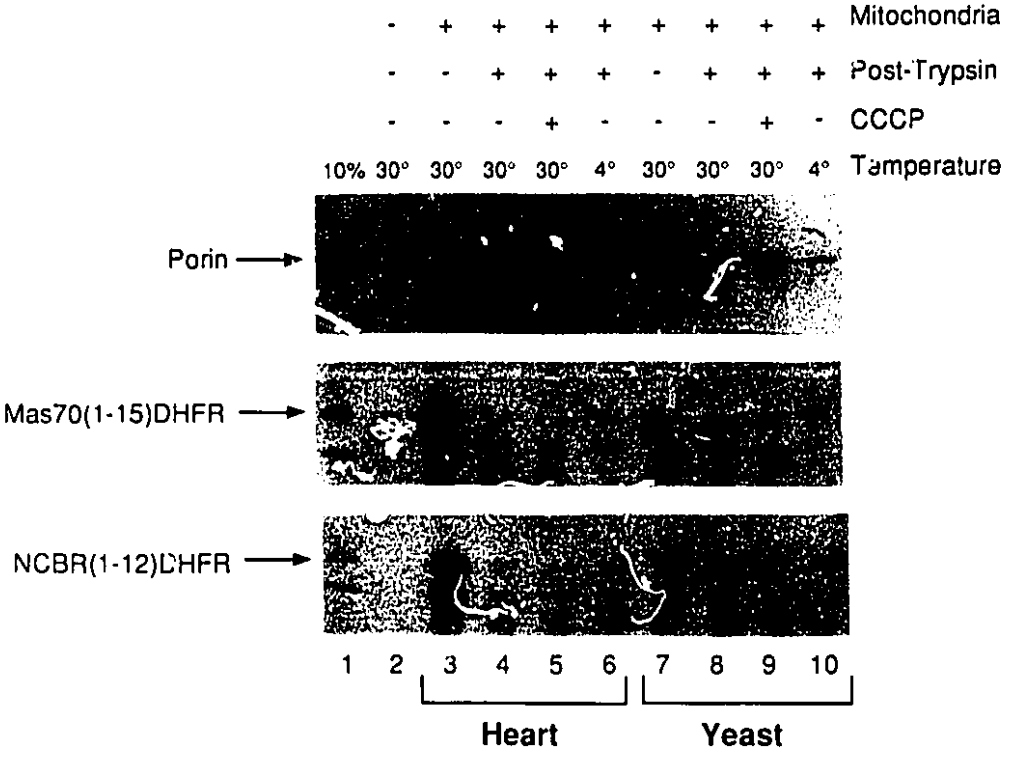
Following import incubations with yeast mitochondria, Mas70(1-15)DHFR and NCBR(1-12)DHFR were recovered with the organelle (Fig.1B, lane 7) and a significant fraction was resistant to subsequent treatment with trypsin (lane 8). Acquisition of protease-resistence was dependent on the presence of mitochondria (lane 2), on $\Delta\psi$ (i.e., it was abolished by CCCP, lane 9) and physiological temperature (lane 10), and the protein extractable at alkaline pH (not shown), all of which are consistent with import to the matrix compartment. In marked contrast, negligible protease-resistant precursor was recovered with rat heart mitochondria (Fig.1B, compare lane 4 with lanes 5 and 6) or mitochondria

Figure 1. Mas70(1-15) and NCBR(1-12) direct protein import into yeast, but not rat heart, mitochondria . *A*, Residues 1-15 of Mas70p (*left panel*) and 1-12 of NCBR (*right panel*) are shown as predicted α-helices, with amino acids indicated in the single letter code. Positively charged amino acids are circled in bold and indicated by a plus sign. *B*, [^{35}S]porin (*top panel*), [^{35}S]Mas70(1-15)DHFR (*middle panel*) and [^{35}S]NCBR(1-12)DHFR (*bottom panel*) were generated by transcription-translation of recombinant plasmids and the resulting reticulocyte lysate incubated with mitochondria (0.5 mg/ml protein) purified either from rat heart (lanes *3-6*) (Li and Shore, 1992b) or from yeast strain D273-10B (lanes *7-10*) (Glick *et al.*, 1992), in the presence (lanes *5* and *9*) or absence (lanes *2-4*, *6-8*, and *10*) of 1.0 μM CCCP, and incubated under standard import conditions for 30 minutes either at 30° (lanes *2-5* and *7-9*) or 4° (lanes *6* and *10*). Organelles were recovered from reaction mixtures by centrifugation either directly (lanes *3* and *7*) or following treatment with 0.125 mg/ml trypsin for 20 minutes at 4° followed by a 10 minute incubation with 1.25 mg/ml soybean trypsin inhibitor (lanes *4-6* and *8-10*) (McBride *et al.*, 1992). They were subjected to SDS-PAGE and fluorography. Lane *1*, 10% input translation product; lane *2*, no mitochondria added to import reactions.

A


Mas70(1-15)
NCBR(1-12)
MKSFITRNKTAILAT.....
MFSRLSRSHSKA .....

B

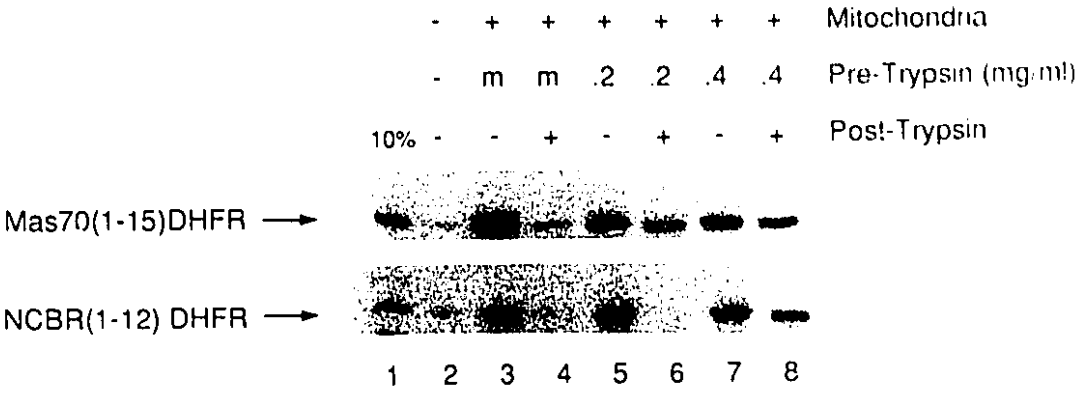

Mitochondria
Post-Trypsin
CCCP
Temperature
10% 30° 30° 30° 30° 4° 30° 30° 30° 4°
Porin
Mas70(1-15)DHFR
NCBR(1-12)DHFR
1 2 3 4 5 6 7 8 9 10
Heart
Yeast

from human cells (not shown), despite the fact that, like yeast mitochondria (Fig.1, lanes 7-10), the heart mitochondria supported robust import of porin (lanes 3-6), as well as the entire Mas70p signal-anchor (McBride *et al.*, 1992) and many other proteins. Import and insertion of porin into the outer membrane of yeast and heart mitochondria was measured by assaying temperature-sensitive and $\Delta\psi$–independent acquisition of resistance to trypsin (Mihara *et al.*, 1982, Gasser and Schatz, 1983) (Fig.1B).

Activation of Import of Mas70(1-15)DHFR and NCBR(1-12)DHFR into Heart and Liver Mitochondria

Because the NH_2-termini of Mas70(1-15)DHFR and NCBR(1-12)DHFR have the potential to form positively-charged amphiphilic helices typical of matrix-targeting signals (Fig.1A), it was predicted that these domains might in fact constitute cryptic signals that are denied entrance to heart mitochondria due to a screening mechanism located on the surface of the organelle. Consistent with this possibility, Fig.2 demonstrates that pre-treatment of intact rat heart mitochondria with trypsin permitted subsequent uptake of Mas70(1-15)DHFR and NCBR(1-12)DHFR, resulting in the acquisition of protease protection for both proteins (compare lane 4 with lanes 6 and 8). Interestingly, NCBR(1-12)DHFR required a higher concentration of trypsin in the pre-treatment period than did Mas70(1-15)DHFR (Fig.2). In both cases, however, trypsin pretreatment reduced the amount of total precursor that was subsequently recovered with the surface of the organelle (Fig.2, compare lane 3 with lanes 5 and 7), but had no effect on the level of a marker protein in the inter-membrane space, sulfite oxidase (not shown, see Nguyen *et al.*, 1993), indicating that trypsin had not broached the outer membrane. When exposed, sulfite oxidase is otherwise highly sensitive to trypsin (Nguyen *et al.*, 1993, Ono and Ito, 1984). It

Figure 2. Activation of import of Mas70(1-15)DHFR and NCBR(1-12)DHFR by pretreatment of heart mitochondria with protease. Mitochondria purified from rat heart (0.5 mg/ml protein) were incubated with 0.2 mg/ml (lanes *5* and *6*) or 0.4 mg/ml (lanes *7* and *8*) trypsin for 45 minutes at 4° at which time soybean trypsin inhibitor (SBTI) was added in 50 fold excess for a further 20 minute incubation at 4°. As a mock control (*m*) (lanes *3* and *4*), incubation with 0.8 mg/ml trypsin was carried out in the presence of SBTI. The mitochondria were recovered by centrifugation and incubated with [^{35}S]Mas70(1-15)DHFR (*top panel*) or [^{35}S]NCBR(1-12)DHFR (*bottom panel*) under standart import conditions for 30 minutes at 30° (lanes *3-8*). Following the import reaction, the mitochondria were centrifuged through a 0.5 ml 250mM sucrose/10mM Hepes, pH 7.5 cushion, and either resuspended directly in SDS sample buffer (lanes *3*, *5* and *7*) or resuspended in import medium to 0.5 mg/ml protein and incubated with 0.125 mg/ml trypsin for 20 minutes at 4°, followed by 10 minutes in the presence of SBTI. Mitochondria were recovered, subjected to SDS-PAGE, and analyzed by fluorography. Lane *1*, 10% of input [^{35}S]precursor protein; lane *2*, import in the absence of organelles.

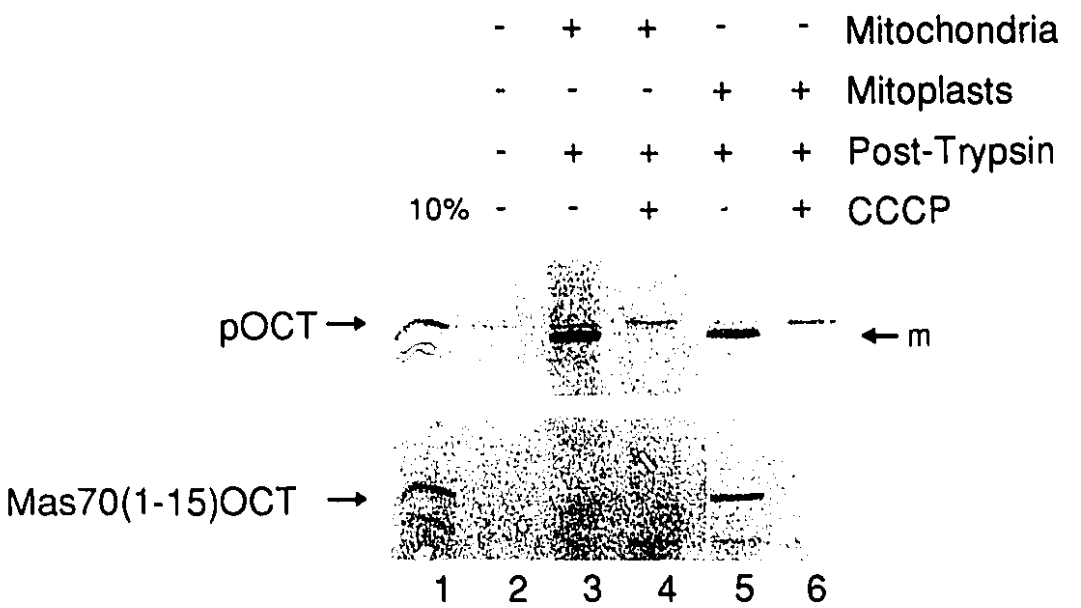

- + + + + + + Mitochondria
- m m .2 .2 .4 .4 Pre-Trypsin (mg/ml)
10% - - + - + - + Post-Trypsin
Mas70(1-15)DHFR
NCBR(1-12) DHFR
1 2 3 4 5 6 7 8

would appear, therefore, that one or more proteins on the surface of heart mitochondria promote binding of these precursor proteins to the surface of the organelle. However, a surface-exposed protein(s) also prevents these precursors from subsequently passing across the outer membrane. Under the conditions employed, trypsin presumably removed this barrier, while leaving other components of the import machinery (e.g., Tom40p) at least partially functional.

The inability of Mas70(1-15) (Fig.3) and NCBR(1-12) (not shown) to support import of a passenger protein into intact mitochondria was also observed for rat liver mitochondria and was observed when the passenger protein, DHFR, was replaced by the mature portion of pOCT, a mitochondrial matrix protein which is efficiently imported in vitro (Fig.3). The inability of these precursors to import into intact mammalian mitochondria, therefore, is a property of the cryptic targeting signal rather than due to the type of passenger protein employed.

In addition to trypsin pre-treatment of mammalian mitochondria as a means of activating protein import directed by Mas70(1-15) or NCBR(1-12), Figs. 3 and 4 also show that mitoplasts, in place of intact mitochondria, permit efficient import mediated by these sequences. Whereas intact mitochondria and mitoplasts were equivalent in their ability to import pOCT (Fig.3, upper panel), only the mitoplasts sustained import under the direction of Mas70(1-15) (Fig.3, lower panel) or NCBR(1-12) (Fig.4, bottom panel). Again, import into mitoplasts was assayed by the acquisition of protease protection that was dependent on the presence of the organelle (Fig.4, compare lanes 2 and 4), on an intact electrical chemical potential (Fig.4, compare lanes 4 and 5), and on physiological temperature (compare lanes 4 and 6). As expected, import directed by Mas70(1-15) into mitoplasts was ablated when the positively-

Figure 3. Amino acids 1-15 of Mas70p mediate import of a reporter protein into mitoplasts but not into intact mitochondria. [^{35}S]pOCT and [^{35}S]Mas70(1-15)OCT were incubated with intact mitochondria or mitoplasts purified from rat liver (McBride *et. al*, 1995) (0.5 mg protein/ml) under standard import conditions for 30 min. at 30° (lanes *3-6*), in the presence (lanes *4* and *6*) or absence (lanes *3* and *5*) of 1.0 µM CCCP. Organelles were recovered from reaction mixtures by centrifugation either directly (*upper panel*) or following treatment with 0.125 mg/ml trypsin for 20 minutes at 4°, followed by a 10 minute incubation with 1.25 mg/ml soybean trypsin inhibitor (*bottom panel*, lanes *3-6*). They were subjected to SDS-PAGE and fluorography. Lane *1*, 10% of input [^{35}S]precursor protein; lane *2*, import in the absence of organelles. *m*, processed pOCT.

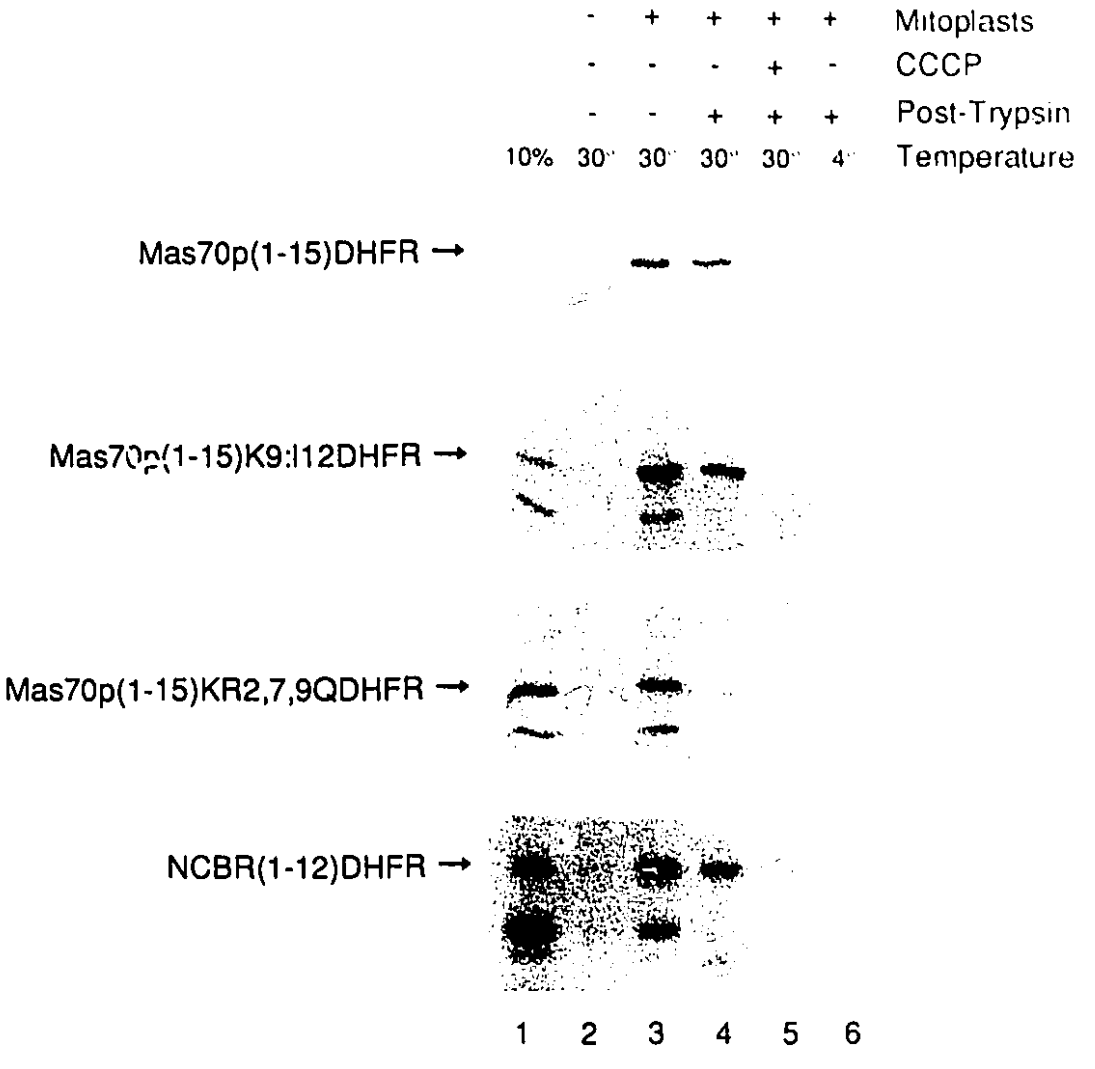

- + + - - Mitochondria
- - - + + Mitoplasts
- + + + + Post-Trypsin
10% - - + - + CCCP
pOCT
m
Mas70(1-15)OCT
1 2 3 4 5 6

charged amino acids at positions 2, 7, and 9 were converted to uncharged glutamine residues (Fig.4, compare first and third panels), a finding consistent with the evidence that import across the inner membrane is initiated by an electrophoretic force that is imposed by $\Delta\psi$ on a positively-charged signal sequence (Martin *et al.*, 1991, McBride *et al.*, 1995). Import, however, was sustained following a rearrangement of the positively-charged amino acids within Mas70(1-15) (Fig.4, second panel). Finally, import of Mas70(1-15)DHFR into mitoplasts was competed by a sequence corresponding to the matrix targeting sequence of pOCT, either in the form of a synthetic signal peptide (Gillespie *et al.*, 1985) (Fig.5, left panel) or in the form of a bacterial expression product, pODHFR (Sheffield et al.,1990), in which the pOCT signal sequence has been fused to DHFR (Fig.5, right panel). The extent and pattern of competition for import of the transcription-translation products of Mas70(1-15)DHFR and pODHFR were very similar (Fig.5), strongly suggesting that the two proteins were imported into mitoplasts by a common mechanism.

Substitution of yTom20p with hTom20p in Yeast Mitochondria Prevents Import of Mas70(1-15)DHFR and NCBR(1-12)DHFR

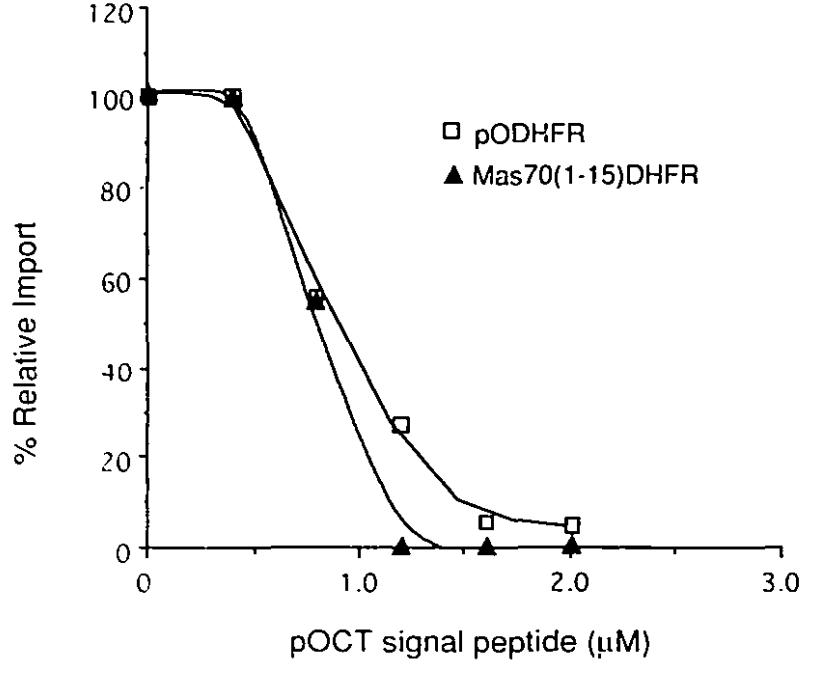
We previously cloned and partially characterized the human homolog of Tom20p and demonstrated that *hTOM20* complemented the respiratory defect of *Δtom20* in yeast (Goping *et al.*, 1995), indicating that hTom20p can functionally substitute for the yeast receptor and sustain import of yeast precursor proteins in vivo. As shown in Fig.6 (upper panel), mitochondria isolated from the *hTOM20* strain of yeast supported import of porin into the mitochondrial outer membrane in vitro, with characteristics very similar to import of porin into wt yeast mitochondria. In both cases, most of the porin that was recovered with the wt or hTom20p yeast mitochondria was resistant to protease

Figure 4. Import of Mas70(1-15)DHFR and NCBR(1-12)DHFR into mitoplasts. [^{35}S]-labeled Mas70p(1-15)DHFR (*top panel*), Mas70p(1-15)K9:I12DHFR (*second panel*), Mas70p(1-15)KR2,7,9QDHFR (*third panel*) and NCBR(1-12)DHFR (*bottom panel*) were obtained by transcription-translation and their import into rat liver mitoplasts assayed. Conditions and manipulations are described in Fig. 3. Lane *1*, 10% of input precursor protein; lane *2*, import in the absence of mitoplasts; lanes *3-6*, import in the presence of mitoplasts; lanes *4-6*, treatment with trypsin following the import reaction; lane *5*, import in the presence of 1.0 μM CCCP; lane *6*, import at 4°.


- + + + + Mitoplasts
- - - + - CCCP
- - + + + Post-Trypsin
10% 30' 30' 30' 30' 4' Temperature
Mas70p(1-15)DHFR →
Mas70p(1-15)K9:I12DHFR →
Mas70p(1-15)KR2,7,9QDHFR →
NCBR(1-12)DHFR →
1 2 3 4 5 6

treatment following import at 30° but not at 4°. In contrast, temperature-sensitive import of Mas70(1-15)DHFR was observed for wt yeast mitochondria (Fig.6, lower panel, lanes 3-5), but not for hTom20p yeast mitochondria (lanes 6-8). Most of the Mas70(1-15)DHFR that was recovered with wt yeast mitochondria following import at 30° was resistant to protease (compare lanes 3 and 4), whereas this acquisition of protease-resistance was significantly reduced following import at 4° (lane 5). Following import into hTom20p yeast mitochondria at 30°, however, acquisition of protease-resistance of Mas70(1-15)DHFR was low (compare lanes 6 and 7) and, in fact, was near the level of the 4° control (lane 8). Similar results were also obtained with NCBR(1-12)DHFR (not shown). Thus, hTom20p yeast mitochondria exhibit similar characteristics to rat heart and liver mitochondria with respect to the lack of protein import under the direction of Mas70(1-15) and NCBR(1-12) signal sequences (Figs.1 and 3). Also, mitochondria from *Δtom20* yeast (Ramage *et al.*, 1993) failed to import Mas70(1-15)DHFR and NCBR(1-12)DHFR (not shown). However, it is not known if this means that yTom20p directly contributes to positive recognition of these precursors or if deletion of yTom20p indirectly results in disruption of components that otherwise interact with yTom20p and are required for import.

Figure 5. Synthetic matrix-targeting signal peptide and bacterial-expressed matrix precursor protein compete for import of Mas70p(1-15)DHFR into mitoplasts. Solutions containing 7M urea, 10mM Hepes, pH 7.4, and various concentrations of either the peptide, pO(1-27)cys (Gillespie *et al.*, 1985), or purified pODHFR (amino acids 1-36 of pOCT fused to amino acids 4-186 of DHFR) obtained from expressing bacteria (Sheffield *et al.*, 1990), were flash diluted 25-fold into a standard import reaction lacking translation product, and incubated for 10 minutes at 4°. Reactions contained a final concentration of 0.28 M urea and the indicated concentration of pO(1-27)cys (0-2 μM, *left panel*) or pODHFR (0-6 μM, *right panel*). Import into mitoplasts was initiated upon addition of reticulocyte lysate containing [^{35}S]pODHFR or [^{35}S]Mas70p(1-15)DHFR, and the mixture incubated at 30° for 15 min. Trypsin (0.125 mg/ml) was added and the incubation continued for 20 min. at 4°, followed by a further incubation with 1.25 mg/ml SBTI for 10 min. Mitoplasts were recovered by centrifugation and analyzed by SDS-PAGE and fluorography. Bands corresponding to trypsin protected Mas70p(1-15)DHFR and processed pODHFR were quantified using a FUJI BAS 2000 Bioimager, with the products obtained in the presence of CCCP (see Figs. 1 and 3) employed as baseline. Imported products obtained in the absence of competitor peptide or bacterial pODHFR were arbitrarily set at 100%.


120
100
80
60
40
20
0
% Relative Import
pODHFR
Mas70(1-15)DHFR
0
1.0
2.0
3.0
pOCT signal peptide (μM)

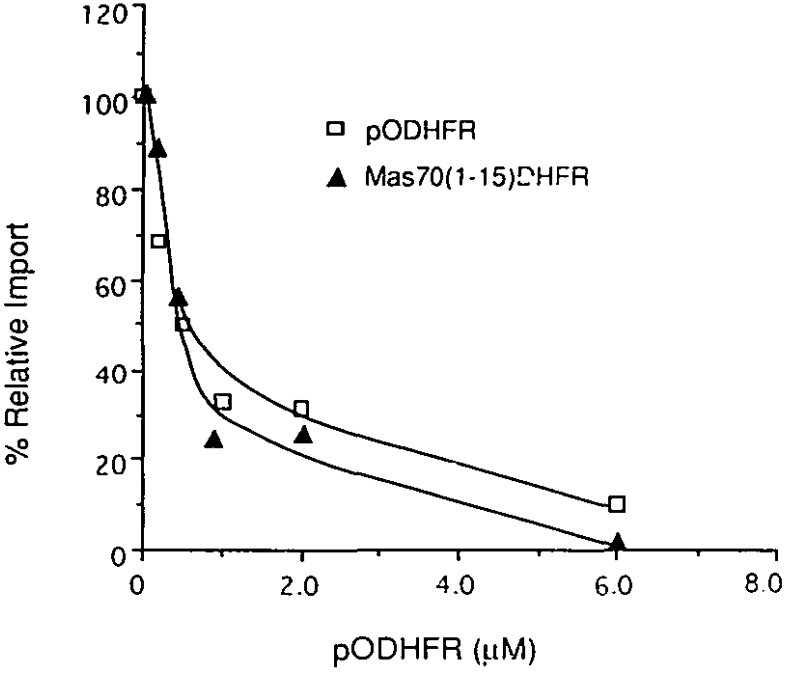
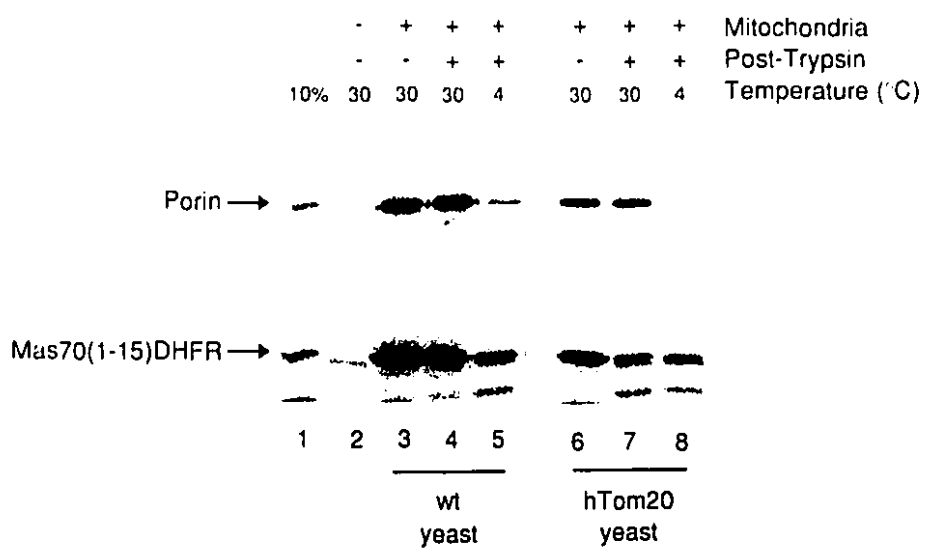
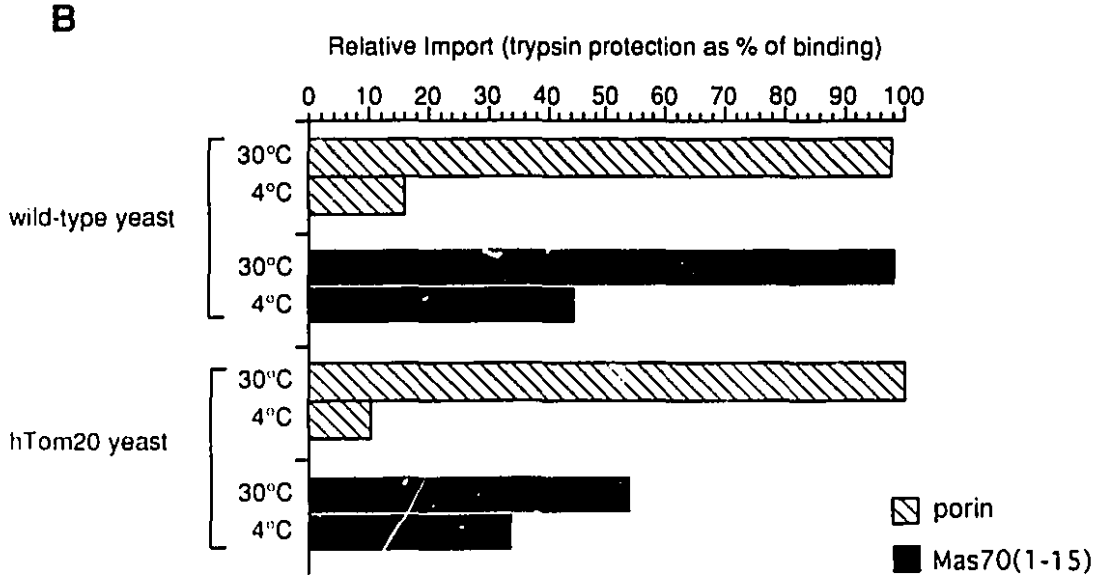

120
100
80
60
40
20
0
% Relative Import
pODHFR
Mas70(1-15)DHFR
0
2.0
4.0
6.0
8.0
pODHFR (μM)

Figure 6. hTom20p prevents import of Mas70(1-15)DHFR into yeast mitochondria. *A*, [^{35}S]porin (*top panel*) or [^{35}S]Mas70(1-15)DHFR (*bottom panel*) were incubated with mitochondria purified either from wild-type yeast (lanes *3-5*) or from *Δmas20* yeast complemented with the hTom20 receptor (Goping *et al.*, 1995). Incubation was performed for 30 minutes at either 30° (lanes *3,4,6* and *7*) or 4° (lanes *5* and *8*) under standard import conditions. Mitochondria were collected either directly (lanes *3* and *6*) or following trypsin treastment (lanes *4,5,7* and *8*). Lane *1*, 10% of input [^{35}S]precursor protein; lane *2*, import in the absence of organelles. *B*, Autoradiograms in *A* were quantified by laser densitometry and analyzed using NIH Image v.1.57 image analysis software. The radioactive bands in panel *A*, lanes *3* and *6* were arbitrarily set at 100. Porin, *hatched bars* ; Mas70(1-15)DHFR; *solid bars.*

A


- + + + + + + Mitochondria
- - + + - + + Post-Trypsin
10% 30 30 30 4 30 30 4 Temperature (°C)
Porin
Mas70(1-15)DHFR
1 2 3 4 5 6 7 8
wt yeast
hTom20 yeast

B


Relative Import (trypsin protection as % of binding)
0 10 20 30 40 50 60 70 80 90 100
wild-type yeast
hTom20 yeast
30°C
4°C
30°C
4°C
30°C
4°C
30°C
4°C
porin
Mas70(1-15)

Conclusions

We have shown that a sequence which can function as an active matrix-targeting signal in yeast mitochondria, is a cryptic signal in mammalian mitochondria, and is denied access to the translocation machinery of the mammalian organelle by Tom20p. Thus Tom20p has two functions: it contributes to positive recognition of precursor proteins and, as well, constitutes a barrier, or guardian, against sequences that otherwise can be recognized by distal components of the import machinery and be functionally imported into the organelle. While the underlying mechanism of such screening by Tom20p is not known, there are two obvious explanations. Tom20p might be situated within the receptor complex in such a way that it shields the translocation pore and physically prevents cryptic targeting sequences from by-passing the receptor complex and gaining direct access to downstream components within the import machinery with which the cryptic signal can functionally interact. Alternatively, Tom20p, perhaps in cooperation with other components of the import machinery, may recognize both specific and related cryptic signals but vectorially release into the translocation pore only those proteins bearing the correct sequence. This is consistent with the finding that binding of Mas70(1-15)DHFR and NCBR(1-12)DHFR to rat mitochondria was reduced following pretreatment of the organelle surface with protease (Fig.2). Such discrimination between different ligands is common during signal transduction events controlled by GTP binding proteins, including the release of ER-targeted proteins from SRP to the translocon following binding of the SRP-signal sequence complex to SRP receptor (Miller and Walter, 1993). An analogous ATP-dependent transfer of precursor proteins between components of the mitochondrial receptor complex has been documented for precursors that are complexed with MSF (Hachiya *et al.*, 1995, Komiya et al., 1996, Mihara and

Omura, 1996). Whatever the mechanism, however, screening of potentially cryptic targeting sequences by Tom20p may play an important role in preventing mis-sorting of proteins to mitochondria in the eukaryotic cell.

Finally, our results may be relevant to post-receptor sorting of precursor proteins within the mitochondrion. For example, cryptic matrix-targeting sequences would be expected to remain topogenically silent until they pass beyond Tom20p and, therefore, would not interfere or compete with other topogenic sequences within the polypeptide that are recognized by this receptor. One class of proteins for which this may be relevant are polytopic integral proteins of the inner membrane, where it has been observed that positively-charged domains within the polypeptide are preferentially located on the matrix side of the inner membrane (Gavel and von Heijne, 1992). Recognition of these domains as cryptic matrix-targeting signals at the inner membrane during import could represent an important determinant for proper transmembrane topology, by driving paired transmembrane helices into the bilayer (Liu *et al.*, 1988). Conversely, the lack of recognition of these sequences at the outer membrane may allow the polypeptide to adopt a conformation which is incompatible with integration into the lipid-bilayer of the outer membrane, thereby resulting in proper sorting to the inner membrane.

Acknowledgments

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[1]The abbreviations are: DHFR, dihydrofolate reductase; NCBR, NADH cytochrome b_5 reductase; pOCT, pre-ornithine carbamyl transferase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SBTI, soybean trypsin inhibitor.

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Chapter 5: General Discussion

The work described in this thesis can be divided into three main sections, a) the identification of a functional signal-anchor sequence selective for the mitochondrial outer membrane, b) the role of the electrochemical potential as an electrophoretic force in protein import into or across the inner membrane and c) the elucidation of a new functional role for receptor proteins on the surface of the mitochondria to screen out cryptic mitochondrial signals. The general discussion will focus on aspects and implications of each chapter not included in the discussions and will place each chapter in the context of the field as it is today, rather than when the experiments were performed.

The signal-anchor sequence.

The amino terminal region of the yeast outer membrane receptor protein yTom70 contains a hydrophobic sequence responsible for a number of distinct interactions which target and insert the protein into the outer membrane. It can target the protein to the surface of the organelle, initiate translocation across the lipid bilayer and abrogate translocation resulting in the lateral release of the domain into the surrounding membrane. This hydrophobic region has been termed a signal-anchor sequence since similar topogenic sequences are found in the endoplasmic reticulum. The signal-anchor domain is now accepted in the field as a *bone fide* topogenic domain and can be found in a growing number of outer membrane proteins. The presence of charged residues on the N- or C-terminus of the hydrophobic signal anchor function to enhance the rate of import, but are not required for proper targeting. For example, yTom20 contains a 28 amino acid region of hydrophobicity at the immediate N-terminus, without an extended flanking region, and this hydrophobic region functions as a signal-anchor sequence (Goping *et al.*, 1995). The case of Bcl-2 demonstrates that a signal-anchor sequence is not limited to the N-terminus, but that the 22 amino

acid hydrophobic C-terminal domain can function alone at the C-terminus of the passenger protein DHFR and target the protein to the mitochondrial outer membrane (Nguyen *et al.*, 1994). Tom6 contains two predicted transmembrane domains, one at either end, and it has recently been shown that the signal-anchor like C-terminal region of Tom6 can drive import of a passenger protein (Cao and Douglas, 1995). Other proteins of the outer membrane, such as Tom37 (Kassenbrock *et al.*, 1995) contain regions resembling a signal-anchor sequence, although the definitive experiments have not been done. The transmembrane region of the outer membrane receptor protein, Tom22 is located internally, and it is unknown whether such an internal region could function as a signal-anchor sequence (Kiebler *et al.*, 1993).

Another interesting feature of the signal-anchor sequence is the fact that the transmembrane domain alone can functionally insert the protein into the lipid bilayer without the presence of any charged residues. This point is relevant when one considers the present model of protein/receptor interactions, which are based primarily on electrostatic interactions between the positively-charged targeting signals and acidic regions located within the cytosolic domain of receptors. Does this imply that without charged residues, the signal-anchor sequence can bypass the receptors and interact directly with the translocating machinery of the GIP? Interestingly, competition experiments using bacterially expressed pODHFR against both a matrix fusion construct pODHFR and the yTom70 fusion construct pOMD29, demonstrated that the rate limiting step for import of pODHFR was distal to that of pOMD29 (McBride *et al.*, 1992). Whether or not this rate limiting step is at the level of the receptors is unknown. It is likely that the enhancement of the rate of import due to the charged amino terminal region is a direct result of increased binding to the

mitochondrial surface, to either lipid or protein, thereby facilitating increased interactions with downstream components of the translocation machinery.

One of the critical consequences of a signal-anchor sequence is that this domain sorts the protein into the first lipid-bilayer it encounters. This concept is further verified by the fact that upon direct exposure to the inner membrane of the mitochondria by incubating the construct with mitoplasts, pOMD29 is efficiently imported into the inner membrane with a newly acquired dependence on $\Delta\psi$ (Li and Shore, 1992a, McBride *et al.*, 1995). It is known that many transmembrane regions destined for the inner membrane are not arrested at the level of the outer membrane, so mere hydrophobicity cannot be the requirement for lateral release into the outer membrane lipid bilayer. It is also known that the primary sequence of the transmembrane domain follows no discernible consensus which would distinguish between outer vs. inner transmembrane domains. An earlier model proposed that a membrane region would be laterally released into the outer membrane unless the region N-terminal to it has engaged the inner membrane (Nguyen *et al.*, 1988, Shore *et al.*, 1995). This model continues to apply to bitopic outer membrane proteins containing signal-anchor sequences since the number of residues flanking the signal-anchor could not merit irreversible interactions with the inner membrane. The case of polytopic inner membrane proteins such as UCP or AAC are more complicated since the first transmembrane region is at the N-terminus, but does not function as a signal-anchor sequence. It will be interesting to learn how these regions escape insertion into the outer membrane.

Functional role of the electrochemical potential.

To date, all but one protein imported into or across the inner membrane require the presence of the electrochemical potential across the membrane.

The dependence on the electrochemical potential is shown in Chapter 3 to be a result of positively-charged residues within the presequence, moreover, it is suggested that the potential is required to vectorially clear these charged residues from the translocation pore (McBride *et al.*, 1995). It is interesting to speculate on the nature of the interactions which would require such a force in order to disengage them. Two lines of evidence point to a model whereby the positively charged presequence may interact with regions of protein exposed to the intermembrane space. The first is that import into protease pretreated mitoplasts is inhibited for pODHFR, UCP and pOMD29, however, import is unaffected for the charge-deficient mutants (data not shown). Furthermore, although import of pODHFR, UCP and pOMD29 is competed for by the addition of matrix signal-peptide, the import of the charge deficient mutants remains unaffected (McBride *et al.*, 1995). These data suggest that import of the wild type signal anchor into mitoplasts requires the presence of protease accessible components at the level of the inner membrane. Furthermore, the charge deficient mutants do not require such components and are capable of direct interaction with the translocation machinery of the inner membrane. The fact that wild-type pOMD29 is competed for by matrix signal peptide and the mutants are not also suggests that the presence of the charged residues result in a rate limiting step distal from that used by the signal-anchor domain alone. Such a rate-limiting step may be related to the protease accessible component. As suggested in Chapter 3, Tim44 is predicted to bind positive presequences, however, another candidate protein is Tim23, which contains a large acidic region exposed to the inner membrane space. Subsequent work on the mechanism of Tim44 has demonstrated that this protein functions in co-operation with the matrix chaperone Hsp70 as an "import motor" to pull in translocating polypeptides. The motor activity is known to function downstream

of the requirement for $\Delta\psi$, therefore a more likely candidate for interactions with charged presequences is Tim23. The predicted interaction between such a component of the inner membrane machinery and the charged presequence is necessarily much stronger than those encountered at the level of the outer membrane since an electrochemical potential is not needed at the outer membrane to release such binding steps.

Another interesting observation taken from these results is that positive charges are not necessarily required to initiate translocation across the inner membrane, and that a signal-anchor sequence can function at this stage. This may be important in the sorting of downstream transmembrane domains of polytopic inner membrane proteins.

Mammalian specificity, inner membrane sorting and NCBR.

Although the constructs used in Chapter 4 may be quite unique in demonstrating a difference between yeast and mammalian mitochondria, it is interesting to note that differences between mitochondrial species have been observed previously. For example, the mitochondrial import stimulating factor MSF (Hachiya *et al.*, 1993), which functions as a cytosolic chaperone protein, has been identified in rat liver but no homologues exist in yeast to date. An increased requirement for precursor specificity may be due to the increased complexity of the mammalian cell. For example, mammalian and yeast mitochondria are faced with a tremendous number of cellular proteins which are not destined for import. Mis-sorting aberrations are found in human disease conditions where, most commonly, peroxisomal proteins become sorted to the mitochondria (Purdue *et al.*, 1991). In order to screen out peroxisomal, and other proteins, the mitochondria must have evolved a very precise mechanism to discriminate against non-mitochondrial precursors. It remains unclear

whether or not yeast have also evolved an ablity to screen out cryptic signals. In a mammalian system, the receptor hTom20 is shown to have a pivotal role in a screening function, however the mechanism whereby it performs this function is unknown. It is probable that Tom20 functions within the receptor complex in order to achieve proper fidelity. It will be interesting to learn if other constituents of the import machinery have evolved screening functions and if yeast are capable of discriminating against cryptic signals.

The fact that the import machineries of the outer and inner membrane are dynamic structures, each with the potential to function independently of the other strongly implies that both machineries have mechanisms to recognize targeting signals. Data presented in Chapter 4 demonstrate the ability of mammalian mitochondrial outer membrane to recognize only very specific targeting signals, however little is yet known about the signal-recognition properties of the inner membrane import apparatus. The unexpected observation that the relatively short sequences at the amino terminus of yTom70 or NADH cytochrome b5-reductase can function as matrix-targeting signals when presented directly to the exposed inner membrane of mitochondria, but not when it encounters the outer membrane of the intact organelle, suggests that the inner membrane machinery demonstrates a distinct specificity for signals. Minimal structural requirements for recognition by the inner membrane were investigated by constructing mutants to test the role of positive charges vs. the hydrophobicity. The results suggest that positive charges are required, however the loss of hydrophobicity does not affect import efficiency.

The potential *in vivo* consequences of the inner membrane receptor machinery possessing a more relaxed specificity for signals may be two-fold. First, it may be of kinetic importance. It has been shown that contact sites are

not formed until the appearance of the precursor at the trans side of the outer membrane. As the precursors are presented to the inner membrane, they would become stalled until such time as the inner membrane machinery contacts either the presequence in transit or the outer membrane machinery itself. Relaxed signal specificity would allow this to occur at an increased rate.

The second consequence of relaxed specificity of the inner membrane import machinery, as mentioned in Chapter 4, may be of importance for protein sorting between subcompartments. Proteins translocated across the outer membrane may be recognized by the inner membrane receptor complex due to a unique secondary structure previously concealed from the outer membrane apparatus. This phenomenon would explain why proteins destined for the inner membrane are not arrested in transit by the outer membrane. These results may shed new light on previously elusive mechanisms of insertion of inner membrane proteins such as the family of ion channels which include UCP and AAC by considering their targeting and insertion signals in the context of each membrane system separately.

Finally, with respect to Mas70p itself, it is evident that the cryptic mitoplast specific matrix-targeting sequence at its amino-terminus does not interfere with the signal-anchor function of the transmembrane segment, with the result that Mas70p is imported exclusively into the outer membrane (McBride et al., 1992). In the case of NADH cytochrome-b5 reductase, however, the combination of the mitoplast matrix-targeting signal with a weak signal-anchor function of the transmembrane segment results in almost 60% of the molecules sorted to the intermembrane space rather than into the outer membrane like Mas70p (Hahne *et al.*, 1994). This demonstrates the pivotal role the inner membrane machinery plays in recognizing and sorting signals to proper sub-compartment within the mitochondria. However, our results also demonstrate that the amino terminal

region of NADH cytochrome b5-reductase is not responsible for the initiation of translocation across the outer membrane, which further suggests that the signal-anchor sequence engages the machinery which has been observed previously in the case of yTom70 (McBride *et al.*, 1992).

Unanswered questions

The topogenic signal-anchor sequence is now though to be responsible for most outer membrane protein sorting, however there remain proteins whose biogenesis is uncertain, such as beta-barrel containing proteins, like porin, and others like the receptor Tom22. This receptor does not contain any stereotypical mitochondrial targeting signals and the transmembrane region is internal. It has also never been tested whether or not transmembrane regions of inner membrane proteins contain any targeting information, but has been predicted that they function strictly as passive stop-transfer domains. A most intriguing aspect of protein sorting has always been the mechanism by which transmembrane regions escape insertion into the outer membrane and studies such as these will undoubtedly lead to the answers.

The recent identification of the components of the inner membrane machinery has resulted in a heightened understanding of the events at this membrane, however, there remain many experiments to be done. For example, precursor/receptor interactions and the nature of sequential binding events are finally beginning to be addressed in isolated systems using both membranes of the mitochondria. It will be interesting to determine if Tim23 binds tightly to incoming presequences. Furthermore, whether or not the contact site is formed between the machineries of each membrane translocase machinery, or if it is formed as a result of the bridging polypeptide. The future will also undoubtedly hold the answers to the mechanism of how Tom20 functions within the receptor

complex to systematically evaluate incoming proteins. Mutations within the cytosolic region of hTom20 are now being made and their ability to complement the *Δtom20* yeast strain will be tested as will their ability to import various signals.

The last 5-10 years have seen the elucidation of the machinery which translocates mitochondrial proteins into the organelle and new understandings of the pathways on which proteins are imported. We have identified receptor proteins and now know that they are responsible for binding incoming polypeptides and are indispensible for most mitochondrial proteins. However, the mechanism whereby the cytosolic regions of these receptors is capable of mediating protein-protein interactions remains a mystery. A detailed understanding of the molecular interactions which mediate successful translocation of large polypeptides is the next great challenge of the mitochondrial import field.

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Original contributions to knowledge.

The following findings contained within this thesis constitute original contributions to the field.

1. The import of a Tom70 fusion construct pOMD29 into the outer membrane requires ATP, protease-sensitive components on the surface of the organelle and physiological temperature.

2. The identification of a novel class of mitochondrial targeting signals, termed the signal-anchor sequence, which are selective for the outer membrane.

3. The role of positively charged residues within a signal-anchor sequence functions to enhance the rate of import.

4. The role of the electrochemical potential in protein import into the inner membrane is due to an electrophoretic pull on positively-charged presequences in order to clear them from the translocation machinery.

5. Yeast mitochondria are capable of importing some signals which are not admitted into mammalian mitochondria.

6. The inner membrane translocation machinery demonstrates a distinct specificity for matrix targeting signals relative to the outer membrane.

7. The human receptor Tom20 is capable of transferring the mammalian ability to screen specific cryptic mitochondrial signals to yeast mitochondria.

8. The GIP of the mitochondrial outer membrane, when acting without receptor proteins, does not contain the requisite ability to discriminate against cryptic signals.