How membrane proteins travel across the mitochondrial intermembrane space

Carla M. Koehler, Sabeea Merchant and Gottfried Schatz

A newly discovered family of small proteins in the yeast mitochondrial intermembrane space mediates import of hydrophobic proteins from the cytoplasm into the inner membrane. Loss of one of these chaperone-like proteins from human mitochondria results in a disease that causes deafness, muscle weakness and blindness.

MITOCHONDRIAL PROTEIN IMPORT has been studied intensively for the past two decades and, therefore, it was assumed that the basic mechanisms of the import machinery were known. However, new components that play a role in the import of integral proteins of the inner membrane have now been discovered in the mitochondrial intermembrane space.

Import of cytoplasmically synthesized precursors starts with their binding to chaperones in the cytoplasm, followed by binding to import receptors on the mitochondrial surface (Fig. 1)1-3. The precursors then pass through the translocon of the outer membrane (the TOM complex)4. The TOM complex consists of the receptors Tom20p, -22p, -37p, -59p and -70p (which have overlapping roles in recognizing mitochondrial precursors), the pore-forming component Tom40p and the three small Tom proteins, Tom5p, -6p and -7p. After passing through the TOM complex, the precursors engage the translocon of the inner membrane (the TIM complex), in which Tim17p and Tim23p form a pore. ATP hydrolysis by a translocation motor, composed of Tim44p, mitochondrial heat shock protein 70 (mhhsp70) and the nucleotide exchange factor mitochondrial GrpE (Mgep1p), drives the translocation process to completion. An arrested translocation intermediate can span both the TOM and TIM complexes simultaneously5. Finally, the targeting sequence is removed by the matrix processing peptidase and a battery of chaperones in the matrix mediates the folding of the imported protein.

It seemed reasonable to assume that mitochondrial membrane proteins follow a similar import route. Studies on protein import in the endoplasmic reticulum are mechanistically identical to protein transport across a membrane except that, for insertion, further transport is prevented by a hydrophobic stop-transfer sequence in the transported protein. Why should this principle not also hold for mitochondrial protein import? The proteins would follow the same import pathway as outlined above but remain in the TIM complex because of the stop-transfer sequence, and then escape laterally into the lipid bilayer of the inner membrane. Because the 'matrix import' pathway involves temporary linking of the TOM and TIM complexes, there was no need to explain how a hydrophobic inner-membrane protein would negotiate the aqueous intermembrane space. Indeed, such an explanation would have been difficult, given that all efforts to find conventional chaperones in the intermembrane space had failed. The import pathway of the inner-membrane ATP/ADP carrier (AAC) had been characterized biochemically more than ten years ago1 but only in the past two years have some of the key components of this pathway been defined. The newly discovered components are located in the intermembrane space and the inner membrane.

A protein import machinery in the intermembrane space

The important role of the intermembrane space in mitochondrial protein import came to light in an unrelated study. Schwery and colleagues identified two essential proteins of similar sequence, mitochondrial RNA splicing protein 5 (Mrs5p) and Mrs11p, of which overexpression suppressed respiratory defects caused by the loss of mitochondrial RNA splicing (Fig. 2)6,7. Both proteins were located in the intermembrane space. Because most essential mitochondrial proteins participate in protein import8, Mrs5p and Mrs11p were attractive candidates for novel components of a protein import system. Indeed, mttrogenation of these small proteins, either through the use of temperature-sensitive versions11 or by downregulation of the wild-type proteins12, resulted in mitochondria that were depleted specifically of several integral inner-membrane proteins, including AAC, the inorganic phosphate carrier (P'C) and the dicarboxylate carrier (DC). The two proteins proved to belong to a second TIM system that specializes in the transport of hydrophobic proteins into the inner membrane. Accordingly, Mrs5p and Mrs11p were renamed Tim11p and Tim12p.

Early evidence for a second TIM system

Sirenenberg et al.13 caught the first glimpse of a new import pathway two years ago when they identified Tim22p, an essential 22 kDa inner-membrane protein, on the basis of its similarity to the mitochondrial ADP/ATP carrier (AAC). The new protein seemed to participate in mitochondrial import, it was not part of the well-characterized Tim17p-Tim23p complex. Rather, Tim22p was recovered from detergent-solubilized mitochondria in a separate high-molecular-weight complex14. Tim54p, the first partner of Tim22p, was identified by Kerscher et al.14. Activation of Tim54p in a temperature-sensitive mtt54 mutant inhibited import of AAC into isolated mitochondria, and Tim54p interacted genetically and physically with Tim22p (Ref. 14).

These findings demonstrated that import of AAC and other inner-membrane carriers requires a complex comprising...
Tim22p and Tim54p. It was also sug-
gested that carrier import bypasses the
Tim17p-Tim23p system of the general
protein import pathway. Three pieces
of evidence showed directly that Tim22p
is not required for import of AAC. First,
saturation of the Tim17p-Tim23p com-
plex with an arrested precursor did not
significantly inhibit AAC import. Sec-
ond, inactivation of Tim22p in a tem-
perature-sensitive tim22 yeast mutant
did not block import of AAC but did block
that of proteins imported by the same
matrix import pathway. Finally, whereas
precursors with an N-terminal
peptide sequence bound to the inter-
membrane space domain of Tim23p,
AAC did not. Interaction of the prese-
tence with Tim22p presumably allows
docking of the TOM complex in the outer
membrane to the TIM complex in the
inner membrane. The absence of an
interaction between AAC and Tim22p
raised the possibility that transfer of
AAC from the TOM complex to the inner
membrane necessitated either some
other linker molecules or transport of
the hydrophobic AAC precursor across
the aqueous intermembrane space.

**Different roles of Tim10p and Tim12p**

The functions of Tim10p and Tim12p
were deduced by two slightly different
approaches that yielded similar conclu-
sions. Fractionation of yeast mito-
chondria showed that most of Tim10p
was located in the soluble intermem-
brane space, whereas Tim12p was pe-
ripherally bound to the outer surface of
the inner membrane. Both proteins could
be crosslinked chemically to a partly
imported AAC precursor, which indicates
that they interact directly with the im-
ported protein. However, the different
intramitochondrial locations of Tim10p
and Tim12p reflect their different func-
tions in the import pathway. Inactivation
or depletion of Tim12p did not interfere
with import of AAC into the inter-
membrane space but prevented inser-
tion of AAC into the inner membrane. In
contrast, inactivation or depletion of
Tim10p blocked import of AAC, P, and
Tim22 across the outer membrane. Thus,
Tim10p functions before Tim12p, prob-
ably by binding the incoming precursor
as it emerges from the TOM complex.

**A 300 kDa ‘insertion complex’ in the
inner membrane**

Partner proteins (i.e. interacting pro-
teins or components that are part of the
same complex) for Tim10p and Tim12p
were found by genetic and biochemical
approaches. A multiplicity
suppressor screen with the temperature-sensitive
Tim12p mutant identified
Tim22p (Ref. 11). The very
same protein that had al-
ready been implicated in the
import of AAC (Ref. 13).

The genetic interaction between
Tim12p and Tim22p corre-
sponded with a physical in-
teraction as shown by co-
immunoprecipitation and
affinity purification with
hexahistidine-tagged Tim10p.

Further analysis showed that the majority of
Tim10p was present as a
70 kDa soluble complex in
the intermembrane space and
a minor fraction was found as a
300 kDa membrane-bound complex,
together with Tim12p, Tim54p
and Tim22p (Refs 18,19). This
membrane complex is thus
a distinct TIM complex spe-
cialized in the insertion of
imported proteins into the
inner membrane. The exact
composition of the 300 kDa
‘insertion complex’ is not
yet known.

**Two 70 kDa TIM complexes in the
soluble intermembrane space**

A partner protein for
Tim10p was found as a spon-
taneous, extragenic sup-
pressor of the temperature-
sensitive Tim12p mutant
and, in a separate study, by
co-immunoprecipitation. The yeast genome
encodes three small proteins that resem-
ble Tim10p and Tim12p (Refs 18,19); the rest
of the yeast genome contains loosely
associated Tim8p and three small Tom proteins, Tom5p, -6p,
-22p, -37p and -70p, the pore-forming
component Tom40p and Tom20p, and an associated
ATP-driven protein transport
motor on the inner face of the inner membrane. The latter
component Tom20p, -22p, -37p and -70p, the pore-forming
component Tom40p and three small Tom proteins, Tom5p, -4p,
-7p, and that of proteins imported by the
matrix import pathway. Three pieces
of evidence showed directly that Tim22p
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that of proteins imported by the
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other linker molecules or transport of
the hydrophobic AAC precursor across
the aqueous intermembrane space.

The nature of this interaction was
revealed by measuring the import of a
broader spectrum of integral inner-
membrane proteins in strains that lack
Tim10p or Tim11p (Ref. 22). Tim22p
and Tim11p were imported less efficiently
into Tim10p-deficient mitochondria,
The import pathway could be even simpler than shown in Fig. 3. It is possible that the small Tim proteins are not essential for the imported substrates to viability even though they mediate the import of an essential protein. It is conceivable that the intermembrane space has a battery of chaperones with overlapping function (like the chaperones in the cytosol) to facilitate import across the aqueous compartment. The essential function of Tim1p and Tim1p might lie at the inner membrane rather than in the intermembrane space. If so, one might expect to find even more chaperone-like proteins in the intermembrane space.

How do the 70 kDa TIM complexes recognize their substrate proteins? The amino acid sequences of the small Tim proteins are 25% identical and 50% similar to each other. They also share a ‘twin Cx,C’ motif, in which two cysteine residues are separated by three amino acids and each Cx,C sequence is separated from its twin by 11–16 amino acids. This motif is reminiscent of a canonical zinc finger but with a longer spacer. Recombinant Tim1p and Tim1p fusion proteins bind and Tod interaction between Tim1p and AAC is inhibited by zinc chelators. This

which suggests that their import is aided by the Tim1p-Tim1p complex. Indeed, a Tim1p translocation intermediate could be crosslinked to the Tim8p–Tim13p complex. Because Tim1p is an essential protein, Tim1p and Tim1p are therefore also expected to be essential. However, the residual import of Tim1p in the absence of Tim1p and Tim1p seems to be sufficient to keep yeast cells alive. Thus, the Tim1p–Tim13p complex works in parallel with the Tim1p–Tim13p complex by mediating the import of a subset of integral inner-membrane proteins.

Import models

Figure 2

Proteins related to Tim1p of the small Tim (translocase of the inner membrane) protein family in Saccharomyces cerevisiae and humans (h). Black bars mark the ‘twin Cx,C’ motif. Identical residues are blue, similar ones are green (0.4 was set as the minimum for identity and similarity, using the program Boxshade at the European Molecular Biology net, http://www.ebi.ac.uk/).

Figure 3

Import of proteins into the mitochondrial inner membrane (IM). As the precursor emerges from the translocation contact site (Fig. 2), it binds to the Tim1p–Tim1p or Tim1p–Tim13p complex (both in yellow) of the intermembrane space (IMS). The import intermediate is partially degraded by added protease. It predicts a transient complex in which the TOM complex as well as the small Tim proteins are bound to the precursor.

Both models raise intriguing questions. What is the mechanism by which the precursor is handed from one complex to another? Do the two 70 kDa complexes select the imported substrates as they emerge from the TOM complex? Is the transfer of the precursor between the different Tim complexes mediated by conformational changes of those proteins?

The import pathway could be even simpler than shown in Fig. 3. It is possible that the small Tim proteins are not essential for the imported substrates to reach the inner membrane but they might only facilitate the process. This possibility is supported by the finding that Tim1p and Tim13p are not essential for viability even though they mediate the import of an essential protein. It is conceivable that the intermembrane space has a battery of chaperones with overlapping function (like the chaperones in the cytosol) to facilitate import across the aqueous compartment. The essential function of Tim1p and Tim1p might lie at the inner membrane rather than in the intermembrane space. If so, one might expect to find even more chaperone-like proteins in the intermembrane space.

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suggests that the small Tim proteins bind zinc and that zinc binding is re-
quired for their function in vivo. A role for zinc in chaperone function has
already been suggested because the molecular chaperones DnaJ and Hsp33
each possess a zinc-finger-like domain. That of DnaJ is required for recognition
and binding of denatured proteins22, whereas the chaperone function of
Hsp33 is induced by the release of zinc from the zinc-finger-like domain23. The
zinc-binding regions of Tim11p and Tim12p might be structurally important,
either for formation of the correspond-
ing oligomeric complexes or for the interaction with substrate proteins. The zinc-binding motif of Tim11p and
Tim12p could interact with those extra-
membrane loops of AAC that appear to
contain mitochondrial targeting infor-
mation12,21. An analogous study with Tim23p also revealed that positively
charged residues in the extramembrane
loops are required to mediate insertion
into the inner membrane25.

Although it is possible that the small
Tim proteins recognize specific se-
quence motifs12,21, it is equally plausible
that they simply recognize unfolded proteins. The latter hypothesis is
supported by the finding that the
Tim9p-Tim10p complex mediates the
import of Coq2p, Tim11p and Tim17p,
all of which lack sequence similarity to
the metabolite transporters22. In this
case, it could also be relevant that the
import receptor Tom70p binds to
different regions of the P/C
(Ref. 30).

Biogenesis of this import machinery
itself is complicated because individual
subunits use different pathways21. Tim54p is imported via Tim11p-Tim19p
(Ref. 22) and inserted into the inner
membrane through the Tim17p-Tim23p
machinery21, whereas Tim22p is im-
ported via the Tim22p-Tim54p com-
plex21,22. Import of the small Tim pro-
tiens bypasses the Tim machinery
altogether: it requires Tom5p but no
membrane potential22. The complex
interplay between the different ma-
nachines might ensure coordinate regu-
lation of the assembly of the mitochon-
ndrial-protein import systems.

Defective protein import: a novel type of
mitochondrial disease

Humans contain at least five hom-
ologs of the small Tim proteins found in the yeast mitochondrial
membrane. One of these homologs had al-
ready been termed deafness-dystonia
peptide (DDP) because its loss results in the
severe X-linked Mohr-Tranebjærg
syndrome, which is characterized by
dearness, dystonia, muscle weakness,
dementia and blindness32,33. The other
four homologs are encoded auto-
sonically. Because one of these four homologs is closely related to DDP, the
initially discovered isoprotein, DDP, is
referred to as DDP1, and the second one as DDP2 (Ref. 20). The remaining three
human homologs are named hTim9,
hTim10 and hTim13 because of their
sequence relationships to the correspond-
ing yeast proteins.

DDP1 is most similar to yeast Tim1p
(Fig. 4) and, when expressed in monkey
cells, is located in mito-
ochondria. Mohr–Tranebjærg syndrome is, therefore, almost certainly a new
type of mitochondrial disease caused by
a defective protein import system of
mitochondria. Loss of DDP1 function
probably lowers the mitochondrial
abundance of some inner-membrane
proteins that are crucial for the func-
tion, development or maintenance of
the sensorineural and muscular systems
in mammals. The findings from yeast
suggest that DDP1 functions as a com-
plex with related partner proteins. On
the basis of sequence homology and iso-
electric point, hTim13 might be a part-
ner of DDP1 or DDP2. As mutations in
DDP1 partner proteins could also be
deleterious and because all potential
partner proteins are encoded auto-
sonomically, non-X-linked diseases with
symptoms resembling those of Mohr–
Tranebjærg syndrome might well have
a related etiology. Further, the link
between a mitochondrial import defect
and a neurodegenerative disease could
provide insights into the molecular
basis of other, more frequent neurologi-
cal diseases, such as parkinsonism, that
have been correlated with mitochondrial
dysfunction.

Concluding remarks

In the past two years, new proteins in
the intermembrane space and inner
membrane have been identified that
mediate the import and insertion of
inner-membrane proteins. Possible hom-
ologs of the small Tim proteins are
encoded in many eukaryotic genomes,
including those of plants, nematodes,
mammals, lungi, plasmodia and ciliates,
which suggests that these proteins are
ubiquitous in eukaryotes. In plants, they
are probably restricted to mitochondria.
Although the complete genome se-
quence of a plant is not yet available, it
appears that the number of possible
homologues encoded in the Arabidopsis
genome, so far, is not significantly
higher than that encoded in mammalian
genomes. This would not be expected if
the proteins functioned also in the
plastid. Furthermore, all the identified
Arabidopsis homologs are as closely
related to the corresponding yeast
proteins as to the human and Caenor-
habditis elegans proteins, which, again,
argues against divergence to a plastid
pathway.

Why does overexpression of Tim11p
or Tim12p suppress defects in the
splicing of mitochondrial RNA? These
defects are also suppressed by over-
expression of Msr3p and Msr4p, two
mitochondrial metabolic carriers of
unknown substrate specificity35. In-
creasing the amounts of Tim11p and
Tim12p might raise the levels of Msr3p
and Msr4p in the mitochondrial inner
membrane and thereby enhance the
transport of a crucial co-factor, such as a
metal ion that is required to attenuate
or bypass the splicing defect.

This protein import pathway still
poses many intriguing questions. One of
them concerns the interaction between
the two 70 kDa complexes and the
300 kDa inner-membrane complex (Fig. 5).

Another unresolved question is the role
of the 300 kDa insertion complex. Is this complex a scaffold that facilitates proper folding of the incoming membrane protein before inserting it en bloc into the inner membrane? Or is the complex a pore through which a multiprotein membrane is ’itched’ into the inner membrane? Molecular dissection of this import pathway will provide further insights into the mechanism by which proteins are inserted into a membrane.

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Recent electron microscopic studies have revealed new details of the supramolecular organization of actin filaments in lamellipodia. The leading edge is characterized by a distinctive, extensively branched network of actin filaments. Consistent with previous models for protrusion, barbed ends were numerous near the leading edge but, surprisingly, free pointed ends were not detectable. Instead, pointed ends were involved in structural association with the sides of other filaments at ~70° angles, resulting in Y junctions and the formation of a diagonal network filling the lamellipodium (Fig. 1a). These structural studies suggested that lamelipodial protrusion needed to be re-interpreted in terms of the formation and recycling of a branched filament network.

Arp2/3 complex

A key component in understanding the origin of the branched actin filament network is the Arp2/3 complex (reviewed in Refs 5, 6). This consists of actin-related proteins 2 and 3 and five other proteins. It localizes to the leading edge of crawling cells and to cortical actin patches in yeast, and it is sufficient to induce actin polymerization at the surface of Listeria cells. The Arp2/3 complex acts on the sides of actin filaments and to their pointed ends and nucleates actin filaments.

REVIEWS

Tatyana M. Svitkina and Gary G. Borisy

The crawling movement of a cell involves protrusion of its leading edge, in coordination with the translocation of its cell body, and depends upon a cytoplasmic machinery able to respond to signals from the environment. Protrusion is now understood to be driven by actin polymerization, and signaling from membrane receptors to actin has been shown to be mediated by the Rho family of GTPases. However, a major gap in our understanding of regulated motility has been how to connect the signalling pathway to the motile machinery itself. Recent structural, biochemical and genetic studies have identified some of the missing links and provided a strong working model for the pathways and mechanisms by which the signals are interpreted and implemented.

TWO MAJOR PROTRUSIVE organelles exist in crawling cells: lamellipodia, which contain a network of diagonally oriented actin filaments, and filopodia, which contain a tight bundle of parallel actin filaments, the filaments invariably oriented with their barbed ends forward. Models that have been proposed to account for polymerization-driven protrusion of lamellipodia involve the nucleation of actin filaments at the membrane and their subsequent release, or the continuous treadmilling of actin filaments by growth at their barbed end and shortening at their pointed end.

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