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Mitochondria-Targeting Sequence, a Multi-Role Sorting Sequence Recognized at All Steps of Protein Import into Mitochondria

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The intracellular sorting of newly synthesized precursor proteins (preproteins) to mitochondria depends on the "mitochondria-targeting sequence" (MTS), which is located at the amino termini of the preproteins. MTS is required, however, not only for targeting newly synthesized preproteins to mitochondria, but also for all the following steps along the mitochondrial protein import pathway. MTS of nascent preproteins is first recognized by a cytoplasmic molecular chaperone, MSF, and then by Tom70 and Tom20 of the mitochondrial outer membrane receptor complex, Tom5 and Tom40 of the outer membrane protein translocation machinery, Tim23 of the inner membrane protein translocation machinery, and finally the processing peptidase, MPP, in the matrix. MTS is a multi-role sorting sequence which specifically interacts with various components along the mitochondrial protein import pathway. Recognition of MTS at multiple steps during the import of preproteins may contribute to the strict sorting of proteins destined for mitochondria.

Key words: mitochondria, mitochondria import stimulation factor, mitochondrial protein import, mitochondria-targeting sequence, processing peptidase.

Most mitochondrial proteins are encoded by nuclear genes, synthesized by cytoplasmic free ribosomes, and then localized to mitochondria post-translationally (*1, 2*). The proteins destined for the inner compartments, inside the outer membrane, of the organelles are usually synthesized as larger precursor peptides with a cleavable peptide extension at their amino termini, which is called the presequence. The information which directs specific targeting of newly synthesized precursor proteins (preproteins) to mitochondria is in the amino-terminal portion of the presequence (*3*). Some mitochondrial inner membrane and matrix proteins which are synthesized as mature-size peptides by cytoplasmic free ribosomes also contain mitochondria-targeting information in the amino-terminal portion of the peptides (*4, 5*). These particular amino acid sequences which are essential for the import of the preproteins into the inside of mitochondria across the outer and inner membranes are called the "mitochondria-targeting sequence" (MTS) or "matrix-targeting sequence."

Recent studies have shown, however, that MTS is required not only for the targeting and specific binding of the preproteins to mitochondria but also for all the steps along the import process; from the first step, recognition of the nascent precursor peptides by the cytosolic import stimulating factor, MSF (*6*), to the last step, processing of the imported preproteins by the mitochondrial matrix processing peptidase, MPP (*7*). Specific recognition of MTS by the cytoplasmic factor and the matrix protease, in addition to the recognition by the import machineries of the outer and inner membranes, raises the intriguing question of how these diverse proteins along the import pathway recognize the same amino acid sequence, MTS, with accuracy. This review summarizes available information concerning the recognition of MTS at various steps of the mitochondrial protein import process.

Mitochondria-targeting sequence (MTS) in the presequences of mitochondrial preproteins

The lengths of the amino-terminal cleavable presequences of mitochondrial preproteins vary, but are usually in the range of 20 to 40 amino acid residues long, although some preproteins have much longer presequences. Various deletion, mutation, and chimera protein experiments have shown that the amino-terminal portion of the presequence consisting of 15 to 20 amino acid residues, which is dotted with a few positively charged amino acid residues, arginine or lysine residues, contains information necessary and sufficient for the targeting and import of the preproteins into mitochondria (*3*). The essential role of the positively charged amino acid residues in the import was first proposed on the basis of the strong inhibitory effects of synthetic model peptides resembling MTS on the mitochondrial protein import (*8*), and then confirmed by site directed mutagenesis of those amino acid residues (*9, 10*).

It was proposed that the amino-terminal portion of the presequence, in which the mitochondria-targeting information resides, forms an amphiphilic α-helical conformation with the positively charged amino acid residues lined on one side and the hydrophobic amino acid residues on the other

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Abbreviations: MPP, matrix processing peptidase; MSF, mitochondria import stimulation factor; MTS, mitochondria-targeting sequence; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane.

side of the helix (*11*). The presequences of some precursor proteins contain regularly repeated basic amino acid residues in their amino terminal portions, and their predicted secondary structures agree with such a view, but we also know of many other presequences whose secondary structures do not form good amphiphilic α-helices. The amphiphilicity of the amino acid sequence seems to be important, but α-helicity does not seem to be an absolute requirement for the function of MTS (*12*). How these MTSs with apparent structural diversity are all correctly recognized at various steps of the mitochondrial protein import process remains to be clarified.

Recognition of MTS of preproteins by cytosolic import stimulation factor MSF

The requirement of cytosolic factors for the import of *in vitro* synthesized precursor proteins into mitochondria had often been described in the literature since early studies. The first identified cytosolic protein factor was a molecular chaperon, hsp70, whose necessity for mitochondrial protein import was confirmed by biochemical (*13*) and genetical (*14*) evidence. However, hsp70 recognizes and binds the exposed hydrophobic sequences of unfolded proteins in general, and hence cannot be specific to the precursors of mitochondrial proteins. Cytosolic protein factors specifically required for the mitochondrial protein import were described in later years (*6, 15-18*), and a factor, first purified from rat liver cytosol and named "Mitochondria Import Stimulation Factor" (MSF), has been studied in detail (*6, 19-25*).

MSF functions as a conformational modulator of mitochondrial preproteins, catalyzing the depolymerization and unfolding of *in vitro* synthesized aggregated preproteins in an ATP-dependent manner, and forming a complex with the unfolded preproteins to keep them in an import-competent loose conformation (*6*). The depolymerization and unfolding of the preproteins catalyzed by MSF are dependent on the MTS of the preproteins and accompanied by ATP hydrolysis, which is also effectively triggered by short synthetic peptides corresponding to various MTS (*21*). MSF is thus a member of the polypeptide chain-binding protein family with unique recognition specificity, and may be regarded as a private molecular chaperone which specifically recognizes the MTS of the companion.

MSF purified from rat liver cytosol was found to be a heterodimer of 30 and 32 kDa protein subunits, whose primary sequences confirmed that MSF is a member of the 14-3-3 protein family (*20*), a highly conserved and widely distributed eukaryotic protein family, whose physiological functions are not yet clearly understood. A few members of the 14-3-3 protein family have recently been implicated in the regulation of metabolic pathways owing to their binding to some intracellular signalling molecules (*26*).

In addition to its molecular chaperone function of keeping the bound preproteins in an import-competent conformation, MSF has another distinct function of targeting the bound preproteins to mitochondria. The MSF-preprotein complex binds specifically to the outer membrane of mitochondria, and this mitochondria-targeting function of MSF is sensitive to *N*-ethylmaleimide (NEM), whereas the ATP-dependent depolymerization and unfolding of preproteins by MSF is not inhibited by NEM (*19*). It was further confirmed by use of a yeast outer membrane system that the MSF-preprotein complex is bound to Tom37-Tom70 of the membrane, a subcomplex of the outer membrane protein translocation machinery consisting of several Tom (Translocase of the Outer Mitochondrial Membrane) (*27*) proteins, and the preprotein is transferred to Tom20-Tom22, another subcomplex, upon ATP-dependent dissociation of MSF (*23, 24*) (Fig. 1). The role of MSF in targeting of the newly synthesized preproteins to the protein translocation machinery of the mitochondrial outer membrane is thus analogous to that of the signal recognition particle, SRP, in the targeting of the nascent peptides with the signal sequence to the protein translocation machinery of the endoplasmic reticulum (*28*). The ATP-dependent transfer of the preproteins from MSF to the mitochondrial outer membrane receptor complex may be compared with the GTP-dependent transfer of the nascent peptides from SRP to their receptor on the endoplasmic reticulum.

In vitro studies on the targeting of newly synthesized preproteins to the receptors on the surface of the mitochondrial outer membrane have revealed the roles of MSF and hsp70 in mitochondrial protein import (*23*). The MSF-dependent mitochondria targeting pathway is NEM-sensitive and requires the hydrolysis of extra-mitochondrial ATP, whereas the hsp70-dependent pathway is NEM-insensitive and does not require extra-mitochondrial ATP. The NEM-insensitive hsp70-dependent mitochondrial protein import becomes NEM-sensitive when increasing amounts of MSF are added to the import reaction mixture. Two separate targeting pathways for the preproteins, one requiring MSF and the other requiring hsp70, are apparently operative in the cell (Fig. 1), and the contributions of the two pathways are possibly determined by the relative affinities of MSF and hsp70 for various preproteins (*23, 24*). The binding of cytosolic hsp-70 to newly synthesized preproteins seems to be co-translational (*29*), and the requirement of hsp70 varies among different preproteins (*30*).

The recognition of MTS by MSF in the cytoplasm can explain why MTS is functional only when placed at the amino-termini of preproteins (*31*). As the amino-terminal

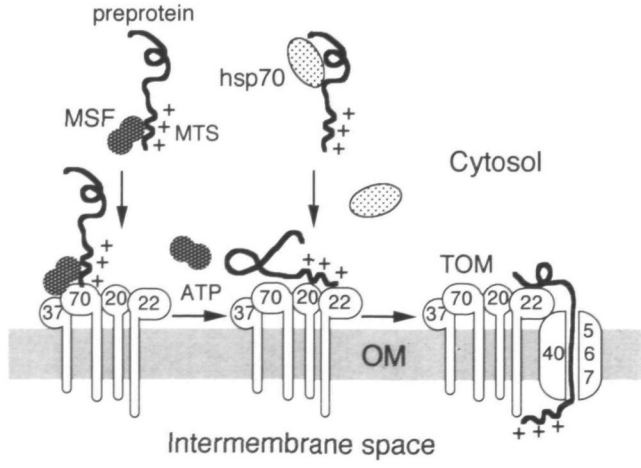

Fig. 1. **Targeting of preproteins to the receptor complex of the mitochondrial outer membrane.** The mitochondria-targeting sequence (MTS) of a preprotein is indicated by positive charges (+ symbols). The numbers in the figure indicate TOM proteins, which constitute the receptor complex and the protein translocation channel of the outer membrane (OM).

MTS emerges from a free ribosome synthesizing the nascent peptide of a mitochondrial preprotein, it is quickly recognized by MSF in the cytosol and forms a MSF-preprotein complex to keep the nascent peptide of the preprotein in an import-competent loose conformation. This may be compared with the recognition of the signal sequence of secretory proteins by SRP as soon as the sequence emerges from a ribosome, although the binding of MSF to the amino-terminal portion of the nascent peptide does not arrest the elongation of the following peptide as in the case of SRP. If MTS is placed at an inner part of the preprotein, it may not be effectively recognized by MSF owing to the interference by the preceding sequence (*31*).

The amino-terminal MTS seems to have an advantage over carboxy-terminal peroxisome-targeting sequence type 1 (PTS1) since rat liver mitochondrial serine:pyruvate aminotransferase is synthesized as a preprotein which has both an amino-terminal MTS and a carboxy-terminal PTS1 but is exclusively sorted to mitochondria *in vivo* (*32*). It is possible that the binding of MSF to the amino-terminal MTS as soon as it emerges from a ribosome gives MTS an advantage over carboxy-terminal PTS1.

The molecular mechanism of interaction between MSF and MTS of mitochondrial preproteins has not yet been clarified. The tertiary structure of a 14-3-3 protein, a mammalian zeta isoform, was determined by X-ray crystallographic analysis, which revealed an amphipathic groove, lined with hydrophobic amino acid residues on one side and polar residues on the other side, in each subunit of the homodimeric protein (*33*). The groove is big enough to accomodate a peptide in an α-helical conformation, and computer graphic analysis showed that an amphipathic peptide helix fits comfortably in the groove (*33*). When the tertiary structure of the MSF-MTS complex is elucidated, it will reveal the details of the interaction between them.

Interaction of MTS with the receptors and protein translocation machinery of the outer membrane

The MSF-preprotein complex is targeted to the Tom37-Tom70 receptor complex on the outer surface of mitochondria. Transfer of the preproteins from MSF to mitochondrial import receptors has been studied by use of the cytoplasmic domains of yeast Tom20 and Tom70 expressed in *Escherichia coli* (*25*). It was found that Tom70 binds the preproteins only in the presence of MSF, yielding a preprotein-MSF-Tom70 ternary complex, from which MSF is released upon ATP hydrolysis, leaving the preprotein-Tom70 complex. In the presence of Tom20, the preprotein is transferred to Tom20 upon ATP hydrolysis. Tom20 itself can also bind preproteins directly in the presence of hsp70, although hsp70 has no affinity for Tom20 and does not form a ternary complex with the preprotein and Tom20, as in the case of MSF (Fig. 1). Therefore, both Tom70 and Tom20 have the binding site for the preproteins, and the binding is dependent on the MTS of the preproteins, although Tom70 functions primarily as the MSF receptor. The MTS-dependent *in vitro* binding of preproteins to the human homologue of Tom20 has also been reported (*34*). The amino-terminal half of Tom20 molecule seems to be sufficient for its function in mitochondrial protein import, suggesting the existence of a MTS-binding site in the amino-terminal half of the molecule (*35*).

The role of MTS in the translocation of preproteins across the outer membrane remains unclear. It was proposed that the presequence of preproteins first binds to the surface receptor, Tom20, on the cytosolic side of the outer membrane (*cis* site), and then is translocated through the protein translocation channel to bind to another specific binding site (*trans* site) on the internal side of the membrane, thus causing the translocation of the following peptide across the membrane (*36*). The outer membrane protein translocation channel is composed of at least four Tom proteins, Tom40, Tom7, Tom6, and Tom5 (*37*). It was recently shown that the presequence of the preproteins becomes in contact with Tom40 after leaving the surface receptor, Tom20, so the *trans* site is possibly the intermembrane space surface of trans-membrane protein Tom40 (*38*). It has also been shown that Tom5 functions as a mediator between Tom20 and Tom40, and mediates the transfer of MTS from the receptor to the translocation channel (*39*). The specific recognition of MTS by these components of the outer membrane protein translocation machinery awaits further clarification.

Role of MTS in protein translocation across the inner membrane

Judging from the ability of the mitoplast to import preproteins into the matrix, the mitochondrial inner membrane has a self-sufficient protein translocation machinery (*40, 41*). The translocation of preproteins across the inner mitochondrial membrane requires the electrochemical membrane potential and is dependent on the MTS of the preproteins. During the translocation of preproteins from the cytosol into the matrix compartment of intact mitochondria, the protein translocation machinery of the inner membrane is connected to its counterpart in the outer membrane by the translocating peptide forming the translocation contact site, although the two protein translocation machineries can function independently (*41, 42*). The protein translocation machinery of the inner membrane consists of several Tim (Translocase of the Inner Mitochondrial Membrane) (*27*) proteins. Tim17 and Tim23 are integral membrane proteins, and are believed to form the protein translocation core of the Tim complex, whereas a peripheral membrane protein, Tim44, is loosely associated with Tim23 and also binds mitochondrial hsp70 (mhsp70) in the matrix. The membrane-bound mhsp70 functions as an ATP-driven import motor that pulls the preproteins into the matrix (*43, 44*).

The role of MTS in the translocation of preproteins across the inner membrane has not yet been clarified. It has been shown that the membrane potential is required for the translocation of the presequence across the inner membrane, but not for the translocation of the mature part of preproteins (*45*). It has also been shown that Tim23 forms a dimer in the inner membrane depending on the membrane potential, and the dimer dissociates upon the interaction with the presequence of the preproteins to trigger opening of the translocation channel for passage of the preprotein peptides across the membrane (*46*). The interaction of Tim23 with MTS seems to be the initial event of the protein translocation across the inner membrane. Another Tim component, Tim22, was recently shown to be required for the import of the ADP/ATP carrier protein into the inner membrane (*47*).

Tim44 on the matrix-side surface of the inner membrane

recruits mhsp70 when a preprotein peptide emerges from the translocation channel of the Tim complex to drive the ATP-dependent translocation of the peptide into the matrix across the membrane (*45*). Whether Tim44 recognizes MTS when the amino-terminal presequence of the preprotein emerges from the protein translocation channel is not yet known. It is highly likely, however, that the recognition of MTS by some components of the Tim complex is necessary for the translocation of preproteins across the inner membrane in addition to its recognition by Tim23 at the outer surface of the inner membrane protein translocation channel.

MTS exhibits specific affinity for lipid membranes containing cardiolipin, which is a characteristic lipid constituent of the mitochondrial inner membrane (*48*). It is not known, however, whether the interaction of MTS with the membrane lipid contributes to the translocation of preproteins across mitochondrial membranes.

Necessity of MTS for the processing of imported preproteins by matrix processing peptidase MPP

As soon as preproteins are imported into the matrix space of mitochondria, their presequences are proteolytically removed to produce the mature size functional proteins (*1, 2*). The processing of most preproteins comprises single step endoproteolytic cleavage of the presequence by matrix processing peptidase MPP, but the presequences of some preproteins are cleaved in two steps; the first cleavage is catalyzed by MPP, and then the resultant intermediate form still bearing a short extra peptide of 8 amino acid residues at its amino-terminus is further processed by a second endopeptidase in the matrix, which is named mitochondrial intermediate peptidase (MIP), to produce the mature size proteins (*49, 50*). Why some preproteins require two step processing of their presequences is not yet known. The MPP of animal, yeast, and fungus mitochondria is a soluble matrix protein, whereas the general processing peptidase of plant mitochondria, the plant counterpart of animal MPP, is a membrane-bound protein integrated into the cytochrome *c* reductase complex (b-c_1 complex) of the inner membrane (*51*). Another distinct protease, which is homologous to *Escherichia coli* leader peptidase, is located on the outer surface of the inner membrane, and catalyzes the processing of the preproteins in the intermembrane space (*52*). Processing of the preproteins in mitochondria is not coupled with their import. Unprocessed preproteins may accumulate in the matrix compartment when the activity of MPP is inhibited by a metal chelator (*53, 54*) or by low temperature (*55*).

Purified MPP preparations were obtained from *Saccharomyces cerevisiae* (*56*), *Neurospora crassa* (*57*), and rat (*58*). MPP is a metalloprotease consisting of two non-identical subunits, α- and β-subunits, and catalyzes the specific endoproteolytic cleavage of the presequences of various mitochondrial preproteins. Its proteolytic activity seems to be specific to mitochondrial preproteins, and it does not show non-specific protease activity. Analysis of a large number of preproteins revealed that a basic amino acid residue, usually arginine, is often present at the -2 or -10 position relative to the cleavage site (*59, 60*). Cleavage of the presequence by MPP followed by the second cleavage by MIP explains the arginine residue at the -10 position. However, it is obvious that the "arginine -2 rule" is not sufficient for explaining the specific proteolysis of mitochondrial preproteins by MPP since arginine residues are present at many positions in various proteins.

A specific structural feature common to the presequences of mitochondrial preproteins is the existence of MTS at their amino termini, although the presequences vary widely in length and exhibit little primary sequence homology. Inhibition studies involving synthetic peptides and site-directed mutations of the basic amino acids in the presequences indicated the recognition of MTS by MPP (*7, 61, 62*). The importance of an ariginine residue present at the -2 position from the cleavage site was also confirmed (*61-63*). It was further shown with the preprotein of mitochondrial malate dehydrogenase that a proline and/or glycine residue must be present in the linker region of the presequence between the cleavage site and the upstream second recognition site, and that a hydrophobic amino acid, particularly Phe or Tyr, is required at the $+1$ position from the cleavage site (*64*). The flexible linker peptide seems to facilitate the simultaneous recognition of the two separate sites by MPP. MPP thus shows unique specificity toward its substrate proteins, recognizing two separate elements in the preproteins, the amino-terminal MTS and the sequence around the cleavage site (Fig. 2), whereas most other sequence-specific endopeptidases recognize only a few amino acid residues around the cleavage site.

An interesting example is the precursor of adrenodoxin, which has a long presequence consisting of 58 amino acid residues. Inhibition of the processing of the preprotein by synthetic peptides corresponding to various portions of the presequence showed that the middle portion of the long presequence, in which several basic amino acid residues are present, and not the amino-terminal MTS, is needed for the processing by MPP (Fig. 2), which was also confirmed by site-directed mutations of the basic amino acid residues in the middle and amino-terminal portions of the presequence (*7*). The length and flexibility of the linker sequence between the cleavage site and the upstream second recognition site, usually MTS at the amino-terminus, seem to be important for efficient processing of preproteins by MPP.

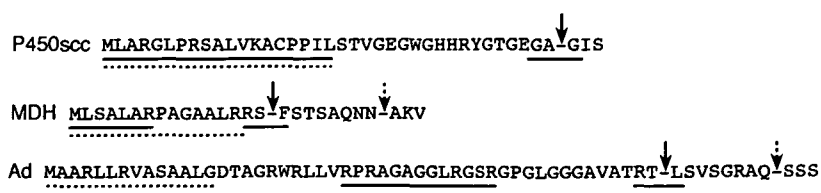

Fig. 2. **Recognition of the amino-terminal presequence of preproteins by matrix processing peptidase MPP.** P450scc, MDH, and Ad stand for bovine cytochrome P450scc, rat mitochondrial malate dehydrogenase, and bovine adrenodoxin, respectively. The processing sites for MPP and the mitochondrial intermediate peptidase, MIP, are indicated by arrows and dotted arrows, respectively. The processing site and the upstream second recognition site of MPP are underlined with solid lines. The MTS of each presequence is underlined with a dotted line.

Both the α- and β-subunits are needed for reconstitution of the proteolytic activity of MPP with the subunits (*57, 62, 65*). The separate roles of the two subunits, one containing the catalytic site and the other having an enhancing function, in the recognition and proteolytic cleavage of the presequence by MPP was suggested (*57*). The β-subunit has a conserved metal-binding sequence, HXXEH, and seems to bear the catalytic site for the proteolysis (*66*). The role of each subunit in the recognition of MTS has, however, not yet been clarified.

Recognition of MTS at multiple steps during the import of preproteins into mitochondria

It is obvious that the "mitochondria-targeting sequence," MTS, is a multi-role sorting sequence, which functions not only at the step of "targeting" of newly synthesized preproteins to the organelle but also at various steps along the mitochondrial protein import pathway. MTS is first recognized by the cytoplasmic molecular chaperone, MSF, to form the MSF-preprotein complex. This complex is targeted to the Tom37-Tom70 receptor subcomplex on the surface of mitochondria, and then MTS is recognized by Tom20 to transfer the preprotein to the Tom20-Tom22 receptor subcomplex. After translocation of the preprotein across the outer membrane through the protein translocation channel, where MTS seems to interact with Tom5 and Tom40, MTS is again recognized by Tim23 of the inner membrane protein translocation machinery. Finally, the MTS of the preprotein imported into the matrix space is recognized by the processing peptidase, MPP, to generate the mature-size functional protein. MTS may also be recognized by some other components of the protein translocation machineries during the translocation of the preproteins across the outer and inner membranes, but the role of MTS in the translocation process is not yet clearly known.

Why is MTS needed for the translocation of peptides across the outer and inner membranes even after the preproteins have been correctly targeted to mitochondria owing to MTS-dependent recognition by the surface receptors? Recognition of the same MTS at multiple steps during the import of preproteins into mitochondria may contribute to the strict sorting of the proteins to be localized to the organelle. The positive charges of MTS may be essential for $\Delta\Psi$-dependent translocation of the preproteins across the inner membrane. It is also possible that the structural requirements for MTS to be recognized at various steps during the preprotein import are not identical. How MTS is recognized by the diverse protein components along the mitochondrial protein import pathway is an intriguing problem to be solved.

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