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Signals and Receptors—The Translocation Machinery on the Mitochondrial Surface[1]

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Most proteins involved in mitochondrial biogenesis are encoded by the genome of the nucleus. They are synthesized in the cytosol and have to be transported toward and, subsequently, imported into the organelle. This targeting and import process is initiated by the specific mitochondrial targeting signal, which differs pending on the final localization of the protein. The preprotein will be recognized by cytosolic proteins, which function in transport toward the mitochondria and in maintaining the import competent state of the preprotein. The precursor will be transferred onto a multicomponent complex on the outer mitochondrial membrane, formed by receptor proteins and the general insertion pore (GIP). Some proteins are directly sorted into the outer membrane whereas the majority will be transported over the outer membrane through the import channel followed by further distribution of those proteins.

KEY WORDS: Import receptor; mitochondria; Tom complex; translocation; preprotein recognition.

INTRODUCTION

Over the past decade much has been learned about the composition of the mitochondrial translocation complex in yeast and *Neurospora crassa* (for review see Neupert, 1997; Pfanner *et al.*, 1997; Schatz, 1996), and a recent publication (Kunkele *et al.*, 1998) describes, for the first time, the low-resolution structure of the general insertion pore (GIP). In addition, the latest findings have increased the understanding of the sorting of proteins in the inner membrane space (for review, see Pfanner, 1998) and the export of mitochondrial proteins into the inner membrane (for review, see Stuart and Neupert, 1996). To date, not much is known about cytosolic sorting of preproteins; however, the function of the sorting and recognition system on the outer membrane has been addressed in recent publications. Investigation of the single components of the receptor complex and GIP has led to a better understanding of the translocation process.

FIRST STEP OF TRANSLOCATION–CYTOSOLIC SORTING

Until now, there has been little evidence of cotranslational import of proteins into mitochondria (Verner, 1993). Recent work (Crowley and Payne, 1998) has discussed the possibility of a GTP-dependent mediation between a ribosome complex and the mitochondrial surface involving an unidentified *receptor* protein. If this receptor is involved in the import machinery and if the targeting of the ribosome to the mitochondrial surface leads to cotranslational import remains to be further investigated.

Most experiments have focused on posttranslational import of proteins into mitochondria (Neupert, 1997). Since not all proteins synthesized in the cytosol

[1] Key to abbreviations: BNG, blue native gel; GIP, general insertion pore; HSP, heat-shock protein; Mft52, mitochondrial fusion protein targeting factor of 52kDa; MSF, mitochondrial stimulation factor; NAC, nascent polypeptide-associated complex; PBF, presequence binding factor; pOCT, preornithine carbamyltransferase; TF, targeting factor.

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are transported to the mitochondrial surface, an earlier sorting event than that of the Tom[4] machinery seems to be required. Several proteins have been identified to be involved in sorting and transport of preproteins to the mitochondrial membrane. These are the chaperones of the Hsp70 family (Ssa1-2p/Hsc70) (Deshaies *et al.*, 1988; Sheffield *et al.*, 1990), proteins of the DnaJ family (Rassow *et al.*, 1997), the mitochondrial-stimulation factor (MSF) (Hachiya *et al.*, 1993), a so-called targeting factor (TF) (Ono and Tuboi, 1988), a presequence binding factor (PBF) (Murakami and Mori, 1990), a mitochondrial fusion protein targeting factor (Mft52) (Cartwright *et al.*, 1997), and the nascent polypeptide-associated complex (NAC) (George *et al.*, 1998).

HSP70 proteins have a general function for protein folding rather than a specific function for proteins, which have to be imported into the mitochondria. However, import of pOCT into mammalian mitochondria is drastically decreased in the absence of Hsc70, a protein of the HSP70 family (Terada *et al.*, 1996), demonstrating the importance of those chaperones for insertion of preproteins into the mitochondria. Further, Hsp70 proteins act in cooperation with DnaJ proteins. Two members of this class, Ydj1p (yeast) (Cyr *et al.*, 1994) and hdj2 (human), were identified to be important for import (Terada *et al.*, 1997).

MSF is a member of the 14-3-3 family of proteins and was found to act as a chaperone specific for mitochondrial preproteins by interaction with their import signals. MSF is recognized by Tom70, a receptor of the import complex, in an ATP and preprotein-dependent manner (Mihara and Omura, 1996). Earlier, a 28-kDa protein was found to stimulate protein import into mammalian mitochondria and was named targeting factor (TF) (Ono and Tuboi, 1990a). Furthermore, a 50-kDa mammalian protein named preprotein binding factor (PBF) was identified to bind to the presequence of mitochondrial preproteins (Murakami and Mori, 1990). Subsequent investigation has demonstrated that PBF does not stimulate import for all targeting signal-containing proteins (Murakami *et al.*, 1992) and its function remains unclear. In parallel, a cytosolic protein in yeast (Mft52) interacts with preproteins. It was speculated that this interaction is mediated by a so called *acid bristle*, a motif first proposed to be formed by the Tom20/Tom22 complex (Lithgow *et al.*, 1997). This protein might be the yeast homolog of the earlier identified PBF, since similar function and biochemical properties were observed. NAC was reported to be important for delivery of preproteins to the mitochondrial surface, but this was found to be a general function for the transport of proteins to organelles rather than a specific function for preproteins targeted to the mitochondria.

Even if the interplay between these cytosolic factors is not yet known, it is becoming clear that the recognition of preproteins by the import complex is not the first sorting event. However, after transport of the preproteins to the mitochondrial surface, they are recognized by the receptor complex of the outer mitochondrial membrane. Since the discovery of the first receptor protein, a multicomponent complex has been found to be involved in the recognition of proteins to be translocated into the organelle. In addition, the biochemical properties and the structure of the import signal have been studied.

TERMINAL TARGETING SIGNAL OF PRECURSOR PROTEINS

Preprotein recognition by the receptor complex is initiated by specific import signals. Two major classes of signals exist: internally and terminally (mostly N-terminal) located signal sequences. Terminal targeting signals are subdivided into three classes: signal anchor, matrix targeting, and stop transfer sequences. The signal anchor sequence is found in proteins anchored to the outer mitochondrial membrane by one transmembrane domain. Stop transfer signals warranty insertion into the inner membrane and the matrix targeting signal directs proteins over both mitochondrial membranes into the matrix of the mitochondria.

The Signal Anchor Sequence

Proteins imported into the outer membrane contain so-called signal anchor sequences (Blobel, 1980). These sequences are composed of a positively charged, hydrophilic part, followed by a hydrophobic sequence and are located either at the N- or C-terminus of the preprotein. The hydrophobic region causes arrest in the outer membrane; as well, it acts as a membrane anchor for the imported protein (Shore *et al.*, 1995). Interestingly, the positively charged domain does not mediate the import, but rather the hydrophobic region.

[4] Nomenclature for the import complex (Pfanner *et al.*, 1996).

The amphiphilicity of the positively charged region was found to determine the orientation of the protein, since an increase of the hydrophobic moment within the signal of yTom70 rather then an increase of the net charge results in a reversed orientation of the protein in rat mitochondria (Steenaart *et al.*, 1996). Recognition by hTom20 is dependent on both, the hydrophobic and the charged region of the signal (Schleiff and Turnbull, 1998a) and involves electrostatic interaction (Schleiff *et al.*, 1997b). However, the hydrophobic region was found not to be essential for the recognition of the preproteins by yTom20. The positively charged region alone (the so-called *cryptic matrix targeting signal*) was capable of translocating proteins into yeast mitochondria (Hurt *et al.*, 1985) in a yTom20 dependent manner, since no import could be found into yeast mitochondria lacking yTom20, even after complementation with hTom20 (McBride *et al.*, 1996). In addition, no import of this signal into mammalian mitochondria was observed. This suggests that either the import of signal anchor sequence containing proteins differs between these two species or that the yeast apparatus has a lower specificity than the human apparatus in recognition of presequences. The later explanation is supported by the finding that yTom20 interacts with the cryptic signal, but hTom20 does not (Schleiff *et al.*, 1997b and unpublished observation). Furthermore, the positively charged region of the signal anchor sequence of Tom70 was able to insert into the mitochondria after removal of trypsin-sensitive components from the mammalian mitochondrial outer membrane (McBride *et al.*, 1996). This finding is consistent with the idea that one component of the mammalian system was blocking the access to the GIP either by denying an interaction with the preprotein or by blocking a binding side at the GIP.

Therefore, the signal for correct insertion into the outer membrane of yeast mitochondria can be divided into two functionally distinct parts, one for recognition by the translocation complex, acting like a matrix-targeting signal, and one for insertion into the outer membrane. The recognition of signal anchor sequences by the mammalian receptor complex requires the complete signal, suggesting that those signals can not be divided into regions with distinct functions.

The Matrix Targeting Sequence

Proteins imported into the matrix contain an N-terminal targeting sequence, which usually (but not always) is cleaved after translocation of this protein into the mitochondria. This matrix-targeting signal is comprised of 20 to 70 residues (Hartl *et al.*, 1989) and can form an amphiphilic α-helix in a hydrophobic environment. These signals are basically charged in addition to their amphiphilicity. The amphiphilicity was found to be an important feature of presequences, since artificial sequences (Allison and Schatz, 1986) can only act as import signals if they are amphiphilic (Roise *et al.*, 1988).

Signal peptides lack a defined structure in solution, but adopt a mainly α-helical conformation in a membrane environment (Roise and Schatz, 1988; Tamm, 1991). This conformational change does not depend on whether or not the protein will be processed after import (Hammen *et al.*, 1994). Since a basic amphiphilic helix is a common secondary structural motif, but not every protein containing such a motif will be imported into mitochondria, it can be argued that the signal sequence has to be on the surface of the protein, suggesting that either the protein is fully folded before import or the mature part remains bound to a *transporter* leaving only the targeting signal accessible. The conformational change in a membranelike environment is believed to be an important step in protein translocation. This idea is supported by the finding that peptides representing a signal sequence have a high affinity for membrane surfaces (Skerjanc *et al.*, 1987; Tamm, 1991) and so do precursor-containing proteins (Skerjanc *et al.*, 1988). In addition, the binding of tightly folded mitochondrial precursor proteins to the mitochondrial outer membrane involves a lipid-mediated conformational change (Endo *et al.*, 1989). Further, recent findings have demonstrated that the preprotein recognition domain of hTom20 is in close approximation to the membrane surface (Schleiff and Turnbull, 1998b) and the interaction between receptor and preprotein is increased in the presence of the membrane environment-induced amphiphilic α-helix (Schleiff and Turnbull, 1998a). This parallels earlier findings that the positively charged amino acids of the presequence of cytochrome oxidase subunit IV involved in the electrostatic interaction between the precursor and the receptor are exposed to the surface rather then inserted into the bilayer (Chupin *et al.*, 1996). In addition, an alteration of the secondary structure of the signal in a membranelike environment because of interactions between signal and passenger molecule can significantly inhibit import (Waltner *et al.*, 1996). Together, these results underline the importance of the structural reformation of the signal

sequence and suggest that lipid contact and subsequent formation of an α-helix are important steps in recognition and translocation of preproteins.

Earlier studies have discussed the possibility of import of preproteins induced by the insertion of the signal sequence (Maduke & Roise, 1993), but to date the *in vivo* evidence for such a process is missing.

The cleavage of the precursor occurs at a specific site recognized by the mitochondrial-processing peptidase (MPP), which is discussed elsewhere (Hartl *et al.*, 1989; Niidome *et al.*, 1994). Interestingly, the targeting information is not necessarily restricted to the cleavable precursor since it was found that the recognition of the preprotein F_0-ATPase subunit 9 by the receptor complex is dependent on its mature part (Pfanner *et al.*, 1987b).

The Stop-Transfer Signal

Proteins containing a terminal signal and imported into the inner membrane are translocated due to their stop transfer signals. A classical example is the signal of subunit Va of yeast cytochrome *c* oxidase and the import mechanism is reviewed in Shore et al. (1995). In brief, the N-terminal one-third of the protein contains the targeting information recognized by the receptor complex (Lithgow and Schatz, 1995); the transmembrane domain is located at the C-terminus. Deletion of the transmembrane domain results in mistargeting of the protein into the matrix, suggesting a combined action of the N-terminal matrix-targeting signal and the transmembrane domain. One important difference between signal anchor and stop transfer sequences is the location of the transmembrane domain, since the spacing between the positively charged region of the targeting sequence and transmembrane domain warranties a contact between the translocation machinery at the inner membrane and the N-terminally located targeting sequence. Recent results reveal there is also a difference in hydrophobicity of the membrane sequence between stop transfer and signal anchor sequence. The yTom70 signal anchor sequence could only be altered to a stop transfer signal after downstream fusion to the matrix targeting signal from pOCT, when the net hydrophobicity was increased from 1.17 to 1.74 (hydrophobicity scale, Kyte and Doolittle) (Steenaart and Shore, 1997). Therefore, the targeting sequence of a stop transfer signal is a typical matrix-targeting sequence as described earlier and the arrest in the inner membrane is caused by the strong hydrophobicity of the transmembrane domain.

INTERNAL SIGNAL SEQUENCES OF PRECURSOR PROTEINS

Two classes of proteins contains internal targeting signals—the beta barrel proteins of the outer membrane, *e.g.*, the voltage-dependent anion channel (VDAC or porin) and the integral proteins inserted into the inner membrane, *e.g.*, uncoupling protein (UCP), ATP/ADP carrier (AAC), phosphate carrier (PC), and some proteins involved in the translocation apparatus. In addition, a new form of internal signal has been described for Tom22 (Rodriguezcousino *et al.*, 1998). Recently the first steps toward understanding these import signals have been taken.

The Internal Targeting Information of Inner Membrane Proteins

It had been found that some inner membrane proteins contain cleavable N-terminal sequences. However, these sequences are not important for targeting to the inner membrane (Zara *et al.*, 1992). The newest results suggest that the internal signal for import into the inner mitochondrial membrane might be composed of a hydrophobic sequence and the orientation of the protein is defined by positive charges in loop regions facing the matrix (Davis *et al.*, 1998), similar to the positive-inside rule found for proteins of the bacterial inner membrane (von Heijne, 1992). Tom20 recognizes these proteins via hydrophobic interaction (Schleiff and Turnbull, 1998a) underlining the importance of the hydrophobic region as targeting signal. Earlier studies have demonstrated that neither UCP nor ADP/ATP carrier (AAC) contains an N-terminal targeting sequence (Liu *et al.*, 1988; Pfanner *et al.*, 1987a; Smagula and Douglas, 1988). The first 111 amino acids of AAC, but not the first 72, are able to translocate a hybrid protein into a state resistant to protease treatment (Smagula and Douglas, 1988). In agreement with this finding, the first 105 amino acids of UCP were found to translocate carbamyltransferase into a partly protease and alkali-resistant state, whereas amino acids 1–51 do not. However, UCP lacking the first 102 amino acids was still imported into a protease-resistant state (Liu *et al.*, 1988). Recent results have demonstrated that the region from residue 101 to 201 is

essential for import into the mitochondria and binding experiments have revealed that amino acids 1–95 can act as an artificial signal (Schleiff, in preparation). Although understanding of these signals has improved, their still require further investigation.

Targeting of Outer Membrane Proteins

The signal for outer membrane proteins remains unknown, but it is worthwhile to mention that recent findings suggest that the import pathway of porin might differ from the general import pathway. Porin was found to be self-imported into lipid bilayers in the absence of receptor proteins (Xu and Colombini, 1996). In contrast, the import of porin into mitochondria is dependent on interaction with a receptor protein and this interaction has a hydrophobic character (Pfaller and Neupert, 1987). Later studies have revealed the dependence of its import on the outer mitochondrial receptor Tom20 (Sollner *et al.*, 1989; Moczko *et al.*, 1994; Millar and Shore, 1996) and porin was found to bind directly via hydrophobic interaction to this receptor (Schleiff *et al.*, 1997b). This finding suggests a hydrophobic signal within the protein for recognition by the receptor complex.

In the case of nTom22, an outer membrane protein with one transmembrane helix, an internal basic region (residues 45–75) was identified to be essential for the import of this protein. This region is predicted to be an α-helix followed by a loop (PHD; Rost & Sander, 1993). Therefore, this region might be surface exposed and act as a signal anchor sequence, since the hydrophobic transmembrane part is separated from this helix by only eight amino acids (Rodriguezcousino *et al.*, 1998). In addition, this region contains five basic charges and the α-helix shows an amphiphilic character. The import mechanism might now be explained by the findings of Steenaart et al. (1996), since this helix might comprise a retention signal because of its high amphiphilicity.

THE RECEPTOR COMPLEX

The import signal of a preprotein is recognized by a receptor complex, which is located on the mitochondrial surface, probably in close proximity to the GIP. Recent results suggest that the interaction between the GIP and receptors is dynamic and might be mediated by the signal sequence of the preprotein (Rapaport *et al.*, 1998; Pfanner, 1998). None of the receptor domains on the mitochondrial surface were found to be essential, but the import process is slowed after deletion of certain receptors (Dekker *et al.*, 1998). Today, the receptor complexes of yeast and *N crassa* are best understood. Five receptors have been identified in these organisms, Tom20, Tom22, Tom37, Tom70 and a homolog of Tom70, Tom71(72). Tom71(72) is only present at very low concentrations compared to Tom70 and might be an alternatively spliced form of this receptor (Bomer *et al.*, 1996; Schlossmann *et al.*, 1996). The four major receptors are proposed to form two hetero-dimers, Tom70–Tom37 and Tom20–Tom22, which have somewhat different functions.

Tom70/Tom37

Tom70 was first described in 1983 (Riezman *et al.*, 1983) and later identified as receptor for AAC carrier proteins, the F_1-ATPase β-subunit, and cytochrome c_1 (Sollner *et al.*, 1990; Hines *et al.*, 1990). This receptor was found to interact with several preproteins *in vitro* (Hines and Schatz, 1993; Schlossmann *et al.*, 1994), but is not essential for cell viability since deletion of Tom70 resulted only in growth rate reduction (Riezman *et al.*, 1983). It could have been speculated that Tom20 complements the function of Tom70, since Tom20 and Tom70 were found to act in parallel (Steger *et al.*, 1990). Surprisingly, the deletion of both Tom20 and Tom70 also does not result in a complete loss of import (Lithgow *et al.*, 1994b). It was found that Tom70 interacts with the mature part of preproteins since the import of proteins consisting of a passenger molecule and a targeting signal was not influenced by either the presence or absence of Tom70 (Hines and Schatz, 1993). In addition, *in vitro* binding of preproteins containing internal targeting signals to Tom70 was observed. However, the authors did not control the dependence of this interaction on chaperones present in the lysate (Brix *et al.*, 1997). This would have been important since an earlier study has revealed that Tom70 recognizes MSF (Hachiya *et al.*, 1995). The main function of Tom70 might, therefore, be the recognition of chaperon and the interaction with preproteins containing internal targeting information.

Tom70 was found to interact with both receptor complex components Tom37 and Tom20 in yeast. The interaction between Tom70 and Tom37 was found to be stable, whereas the interaction between Tom70 and

Tom20 is proposed to by dynamic since it is not detectable with blue native gel (BNG) electrophoresis (Dekker *et al.*, 1998). It was suggested that the interaction between Tom70 and Tom20 (Haucke *et al.*, 1996) or Tom37 (Haucke and Lithgow, 1997) is mediated by a so-called tetratricopeptide repeat motif (Goebl and Yanagida, 1991) present in Tom70 (Haucke and Lithgow, 1997) and partially in Tom20 (Moczko *et al.*, 1994) and Tom37 (Gratzer *et al.*, 1995). This interaction might mediate a transfer of preproteins from the MSF/Tom70/Tom37 complex to Tom20. However, Tom70 might also be able to interact with other components of the outer mitochondrial translocation complex, since import of carrier proteins was not disrupted by deletion of Tom20.

Tom37 is only found in yeast (Gratzer *et al.*, 1995) and its precise function remains unclear. Deletion of Tom37 results in a reduction of import efficiency for carrier proteins. The protein was identified to interact with Tom70 and it might be involved in recognition of MSF (Hachiya *et al.*, 1995). Further investigation will have to demonstrate what function Tom37 has in the Tom37–Tom70 complex.

Tom20/Tom22

Tom20 and Tom22 have been identified in *N. crassa* (Sollner *et al.*, 1989; Kiebler *et al.*, 1993) and in yeast (Ramage *et al.*, 1993; Lithgow *et al.*, 1994a); in mammals, only Tom20 has been identified so far (Goping *et al.*, 1995; Hanson *et al.*, 1996; Seki *et al.*, 1995; Iwahashi *et al.*, 1997). BNG electrophoresis has revealed that the receptor complex of plant mitochondria does not contain Tom22 (Jansch *et al.*, 1998). PotatoTom20 has a different morphology than is predicted for Tom20 from *N. crassa*, yeast, or mammals (Heins and Schmitz, 1996).

Both receptors were found to hetero-dimerize (Mayer *et al.*, 1995). BNG electrophoresis has revealed the possibility that Tom20 acts outside of this dimer and is not stable associated with the general insertion pore (GIP) (Dekker *et al.*, 1998; Schleiff, *et al.* 1999). Tom20 was found to interact with all classes of preproteins *in vitro* (Schleiff *et al.*, 1997a; Lithgow and Schatz, 1995) in the absence of mediator molecules. Antibodies against Tom20 inhibit import of all classes of preproteins (Sollner *et al.*, 1989; Ramage *et al.*, 1993; Millar and Shore, 1996) and deletion of Tom20 results in a decrease of import of most preproteins (Moczko *et al.*, 1994; Bolliger *et al.*, 1995). Tom22 only interacts with proteins bearing a terminal targeting sequence (Brix *et al.*, 1997). However, only Tom22 was found to be essential for cell viability (Lithgow *et al.*, 1994a; Honlinger *et al.*, 1995). This may be explained by the fact that Tom22, but not Tom20, was found to be a major component of the GIP (Dekker *et al.*, 1998). This finding is supported by the observation that deletion of both the cytosolic and intermembrane space domain of Tom22 does not result in complete loss of import competence (Moczko *et al.*, 1997), whereas deletion of the transmembrane domain of Tom22 results in loss of cell function and subsequent growth arrest (Nakai *et al.*, 1995).

Interaction with preproteins was proposed to be via an *acid bristle* motif (Bolliger *et al.*, 1995) formed by Tom20 and Tom22. Indeed, Tom20 and Tom22 interact with proteins containing a terminal targeting sequence via electrostatic interaction (Haucke *et al.*, 1995; Komiya *et al.*, 1998; Schleiff *et al.*, 1997b; Brix *et al.*, 1997). Proteins bearing an internal targeting signal are recognized by Tom20 by hydrophobic interaction (Schleiff *et al.*, 1997b). The region proposed to form the acid bristle was not found to be involved in interaction with preproteins in the human homolog (Schleiff and Turnbull, 1998a) and the same region is not essential for the function of rat Tom20 (Iwahashi *et al.*, 1997). The replacement of the charges in the cytosolic domain of nTom22 by noncharged residues did not result in the expected decrease of import or binding (Nargang *et al.*, 1998). However, other components of the receptor complex might have complemented the disfunction of Tom22, since the cytosolic domain of Tom22 was found not to be essential for viability and import (Moczko *et al.*, 1997). The intermembrane space domain of Tom22 plays a more important role in translocation then the cytosolic domain since it was found to be an important binding side on the *trans* site of the GIP (Bolliger *et al.*, 1995). Tom20 bears two distinct binding sites for internal or terminal-located signals (Schleiff and Turnbull, 1998a), but only the domain found to interact with the terminal targeting signal is essential for its function (Iwahashi *et al.*, 1997).

In order to understand the process of recognition of preproteins, it became essential to determine the affinities of the receptors for the precursor proteins. Since no energy-dependent interaction or release has been found, a translocation driven by increasing affinities was proposed and the first evidence for this was shown by Komiya *et al.* (1998). Such a process would require significant differences in affinity between bind-

ing sites. The first dissociation constant reported was for the intermembrane space domain of Tom22 and aspartate aminotransferase (K_d = 220 nM) and chaperonin 10 (K_d = 800 nM) (Bolliger *et al.*, 1995). Consistent with the idea of an affinity-driven process, the dissociation constant for the interaction between preproteins and Tom20 was found to be in the micromolar range (Schleiff and Turnbull, 1998a,b).

The General Insertion Pore (GIP)

The general insertion or import pore is composed of Tom40, the channel-forming protein (Hill *et al.*, 1998), Tom5, Tom6, Tom7, and Tom22.

The Small Toms

In the yeast system, three small Tom proteins were identified: Tom5, Tom6, and Tom7 (Sollner *et al.*, 1992; Kassenbrock *et al.*, 1993). In the plant import machinery, a fourth small Tom protein was identified, called Tom9 (Jansch *et al.*, 1998). The interaction between these small Tom proteins and Tom40 is proposed to be driven by the transmembrane domains and, therefore, hydrophobic forces. Treatment with urea or salt does not influence the complex formation with Tom40 (Dekker *et al.*, 1998).

Tom7 was identified as an integral outer mitochondrial membrane protein (Honlinger *et al.*, 1996), which assembles with GIP. Deletion of this small Tom protein results in a stable assembly of the receptor complex with the translocation pore and the deletion of Tom20 and Tom7 results in a growth defect of yeast, suggesting a function of Tom7 in disassembly of the complex between Tom20–Tom22 and the GIP.

Tom6 (Kassenbrock *et al.*, 1993) was found to be important for assembly of the GIP–Tom20–Tom22 complex. Tom6 deletion results in a destabilization of the receptor GIP complex and, therefore, in a stabilization of the preprotein receptor complex and, subsequently, in a delay of preprotein import (Alconada *et al.*, 1995). Subsequent investigation of the remaining GIP complex revealed that deletion of Tom6 results in disassembly of both receptor proteins, Tom22 and Tom20, from the complex (Dekker *et al.*, 1998). A double deletion of Tom6 and Tom7 results in loss of cell growth (Honlinger *et al.*, 1996), suggesting that the Tom6 and Tom7 regulate the association and dissociation of the receptor proteins from the GIP, which is essential for the translocation and import process. How this mechanism works remains to be investigated.

Tom5 mediates the transport of preproteins from the receptor complex to Tom40 (Dietmeier *et al.*, 1997). A stable association of Tom5 with Tom40 was proposed since the presence of Tom5 results in the protection of the N-terminus of Tom40 against trypsin cleavage. This association is not regulated by Tom6 or Tom7, since deletion of those proteins results in a stable subcomplex between Tom40 and Tom5 (Dekker *et al.*, 1998). Tom5 interacts with all preproteins independent of the nature of the targeting signal and acts as the last general receptor on the *trans* side of the import pore.

Two acidic regions in the cytsosolic domain of Tom22 were found to be important for the regulation of the oligomerization state of the pore complex (Rapaport *et al.*, 1998). As for the small Tom proteins, the interaction of Tom22 with Tom40 is proposed to be mediated by the transmembrane domain, since the association is detergent, but not urea- or salt-sensitive (Dekker *et al.*, 1998). Until now, it remains unclear how the surface exposed regions can regulate the oligomerization behavior of the GIP and how this mechanism is controlled.

A Hole in the Wall—Tom40

This protein was first identified in yeast as an outer mitochondrial membrane protein involved in import, antibodies raised against this protein were able to block import (Ohba and Schatz, 1987). This protein, as well, could be cross-linked to the precursor of the cytochrome oxidase subunit IV (Vestweber *et al.*, 1989). Furthermore, Tom40 was identified as the core protein of a large import-competent complex. The protein is largely resistant to protease treatment and is essential for cell viability (Kiebler *et al.*, 1990; Baker *et al.*, 1990).

At the same time a large conductance cationic channel was found (Thieffry *et al.*, 1988) in the outer mitochondrial membrane (Chich *et al.*, 1991). The channel could be blocked by a peptide possessing the sequence of the targeting sequence of cytochrome *c* subunit IV (Henry *et al.*, 1989) and by antibodies against Tom40 (Juin *et al.*, 1997). From the similar characteristic of the inactivation of this channel and VDAC, a similar structure of the pore was expected (Henry *et al.*, 1996). The findings were confirmed by reconstitution of overexpressed Tom40. Tom40 con-

tains >60% beta sheet structure and the pore diameter was calculated to be 22 A (Hill *et al.*, 1998). Studies of the whole outer mitochondrial complex using electron microscopy have revealed a complex with a pore diameter of 20 A (Kunkele *et al.*, 1998) suggesting that Tom40 is the pore-forming protein of the import complex.

The Stoichometry of the Outer Mitochondrial Complex

The ratio of the components of the receptor complex and GIP has been investigated several times in the past (see Table I). The values derived from various methods differ somewhat from each other. However, it is clear that one to two proteins of the small Tom proteins (Tom5, Tom6, Tom7, compared to Tom40) are present in the GIP, and one Tom22 molecule joins a complex of two Tom40 proteins. Controversy remains over the question of how many Tom40 dimers are present in one GIP complex [(two to three in yeast (Dekker *et al.*, 1998) versus four in *N. crassa* (Kunkele *et al.*, 1998))]. The presence of Tom20 and Tom70 in the complex is also in question (~0.2 Tom20 molecules and no Tom70 molecules in yeast (Dekker *et al.*, 1998) versus two Tom20 molecules and one to two Tom70 molecules in *N. crassa* (Kunkele *et al.*, 1998)]. This discrepancy might result from different preparation and quantification procedures, nevertheless, the association of these two proteins has to be dynamic and, therefore, they might not be considered to be part of the core GIP. The total amount of Tom20 and Tom70 was found to be four times lower than with Tom22 and Tom40 (Dekker *et al.*, 1998; Kunkele *et al.*, 1998), which might explain that the ratio derived by coimmunoprecipitation using Tom20 antibodies resulted in different ratios, as found by direct quantification (see Table I).

FURTHER COMPONENTS INVOLVED IN TRANSLOCATION OF PREPROTEINS OF HIGHER ORGANISMS

The first indication of the presence of components in the mammalian system was presented by Gillespie (1987). A surface-exposed and trypsine-sensitive 30 kDa receptor was identified using the targeting sequence of pOCT as a probe. The binding to pOCT was not inhibited by 1 M KCl, suggesting that the interaction is not electrostatic. Therefore, this receptor is unlikely to be a homolog of Tom20 or Tom22, since both receptors bind to preproteins via electrostatic interactions, nor is it a homolog to Tom70/Tom37, since these receptors were found to be involved in recognition of internal targeting sequences and chaperones. The second evidence for the existence of this receptor was presented by Ono and Tuboi (1990b). Using the targeting signal of pOAT, two proteins were purified from mitochondria, a 29- and a 52-kDa protein. Further investigations have shown that the prese-

Table I. Stoichiometry of the Outer Membrane Complex

Method used	Tom5	Tom6	Tom7	Tom20	Tom22	Tom37	Tom40	Tom70		Reference
Coimmunprecipitation of the complex with hTom20 AB	—	—	—	2	1.2	—	2	1	Ratio	Kiebler *et al.*, 1990[c]
Coimmunprecipitation of the complex with hTom20 AB	2	—	2	2	1.2	—	2	1	Ratio	Sollner *et al.*, 1992[c]
[^{35}S]Labeling and phosphoimaging of purified core complex	—	—	—	2	3	—	8	1.5	Ratio	Kunkele *et al.*, 1998[c]
Immunoblotting of BN gel-purified complex—total complex	—	—	—	60–70	200–300	—	250–300	60–70	pmol/mg[a]	Dekker *et al.*, 1998[d]
Immunoblotting of BN gel-purified complex—GIP	—	—	—	4–12	200–300	—	250–300	<3	pmol/mg[a]	Dekker *et al.*, 1998[d]
Immunoblotting of BN gel-purified complex—GIP	2–4[b]	2–4[b]	2–4[b]	~0.2	3–6	—	4–6	—	Ratio	Dekker *et al.*, 1998[d]

[a] pmol receptor/mg mitochondrial protein determined in the presence of lipids.
[b] Estimated from size differences of the complex of yeast strains lacking these components.
[c] *Neurospora crossa.*
[d] Yeast

quence directly interacts with the 29-KDa protein and antibodies against this protein inhibited import. The interaction of the 52-kD receptor was found to form a complex with a cytosolic component, which was previously identified to be involved in import (Ono and Tuboi, 1988), suggesting a relationship between this 52-kDa protein and the Tom70 proteins.

Further, in mammalian mitochondria the importance or existence of a 66-, a 60-, a 42-, a 34-, (Pchelintseva *et al.*, 1995), a 37- (Komiya *et al.*, 1996) a 70-kDa protein and metaxin (Armstrong *et al.*, 1997) was demonstrated. These findings lead to the question of what the function of these new components might be and if there is a higher specialization of the whole complex in higher organisms. However, it should be mentioned that parts of the machinery are conserved as the finding of the homolog of hTom20 to yTom20 or nTom20 and the identification of the outer mitochondrial pore forming protein mTom40 suggests (Lee Rivera *et al.*, 1999).

Metaxin has been identified to be involved in mitochondrial import for mammalian mitochondria, since antibodies raised against it block import of preproteins (Armstrong *et al.*, 1997). Disruption of the metaxin gene in mice leads to an embryonic lethal phenotype (Bornstein *et al.*, 1995), suggesting an importance of that gene in the early development of the embryo. Which function metaxin has and if metaxin might be the homolog of Tom37 found in yeast remains unknown, since the deletion of Tom37 in yeast can not be complemented by metaxin (Armstrong *et al.*, 1997).

A mammalian mitochondrial protein named Tom34 was identified to be involved in protein translocation, since antibodies raised against this protein blocked import of pOCT (Nuttall *et al.*, 1997). It was suggested that the 34-kDa protein and the 59-kDa protein recognized by the antibodies against the 34-KDa protein pOCT (Nuttall *et al.*, 1997) are identical with the 34- and 60-kDa protein found by Pchelintseva *et al.* (1995). The C-terminus of Tom34 is exposed to the cytosol and the protein is resistant to alkali extraction indicating that Tom34 is anchored to the outer membrane. Tom34 contains a TPR motif, which was reported to be involved in interaction with HSP90 (Young *et al.*, 1998). This might be interesting since HSP90 was found to be a chaperone specific for proteins involved in signal transduction (Jakob and Buchner, 1994) and only under stress conditions involved in refolding of other proteins (Schneider *et al.*, 1996). This opens several questions: Is there a link between signal transduction and import in mammalian mitochondria? Is there a "back up" import mechanism for stress conditions? All these findings reported thus far suggest that the structure of the import machinery in mammalian mitochondria is more complex than that found in yeast.

OVERALL MECHANISM

Preproteins are believed to be transported to the mitochondria via an active pathway rather than by random diffusion. It is known that the mitochondrial-stimulation factor (MSF) interacts with the Tom70/Tom37 complex in the presence of a bound preprotein and ATP hydrolysis induces precursor release toward the receptor. Since MSF was able to interact with chemically synthesized presequences, it is believed that MSF directly interacts with the targeting signal. However, no interaction between components of the translocation complex and HSP70 could be identified.

After transport through the cytosol and subsequent release from the *transporter* preproteins containing terminal targeting signals are then transferred to Tom20. Those preproteins were found to bind via electrostatic interaction to a site located close to the membrane as shown in Fig. 1. This interaction occurs after transformation of the targeting signal into an α-helix induced by the lipid surface, a state that is also required for import into the GIP. The protein is then transferred to Tom22 and, subsequently, to Tom5 in the last stage of recognition by the receptor complex. The transport is believed to be affinity driven, it is also known that recognition of these preproteins also induces a rearrangement of the outer mitochondrial import apparatus. Tom40 forms the first binding site on the *trans* side of the outer mitochondrial complex (Rapaport *et al.*, 1997) and after transport through the GIP the preprotein is transferred to the negatively charged domain of Tom22.

For most proteins bearing an internal targeting signal, the same pathway can be proposed. The only major differences are the binding sites and the receptor proteins involved. A preprotein containing an internal binding site is transferred to a binding side of Tom20 believed not to be in close contact with the membrane in order to prevent an hydrophobicity-driven import of these proteins into the outer membrane. Tom22 was not found to be involved in the translocation of these proteins toward Tom5. Furthermore, the interaction with these proteins was found to be hydrophobic rather

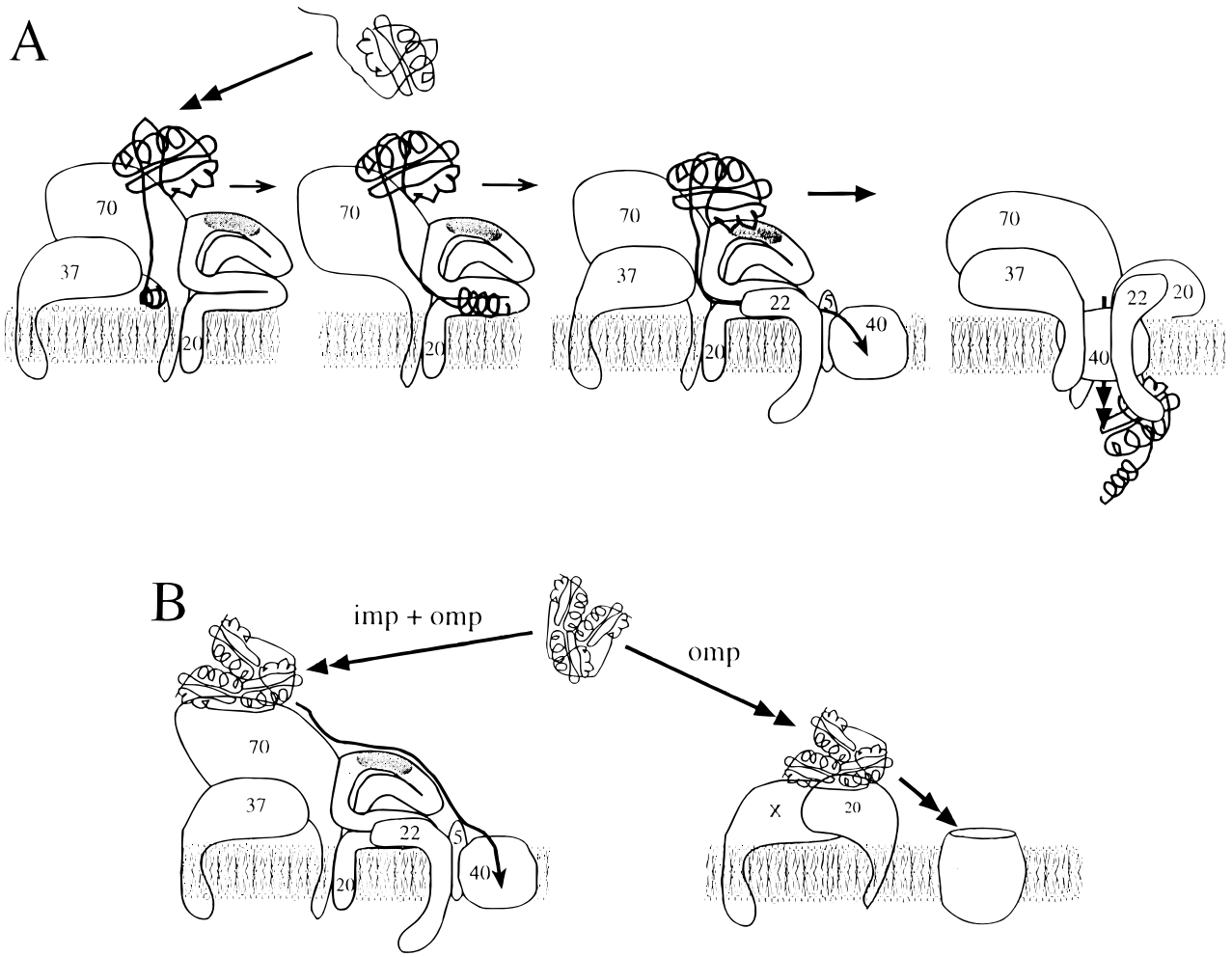

Fig. 1. Preprotein translocation through the outer mitochondrial membrane complex. (A) Translocation of preproteins containing terminal targeting signals. After transport of the preprotein through the cytsosol and recognition by Tom70 in chaperone-complexed form, the targeting signal interacts with the membrane surface and adopts and α-helical state (step 1) and will then be recognized by Tom20 (step 2), transferred toward Tom40 by interacting with Tom22 and Tom5 (step 3), and, subsequently, transferred over the outer membrane (step 4). (B) Import of membrane proteins containing internal targeting signals. The preprotein will either be recognized by Tom70 and transferred over Tom20 and Tom5 toward the translocation channel (left case) or can directly interact with Tom20 and further protein for Tom40-independent insertion into the outer mitochondrial membrane. imp, innermembrane protein, and omp, outer membrane protein.

then electrostatic. Again, Tom5 is the last station of the receptor complex on the *cis* side of the outer membrane.

In addition to the conventional import pathway, proteins imported into the outer mitochondrial membrane can be transported directly into the lipid bilayer. These proteins interact with Tom20 via hydrophobic interactions and Tom20 stimulates the direct import of these proteins into the membrane by recognition. The function of Tom20 might be proposed to be the uptake of those proteins from chaperones as well as the control of the orientation of this proteins (Schleiff *et al.* 1999).

In summary, two pathways of import are proposed. The majority of proteins will be translocated by an affinity and reassembly-driven process via the GIP, whereas proteins translocated into the outer membrane are imported directly in a Tom20-dependent pathway.

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