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The Mitochondrial Import Machinery for Preproteins

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ABSTRACT: Most mitochondrial proteins are transported from the cytosol into the organelle. Due to the division of mitochondria into an outer and inner membrane, an intermembrane space and a matrix, an elaborated system for recognition and transport of preproteins has evolved. The translocase of the outer mitochondrial membrane (TOM) and the translocases of the inner mitochondrial membrane (TIM) mediate these processes. Receptor proteins on the cytosolic face of mitochondria recognize the cargo proteins and transfer them to the general import pore (GIP) of the outer membrane. Following the passage of preproteins through the outer membrane they are transported with the aid of the TIM23 complex into either the matrix, inner membrane, or intermembrane space. Some preprotein families utilize the TIM22 complex for their insertion into the inner membrane. The identification of protein components, which are involved in these transport processes, as well as significant insights into the molecular function of some of them, has been achieved in recent years. Moreover, we are now approaching a new era in which elaborated techniques have already allowed and will enable us to gather information about the TOM and TIM complexes on an ultrastructural level.

KEY WORDS: translocation, TIM, TOM, import receptors.

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I. INTRODUCTION

Compartmentation within any given cell is a prerequisite for maintaining a large variety of anabolic and/or catabolic biochemical pathways in parallel. This is especially true for eukaryotic cells, which have evolved membrane enclosed organelles. An important idea relating to the inheritance of these entities came from George Palade, who proposed that membranes are not formed *de novo* but rather arise from preexisting ones following division of the membranes.[1] This important model explains to some extent the way in which organelle boundaries are preserved. However, although membranes are essential, they alone are not sufficient to maintain the function of an organelle. A well-defined set of proteins must be present within the organelle to facilitate its biochemical function. As all organelles in lower and higher eukaryotic cells are at best genetically semiautonomous and depend on proteins that are nuclear encoded, another problem arose: What determines the subcellular localization of a protein and how is the transport to its destination facilitated. A novel idea directly relating to this problem was formulated by Günther Blobel. The so-called signal hypothesis suggests that proteins carry information within their se-

quence or structure that encodes information as to where this protein should be transported.[2-4] This model implies that other components will assist in the recognition and delivery of the cargo. Moreover, the translocation of these proteins across the membrane barrier must be facilitated by specialized proteins within the membrane that are able to form hydrophilic pores. These proteinaceous channels are the gates for protein entry into a membrane-enclosed compartment. All these proposals have now been supported by experimental evidence.

Mitochondrial research has played an important role for our understanding of the nature of protein transport. Together with work on bacterial protein secretion and translocation of preproteins into the endoplasmic reticulum the analysis of how proteins are targeted to and translocated into mitochondria has significantly contributed to our understanding of the basic mechanisms that underlie these transport processes. In the last 2 decades targeting signals on mitochondrial proteins have been identified, receptors on the outer membrane of mitochondria have been found as well as a general import pore (GIP). Only recently have components of the inner membrane been identified that participate in protein sorting and translocation to and across the inner membrane. Besides these membrane resident protein complexes the translocation of proteins into the matrix requires the assistance of heat shock proteins in the matrix. The components that participate in protein transport into mitochondria have been shown to be well conserved among lower and higher eukaryotes.[5-7]

This article aims to provide an overview on protein transport into mitochondria. A major aspect will be attributed to the work done on *Saccharomyces cerevisiae* and *Neurospora crassa*, both of which have been and still are the model organisms for most analyses in the field.

II. POSTTRANSLATIONAL IMPORT AND CYTOSOLIC FACTORS

Mitochondria are believed to have descended from an endosymbiotic procaryote. Through evolution this endosymbiont has turned into an essential cellular organelle and has lost most of its genetic information to the nucleus during this process. Accordingly, only a small number of mitochondrial proteins are encoded and synthesized within the mitochondrion itself, while the other approximately 99% are nuclear encoded and synthesized on cytosolic ribosomes. It is a generally accepted view that these proteins are released from the ribosome and transported to and into mitochondria in a posttranslational manner.[8-10] However, even though *in vitro* import of most mitochondrial proteins tested occurs posttranslationally, we cannot exclude the possibility that cotranslational import occurs *in vivo* in special cases.[11,12]

After or during the completion of synthesis the newly made mitochondrial preproteins are bound by cytosolic factors. This interaction is necessary to protect proteins from aggregation and to keep them in an unfolded or partly folded conformation, which is essential for the subsequent translocation process. Proteins that assist mitochondrial protein import in a general manner include members of the cytosolic Hsp70 family.[13,14] They have also been implicated in other protein transport pathways. Other cytosolic factors have been described that seem to specifically assist mitochondrial proteins and may even participate in the targeting process. Among these are MSF (mitochondrial import stimulating factor) and PBF (presequence binding factor).

Heterodimeric MSF is an ATPase that belongs to the growing family of 14-3-3 proteins. MSF was isolated from rat liver

cytosol by means of affinity chromatography on presequence peptides. Additional studies demonstrated that MSF stimulates import of proteins into mitochondria as well as dissociating aggregated mitochondrial preproteins, restoring their import competence.[15] Furthermore, there is evidence that precursor-bound rat MSF can bind to yeast Tom37 and the receptor Tom70 on the outside of the mitochondrial membrane.[16]

In contrast to MSF, the properties of PBF have not been well defined. It was initially identified as a reticulocyte lysate protein that binds to the precursor of rat ornithine carbamoyltransferase (OTC) and stimulates its import into mitochondria.[17,18] The current view is that PBS cooperates with Hsp70 in maintaining proteins in an import competent conformation.[17,18]

At this point it is necessary to mention that, while functional data for both MSF and PBF has been obtained in *in vitro* import reactions, an *in vivo* role in protein transport to mitochondria remains to be established.

III. TARGETING SIGNALS FOR MITOCHONDRIAL PROTEINS

Amino acid sequences that are sufficient and necessary to direct a protein into mitochondria are considered to represent mitochondrial targeting signals. These signals were studied in great detail early in the history of mitochondrial protein transport and have been the topic of a number of reviews.[19-21] The general experimental approach taken in order to study whether a sequence functions as a targeting signal is to fuse it to a passenger protein such as the cytosolic protein dihydrofolate reductase (DHFR) from mouse.[22-24] The normally cytosol localized DHFR is then directed to a mitochondrial subcompartment by the mitochondrial signal sequence. Beside DHFR-fusion proteins other reporters have also been applied, among these β-galactosidase or invertase,[25,26] however, DHFR has a number of advantages and thus is used most often. Due to these studies two types of targeting signals have been discriminated: amino-terminal matrix targeting signals and internal targeting signals.

A. Matrix Targeting Signals

Virtually all proteins that are destined for the mitochondrial matrix possess a matrix targeting signal at their amino-terminus, which is often referred to as a presequence. Presequences usually consist of 15 to 30 amino acid segments that are rich in positively charged and often hydroxylated amino acids (Figure 1). Moreover, they are predicted to have the capacity to form amphipathic α-helices.[27-29] The recent NMR solution structure of the cytosolic domain of rat Tom20 bound to the presequence of rat aldehyde dehydrogenase has demonstrated that the receptor bound presequence is indeed in a α-helical conformation.[30] This finding together with *in vitro* binding data[31] indicate that the secondary structure of the presequence is a prerequisite for productive association with the receptors. In these experiments the presequence of cytochrome c oxidase subunit IV (pCoxIV) could compete with presequence-containing preproteins for binding to Tom20 and Tom22. In contrast a peptide with a similar charge to pCoxIV but lacking a hydrophobic face was more or less unable to compete for binding to the receptors. Most presequences are cleaved off by a specific peptidase in the mitochondrial matrix once the presequence has reached this compartment. However, there are some exceptions such as rhodanese,[32] mitochondrial thiolase,[33] the β-subunit of the human elec-

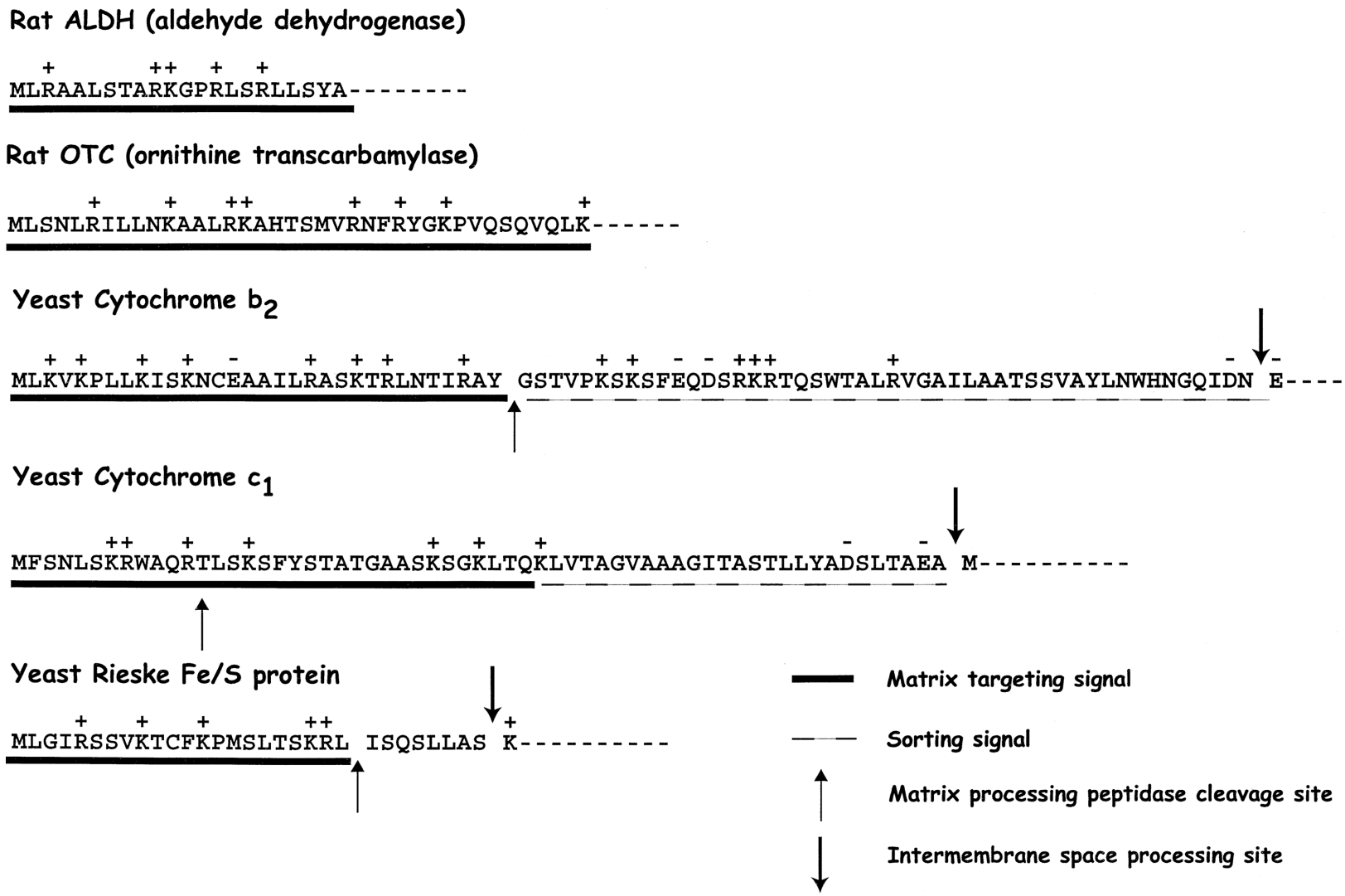

FIGURE 1. Presequences of mitochondrial proteins. Most mitochondrial proteins contain targeting information in their amino-terminus. A matrix targeting signal (underlined in black) directs the protein to the TIM23 complex and into the matrix. A matrix located processing peptidase cleaves the signal off. Proteins destined for the intermembrane space carry a sorting sequence (dashed line) behind the matrix targeting sequence. A peptidase located in the intermembrane space cleaves downstream of the sorting signal and releases the mature protein into the intermembrane space.

tron transfer flavoprotein,[34] and chaperonin 10.[35,36] All of these preproteins contain amino-terminal presequences that are not processed after import.

B. Internal Targeting Signals

A number of proteins that are delivered to mitochondria, among these the mitochondrial carrier protein family,[37] do not contain amino-terminal targeting signals. Elucidation of the topogenic signals responsible for the import of this protein family has drawn much attention. One family member, the ADP/ATP carrier (AAC), has been the "guinea pig" for most targeting studies. However, the experimental approaches that had been employed successfully to study the targeting of presequence-containing proteins were generally unsuitable for the carrier proteins because mutations within the core of the protein can easily affect the structure and/or function of the protein. Nevertheless, experimental evidence indicates that targeting information resides in the amino- as well as the carboxy-terminus of AAC,[38,39] but the signal within these segments has still not been defined. Using a novel experimental approach in which binding of recombinant mitochondrial receptor domains to an immobilized peptide library was employed Brix et al.[40] were able to gather new information. Using 13 mer peptides of the phosphate carrier, they were able to identify a number of interacting sequence motifs within the primary sequence. Both charged and uncharged peptides of the carrier bound to the receptors. This result was in contrast to previous models that indicate that charged patches within the carrier sequence most likely represent the topogenic signals. In summary, these experiments indicate that a complex targeting and recognition process underlies the transport of the carrier proteins. A more detailed description of the carrier transport and recognition process is given below.

IV. THE TOM COMPLEX

A. Import Receptors on the Outer Mitochondrial Membrane

The existence of targeting signals on mitochondrial proteins implies a requirement for a machinery that specifically recognizes them. Outer mitochondrial membrane proteins that possess hydrophilic domains exposed to the cytosol mediate this function (Table 1). The import receptors are at least transiently associated with the general import pore (GIP) through which proteins are eventually imported. The association between receptors and the GIP is mandatory for the transition from recognition of the cargo proteins to the subsequent translocation process. However, given the fact that yeast cells remain viable if any one of the outer membrane receptors is lost, then there must be either redundancy and or backup recognition occurring at the preprotein recognition step.

The three Tom proteins on the mitochondrial outer membrane, Tom20, Tom22, and Tom70, represent without doubt the principal receptors for the two types of mitochondrial preproteins (Figure 2). Tom20 was first identified in *N. crassa*. Antibodies raised against an ~20 kDa outer membrane protein efficiently inhibited the import of a variety of mitochondrial preproteins except for the ADP/ATP carrier (AAC).[41,42] The homologue from *S. cerevisiae* was later identified by a similar approach.[43] Tom20 is a membrane protein that spans the outer mitochondrial membrane once and has a type I topology with the major, carboxy-terminal portion

TABLE 1
The Components of the Translocase of the Outer Membrane (TOM)

Protein	Function	Properties	Ref.
Tom20	Preprotein receptor for presequence containing proteins and some hydrophobic preproteins	Nonessential, N_{in},C_{out}-topology, 1 TPR domain	41,42,43,44
Tom22	Preprotein receptor for presequence containing proteins and hydrophobic preproteins	Nonessential, part of the GIP, Type II membrane protein, potential *trans* binding site	45,48,50,51,52,56
Tom70	Preprotein receptor for hydrophobic preproteins	Nonessential, 7 TPR domains, N_{in},C_{out} topology, integral membrane protein	60,61,62,63
Tom72	Homologue to Tom70	Nonessential, 7 TPR domains, N_{in},C_{out} topology, integral membrane protein	71,72
Tom37	Implicated in protein transport	Nonessential, peripheral outer membrane protein	55, 70
Tom40	Protein conducting channel in the outer mitochondrial membrane	Essential, central component of the GIP	73,74,75,77
Tom6	Promotes GIP assembly	Nonessential, integral membrane protein, part of the GIP	81, 82
Tom7	Promotes GIP disassembly	Nonessential, integral membrane protein, part of the GIP	80
Tom5	Preprotein recognition and transfer into GIP	Nonessential, integral membrane protein, part of the GIP	79

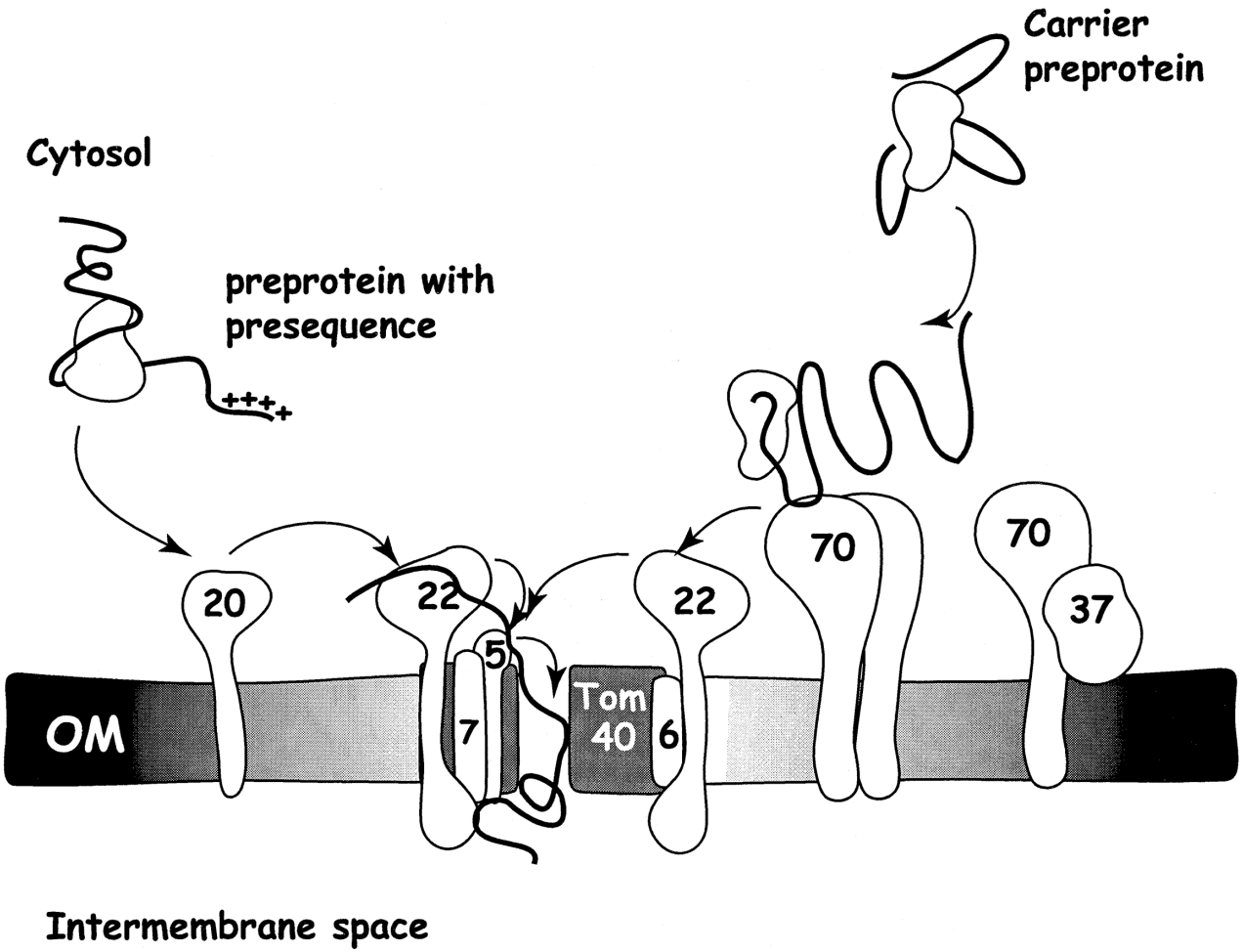

FIGURE 2. Protein transport across the outer mitochondrial membrane. Preproteins with presequences or internal targeting information are transported to the outer surface of mitochondria in a loosely folded conformation. Cytoplasmic heat shock proteins prevent the cargo molecules from folding. The receptor subunits of the translocase of the outer membrane (TOM complex) recognize the preproteins and transfer them to the general import pore (GIP). Tom40 is the channel forming subunit of the GIP. OM, outer membrane.

of the protein protruding into the cytosol.[43,44] The NMR structure of a portion of the carboxy-terminal domain of Tom20 in complex with a presequence peptide was recently determined[30] (for review see Ref. 21). This finding represents the most direct demonstration that Tom20 binds to two turns of the α-helix formed by the presequence. Moreover, it showed that only one side of the presequence was in contact with the shallow binding pocket. This finding might explain how a large variety of presequences, with heterogeneous primary but most likely similar secondary structure, are able to fit into the same binding site.

Tom20 physically associates with Tom22.[45,46] Moreover, Tom20 plays a critical role in the biogenesis of Tom22,[47] leading to significantly reduced amounts of Tom22 in mitochondria from mutant yeast cells lacking Tom20 (*tom20Δ*).[48,49] Accordingly, some of the protein transport defects observed in *tom20Δ* mitochondria might be indirectly caused by the reduction of Tom22. Tom22 was initially identified in *N. crassa* in a screen with antibodies directed against a 22-kDa outer membrane protein[50] and subsequently in *S. cerevisiae* by genetic means.[48,51,52] In contrast to Tom20 and Tom70, Tom22 is a type II membrane protein that consists of a small carboxy-terminal domain that protrudes into the intermembrane space and a larger cytosolic domain. The cytosolic portion interacts with presequences.[46,52] Initial studies suggested that Tom22 is essential for cell viability,[48,52,53] however, recently van Wilpe et al.[45] were able to generate viable *tom22*Δ cells using a gentle procedure. The main functional difference between Tom20 and Tom22 is that Tom22 is a structural component of the GIP,[45] while Tom20 only weakly associates with this complex.[54] Moreover, it is generally believed that the two receptors recognize different surfaces of the amphipathic presequence. While Tom20 binds to the presequence via hydrophobic interactions,[30,31] Tom22 seems to associate via electrostatic interactions.[31] It is the current view that preproteins with amino-terminal presequences are initially recognized by Tom20 and subsequently handed over to Tom22 (Figure 2) from which the preprotein is subsequently fed into the GIP. Another important aspect of Tom22 function is that it not only affects the import of preproteins with amino-terminal targeting signals but also of those with internal signals.[46,50] As Tom70 is the primary receptor for proteins with internal targeting signals,[55] Tom22 receives the proteins from Tom70 and mediates their transfer into the GIP.

In addition to cytosolic receptor domains, that form the so-called *cis* binding site, a requirement for a *trans* binding site has been demonstrated *in vitro*[56] and shown to contribute to the transfer of proteins across the outer membrane. Tom22 not only acts on the cytosolic face of the mitochondrial membrane, but in addition is considered to form the *trans* binding site in the intermembrane space, at the GIP exit site.[57,58] A number of negative charges on the carboxy-terminal domain of Tom22 are believed to represent the region that binds to the positive charges of the presequence as they emerge from the pore.

The third receptor molecule is the type I membrane protein Tom70. Like Tom20, it consists of a large cytosolic domain that participates in recognition of preproteins. Tom70 was initially identified in *S. cerevisiae* as a 70-kDa protein of unknown function.[59,60] Subsequent work on the *N. crassa* homologue, and on the yeast protein itself demonstrated an involvement in protein transport especially for preproteins with internal targeting signals such as the ADP/ATP carrier (AAC) or the phosphate carrier.[61-64] Direct binding of the AAC to Tom70 has been demonstrated by crosslinking[52,55,62,65] and by *in vitro* binding

studies.[31,66,67] While Tom20 was initially shown to possess some affinity for proteins with internal targeting information,[61] it is now clear that Tom70 is the primary receptor for these proteins. However, work by Hines et al.[63] indicated that Tom70 is also involved in import of F_1-ATPase β-subunit and cytochrome c_1. This finding indicates that even though mitochondrial receptor proteins favor binding to certain subgroups of preproteins, they do have overlapping preprotein specificity and hence function.

An additional interesting feature of Tom70 is its ability to dimerize via its membrane anchor (Figure 3).[65,68] Only recently Wiedemann et al.[69] demonstrated that the Tom70 homo-dimer is a functional unit that associates with AAC during import. This process is described in detail in a separate section below.

B. Additional Surface-Exposed Tom Proteins

An additional protein that was originally implicated in import into mitochondria is Tom37.[70] It was identified in a genetic screen for cells with a defect in phospholipid synthesis. Further analysis of the nonessential membrane protein Tom37 indicated that a lack of Tom37 (*tom37Δ*) is synthetically lethal in combination with *tom70Δ* or *tom20Δ*. Moreover, these early studies identified a physical association between Tom70 and Tom37 (Figure 3) and an involvement in protein import. However, more recent experiments demonstrated that Tom37 is only peripherally associated with the mitochondrial membrane and does not stably associate with Tom70. In addition, protein import into *tom37Δ* mitochondria was not affected.[55] In summary, the role of Tom37 in protein transport into mitochondria has not been resolved.

Tom72 was identified by computer searches of the yeast genome that revealed an ORF encoding a protein with significant sequence identity to Tom70.[71,72] Although Tom72 loosely associates with the Tom machinery significant sorting defects have not been attributed to the loss of Tom72.

To reiterate, the receptors Tom20, Tom22, and Tom70 are not functionally fully distinct entities. Instead, they are very dynamic components that form subcomplexes, which associate with the GIP and with each other (Figure 3). Indeed, using different approaches a number of interactions between the receptors themselves as well as receptors and GIP have been found (Figure 3). Some of these interactions are weak or transient and indicate that the TOM complex is not a stable functional unit. In contrast, it needs to be dynamic with regard to association and dissociation of its components in order to fulfill its function.

V. THE GENERAL IMPORT PORE (GIP)

As described above Tom22 is the central receptor to which both Tom20 and Tom70 feed their cargo. In contrast to Tom70 and Tom20, Tom22 is stably associated with Tom40 and the small Toms: Tom5, Tom6, and Tom7 (Figures 2 and 3). Together these five proteins form a 400-kDa complex[54] termed the general import pore (GIP).

A. Tom40 Represents the Protein-Conducting Channel

Tom40 is the major component of the GIP and is the only truly essential Tom

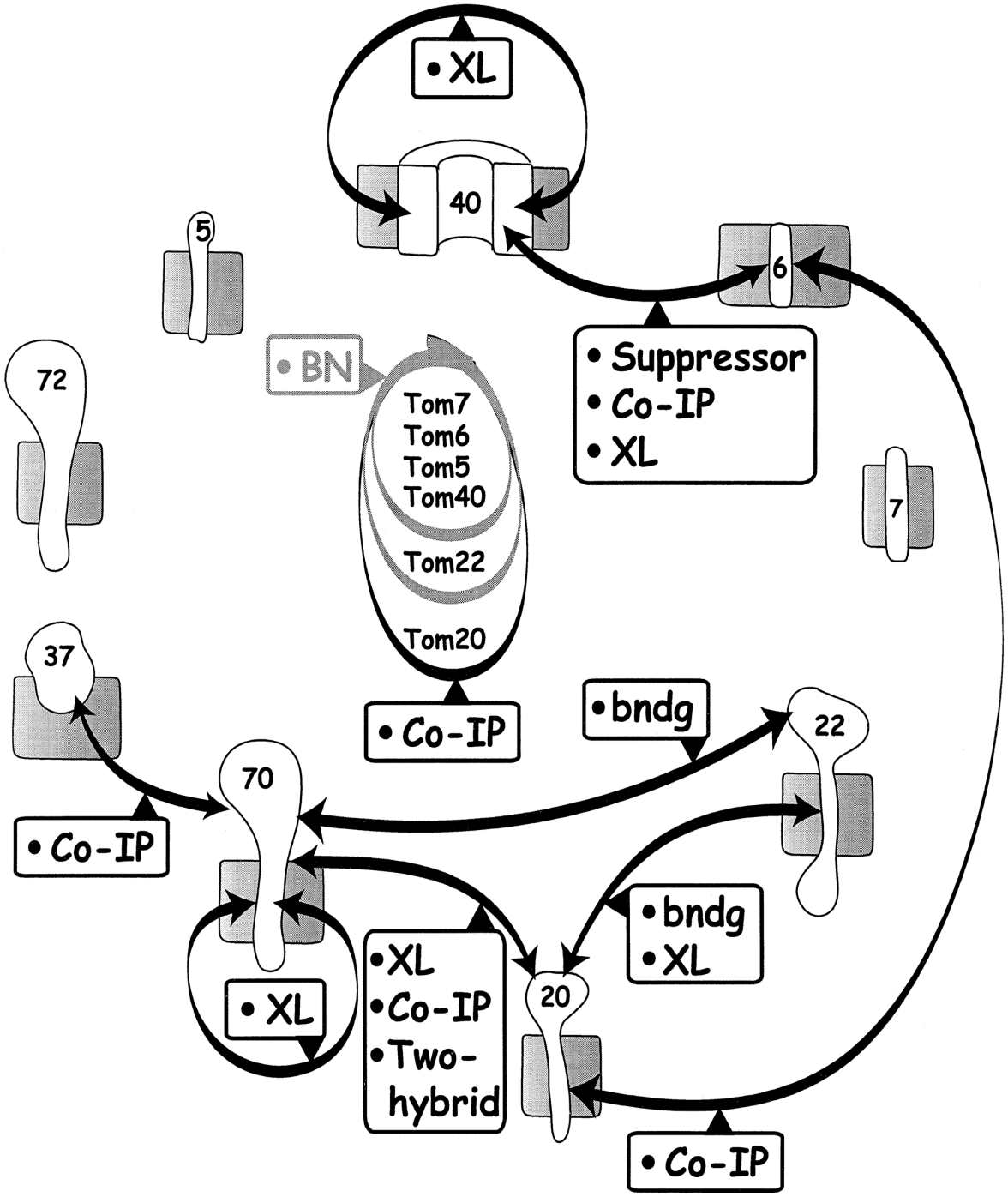

FIGURE 3. Protein interactions between subunits of the TOM complex. Summary of biochemical and genetic approaches that demonstrate interactions among the individual Tom proteins. Chemical crosslinking (XL), coimmunoprecipitation (Co-IP), blue native PAGE and direct binding (bndg) have been used to define TOM subcomplexes and to determine their constituents. In addition, genetic means such as two-hybrid analyses (two-hybrid) and suppressor screens (suppressor) have been applied successfully.

component (Figure 2). It was initially identified by crosslinking using a mitochondrial transport intermediate as bait.[73] Antibodies raised against mitochondrial outer membrane proteins, including Tom40, were employed to isolate the genes encoding these proteins from expression libraries.[74,75] An intimate involvement of Tom40 in protein transport was also demonstrated, as its function was essential for the translocation of all proteins tested. Sequence analyses indicates that Tom40 has the capacity to span the outer mitochondrial membrane several times as β-strands.[76] Hill et al.[77] were able to reconstitute recombinant Tom40 into lipid membranes and demonstrate via electrophysiological methods that it forms cation-selective channels. According to these studies the diameter of the Tom40 channel is approximately 22 Å. The channel thus would be sufficient to accommodate an α-helical peptide or even a protein loop. The size estimation from polymer-exclusion experiments as demonstrated by Hill et al.[77] is corroborated by experiments in which rigid compounds of defined size were chemically crosslinked to preproteins that were subsequently assayed for their ability to be imported.[78]

Electrophysiological experiments also revealed that the reconstituted Tom40 channel was mainly in an open state. In contrast, when Tom40 was isolated from yeast cells together with associated GIP components and reconstituted into lipid membranes, it was primarily in a closed state, indicating that the associated Tom proteins regulate the channel activity of Tom40. Tom22 seems to be important for this regulation because the translocation channel isolated from *tom22Δ* cells was mainly in an open state.[45]

Together, this set of data is in agreement with the idea that Tom40 forms the protein-conducting channel of the outer mitochondrial membrane.

B. The Small Tom Proteins

In addition to Tom22 and Tom40, three small Tom proteins have been identified: Tom5, Tom6, and Tom7. Tom5[79] and Tom7[80] were identified by co-precipitation with Tom40. In contrast, Tom6 was found by genetic means as a suppressor of a *tom40* yeast mutant.[81,82] Even though they are relatively small proteins they all span the outer mitochondrial membrane (Figure 2) and are tightly associated with Tom40 (Figure 3).[83]

1. Tom5 — As Close As It Gets to the Pore

Tom5 plays an important role at the entry site of the outer membrane translocation channel. While Tom5 itself is dispensable for yeast cell viability, every double deletion combination between Tom5 and any of the receptors is synthetically lethal.[79] Moreover, Tom5 is required for the import of both preproteins with amino-terminal presequences and with internal targeting information, respectively. In experiments that addressed where Tom5 acts in the cascade of recognition events for preproteins at the outer mitochondrial membrane, Dietmeier et al.[79] could demonstrate that its function succeeded that of Tom70, Tom20, and Tom22. This finding implies that Tom5 receives preproteins from Tom22 and delivers them to Tom40 (Figure 2). It has been known for a while that after removal of the cytosolic receptor domains by mild trypsin treatment, preproteins can still be imported, although with reduced efficiency.[84] This phenomenon was termed "bypass import" because the import pathway seemed to circumvent the need for preprotein receptors and lead to direct insertion into the GIP.[74,80] Analysis of *tom5Δ* mitochondria revealed that the supposed receptor independent

import of preproteins is largely dependent on Tom5.[79] Further, Tom5 was shown to be unaffected by trypsin treatment conditions that efficiently removes the cytosolic domains of Tom20, Tom70, and Tom22.[79] The amino-terminal domain of Tom5, which is exposed to the cytosol, is rich in negative charges and could potentially bind to incoming mitochondrial proteins. The physical interaction between Tom5 and mitochondrial preproteins[79] supports a direct function of Tom5 in the recognition and or transport of preproteins within the GIP.

2. Tom6 and Tom7 Are Involved in the Dynamics of the GIP

The small Tom proteins Tom6 and Tom7 are not essential for cell viability and the deletion of either leads only to mild import defects *in vitro*. A clue to the function of both proteins came from detailed analyses of the composition and size of the GIP.

As mentioned previously, the GIP is a 400-kDa protein complex as judged by blue native gel electrophoresis.[54] Tom40, Tom22, Tom5, Tom6, and Tom7 are components of the GIP complex (Figure 3). Analysis of the GIP complex in *tom6Δ* mitochondria indicated that the absence of Tom6 leads to destabilization of this protein complex, transforming the 400-kDa complex into a smaller complex with a molecular mass of 100 kDa.[85] The 100-kDa complex contains Tom40, Tom5, and Tom7, but is devoid of Tom22. Interestingly, *in vitro* import of Tom6 into *tom6Δ* mitochondria and thus into the 100-kDa complex leads to partial restoration of the 400-kDa complex.[85] This information supports the idea that the GIP is a very dynamic protein complex in which Tom6 promotes the association between Tom22 and Tom40. Tom6 is not just a structural component of the GIP but actively participates in complex dynamics as it is not just the presence of Tom6 that is required for association between Tom22 and Tom40 but its activity. After solubilization of the outer membrane with the detergent Triton-X100, a stable complex can be detected that still contains Tom22 and Tom40 but lacks Tom6.[85]

Tom7, however, seems to have the opposite activity compared with Tom6. The current data indicate that the deletion of Tom7 stabilizes the 400-kDa complex and also causes a tighter association between Tom22 and Tom40.[80] The action of Tom7 seems to be important for the transport of outer membrane proteins. While deletion of *TOM7* does not have severe effects on most mitochondrial proteins tested, import of porin into the outer membrane was drastically reduced in these cells.[80] Perhaps Tom7 itself enhances the dynamic interactions between GIP complex components and thus allows the lateral exit of proteins out of the GIP. Recently, it has been shown that the Tom6/Tom7-mediated dynamics within the TOM complex are especially important for the biogenesis of the TOM complex.[86] When analyzing the assembly of Tom40 Model et al.[86] found that Tom40 is first transported across the outer mitochondrial membrane. During this process it associates with Tom5 and forms a protein complex of 250 kDa on the intermembrane space side of the outer membrane. Subsequently, a 100-kDa-intermediate-sized Tom40/Tom5 complex assembles into which Tom6 is recruited. This complex serves as an acceptor for Tom22 and Tom7. The association of Tom22 and Tom7 leads to the maturation of the 100-kDa complex into the 400-kDa TOM complex. The action of Tom6 and Tom7 is critical for the binding of incoming Tom40 to preexisting TOM components. Therefore, the small Tom proteins participate in the dissociation of TOM complexes and release of Tom22 as well as in promoting the association of Tom40 with Tom22.

VI. A GLIMPSE AT THE TOM COMPLEX

Using a histidine tagged version of Tom22, Künkele et al.[87] were able to purify the TOM complex from *N. crassa* mitochondria. The isolated protein complex contained Tom40, Tom22, Tom20, Tom70, Tom6, and Tom7 (Tom5 has not yet been identified in *N. crassa*). Following insertion of the TOM complex in liposomes channel properties were detected in the bilayer as determined by electrophysiological methods. The TOM complex also supported residual translocation activity. Moreover, electron micrographs reveal that the purified TOM complex has a diameter of about 140 Å and contains up to three stain-filled pits. These pits might represent the translocation pores formed by Tom40 within the complex. The estimated pore width of these structures was calculated to be around 20 Å, which is in agreement with the electrophysiological estimations obtained for recombinant Tom40.[77] In a later study Ahting et al.[88] isolated a TOM core complex that lacked Tom20 and Tom70 after treatment with the detergent dodecyl maltoside. In contrast to the complete TOM complex, this partial complex, which represents the GIP, formed double but not triple stain-filled ring structures. The orientation of the complex and the distribution of Tom proteins within this structure are yet to be determined.

VII. DRIVING PROTEINS ACROSS THE OUTER MEMBRANE

The forces responsible for driving proteins across the outer membrane are not known for sure. Only one model, which is in agreement with experimental data, has been put forward to explain the underlying mechanism. The so-called "acid chain hypothesis" suggests that the outer membrane receptor domains represent a cascade of binding sites for mitochondrial presequences. According to this model the binding affinity of a given presequence for Tom components increases along the import pathway whereby the first component of the pathway, has the lowest affinity for the presequence and the last component has the highest. Acidic patches on the receptor domains of Tom70, Tom20, Tom22, and Tom5 as well as the intermembrane space domains of Tom40 and Tom22 could well represent elements to which presequences or internal targeting signals associate.[52,79,89] Work by Komiya et al.,[66] who analyzed the binding affinities of presequences to the different receptors, is in accordance with the idea of a sequential increase in binding affinity. However, the recent finding that amino-terminal presequences bind to Tom20 via hydrophobic forces rather than by electrostatic ones[30,31] is a restraint on the model and indicates that a more complex cascade of affinities underlies the process. Still, the principal idea of increasing strengths of interaction could be a means to translocate the protein.

Alterations in the conformation of Tom40 have been suggested to provide an additional force to move a protein through the outer membrane.[90] Binding of the presequence to Tom40 seems to induce changes within Tom40 as judged by crosslinking experiments. It is conceivable that different conformational states lead to the presentation of new preprotein binding sites within the pore. Such a mechanism would potentially represent a means to transport a protein through the channel. Meisinger et al.[91] showed that a preprotein accumulated in the TOM channel is not kept in the channel by ionic forces, but by urea-sensitive interactions such as hydrogen bonds

and hydrophobic interactions. We propose to extend the "acid chain hypothesis" to the "binding chain hypothesis": the TOM machinery provides multiple binding sites for preproteins, including each type of noncovalent interactions, and thus guides preproteins across the outer membrane.

Once a protein destined for the matrix, inner membrane, or intermembrane space has emerged from the channel, it is accessible to components of the intermembrane space or inner membrane that assist subsequent transport steps. The participating forces and components of these processes are described in greater detail in the following sections.

VIII. BIOGENESIS OF THE OUTER MEMBRANE PROTEIN PORIN

In contrast to the transport of mitochondrial matrix proteins, little is known about transport and insertion of mitochondrial outer membrane proteins. With regard to its transport mechanism, one interesting and controversial preprotein is the outer membrane protein porin. Porin is the most abundant mitochondrial outer membrane protein of yeast. It forms a pore in the membrane, which allows the diffusion of small molecules across the membrane. Porin does not possess any obvious targeting signals. There is ample evidence indicating that porin requires Tom20 for its transport.[41,42,92-95] However, whether other receptors and the GIP are also involved is a controversial topic. While some groups found indications for the involvement of receptors and the GIP in porin transport,[45,79,80,96] others reported that only Tom20 is required for membrane insertion and assembly of porin into liposomes.[95] Schleiff et al.[95] also presented data that indicated that Tom40 was not involved in the process because a *tom40* mutant did not affect porin transport. This issue was revisited recently by Krimmer et al.[97] Using both *S. cerevisiae* and *N. crassa* mitochondria and a very detailed set of analyses, they found that the biogenesis of porin strongly depends on Tom20, Tom22, Tom5, and Tom7. Moreover, they were able to demonstrate that saturating amounts of matrix targeted precursor proteins could compete with porin import, suggesting that porin also utilizes the GIP. The finding that a number of new *tom40* alleles are affected in porin biogenesis corroborates this conclusion. Finally, Krimmer et al.[97] were able to chemically crosslink porin to Tom20, Tom22, and Tom40 demonstrating a physical association with receptors and the GIP.

It seems reasonable to conclude that porin is indeed imported via the general import pathway utilizing outer membrane receptors as well as the GIP.

IX. TRANSLOCATION MACHINERIES OF THE INNER MITOCHONDRIAL MEMBRANE

The transport pathways for most, if not all, mitochondrial proteins merge at the level of the GIP. After exit from the GIP, the transport pathways for different proteins diverge and one of two translocation machines of the inner mitochondrial membrane interacts with the incoming cargo. The TIM23 complex, which is also termed the preprotein translocase, mediates the transport of mitochondrial proteins that possess an amino-terminal presequence. A second protein complex termed the TIM22 complex or the carrier translocase is implicated in the transport of mitochondrial membrane proteins that contain internal targeting information.

A. The TIM23 Complex

Identification of outer membrane import components was primarily achieved by biochemical approaches, as described in the sections above. In contrast, components of the TIM23 complex were identified by genetic screens in *S. cerevisiea*. Why genetic means were the right choice to identify components of the mitochondrial inner membrane involved in protein transport becomes obvious if one compares the relative amounts of Tim and Tom components. In *S. cerevisiae* Tom40 and Tom22 are present at a concentration of approximately 200 to 300 pmol/mg mitochondrial protein. The associated receptors Tom20 and Tom70 are less abundant than the GIP constituents but still make up 60 to 70 pmol/mg mitochondrial protein. In contrast, the TIM23 complex constitutes only 15 to 20 pmol/mg mitochondrial protein.[85] Thus, the total yield of Tim proteins from mitochondria in any biochemical purification procedure is significantly lower than that found for the Tom proteins, making the identification of such proteins very difficult.

The two genetic screens that were successfully utilized to identify components involved in mitochondrial import were based on positive selection schemes. In one case a cytosolic enzyme was mistargeted to the mitochondrial matrix by attachment of a mitochondrial presequence. Subsequently, mutants were selected in which the fusion protein remained in its original location, namely, the cytosol and thus was not imported into mitochondria.[98-100] The second screen was applied for the detection of mutants temperature sensitive for growth at 37°C. These temperature conditional mutants were analyzed subsequently for their ability to import the precursor of the β subunit of the F_1-ATPase at 23°C vs. 37°C.[101]

Tim proteins that were identified by these screens were Tim44,[98] Tim23,[100,102] and Tim17[99] (Table 2). Tim23 and Tim17 are homologous proteins, yet they are not able to substitute for each other functionally because deletion of either one is lethal for yeast cells.[99,103] Both proteins reside in the mitochondrial inner membrane and are predicted to span the membrane four times. This leads to a topology in which both the amino- and carboxy-termini are exposed to the intermembrane space.[104] In contrast to these two membrane proteins Tim44 is mainly located on the matrix side of the mitochondrial inner membrane and peripherally associates with the translocation machinery (Figure 4).[105-107] There is clear experimental evidence indicating that all three proteins are involved in protein translocation. Antibodies raised against Tim23 and Tim44 inhibit protein import into mitoplasts.[102,104,105] In addition, Tim17, Tim23, and Tim44 could be crosslinked to preproteins in transit.[104,106,108,109]

Tim23 and Tim17 form a protein complex in the inner membrane that can be extracted from mitochondria under mild detergent conditions. Using blue native gel electrophoresis as a means to analyze protein complexes, Dekker et al.[83] were able to show that the Tim23-17 complex migrates at approximately 90 kDa. These studies also showed that in a temperature-conditional allele of *TIM23 (tim23-2)* the 90-kDa complex is destabilized. This effect reduces the import of cleavable mitochondrial preproteins. Accordingly, the TIM23 complex is intimately involved in the transport of presequence-containing proteins. The formation of the 90 kDa complex seems to be mediated by the transmembrane domains of Tim23 and Tim17.[110] In agreement with this finding, it is a substitution of one amino acid within the first putative transmembrane domain of Tim23 that leads to the destabilization of the complex in *tim23-2* mitochondria.[83]

TABLE 2
The Components of the TIM23 Complex, Preprotein Translocase of the Inner Membrane

Protein	Function	Properties	Ref.
Tim23	Preprotein receptor for presequence containing proteins on inner membrane, potential channel	Essential, inner membrane N_{out},C_{out}-topology, N-terminal coiled-coil, potentially spanning outer membrane	100,102
Tim17	Potential channel component, complex with Tim23	Essential, inner membrane N_{out},C_{out} topology	99
Tim44	Membrane anchor for mtHsp70	Essential, 90% soluble, peripherally associated with Tim23, cleavable presequence	98
mHsp70	Import motor	Essential, Ssc1 in *S. cerevisiae*, soluble matrix protein	153,154
Mge1	Exchange factor for Hsp70	Essential, soluble matrix protein	163

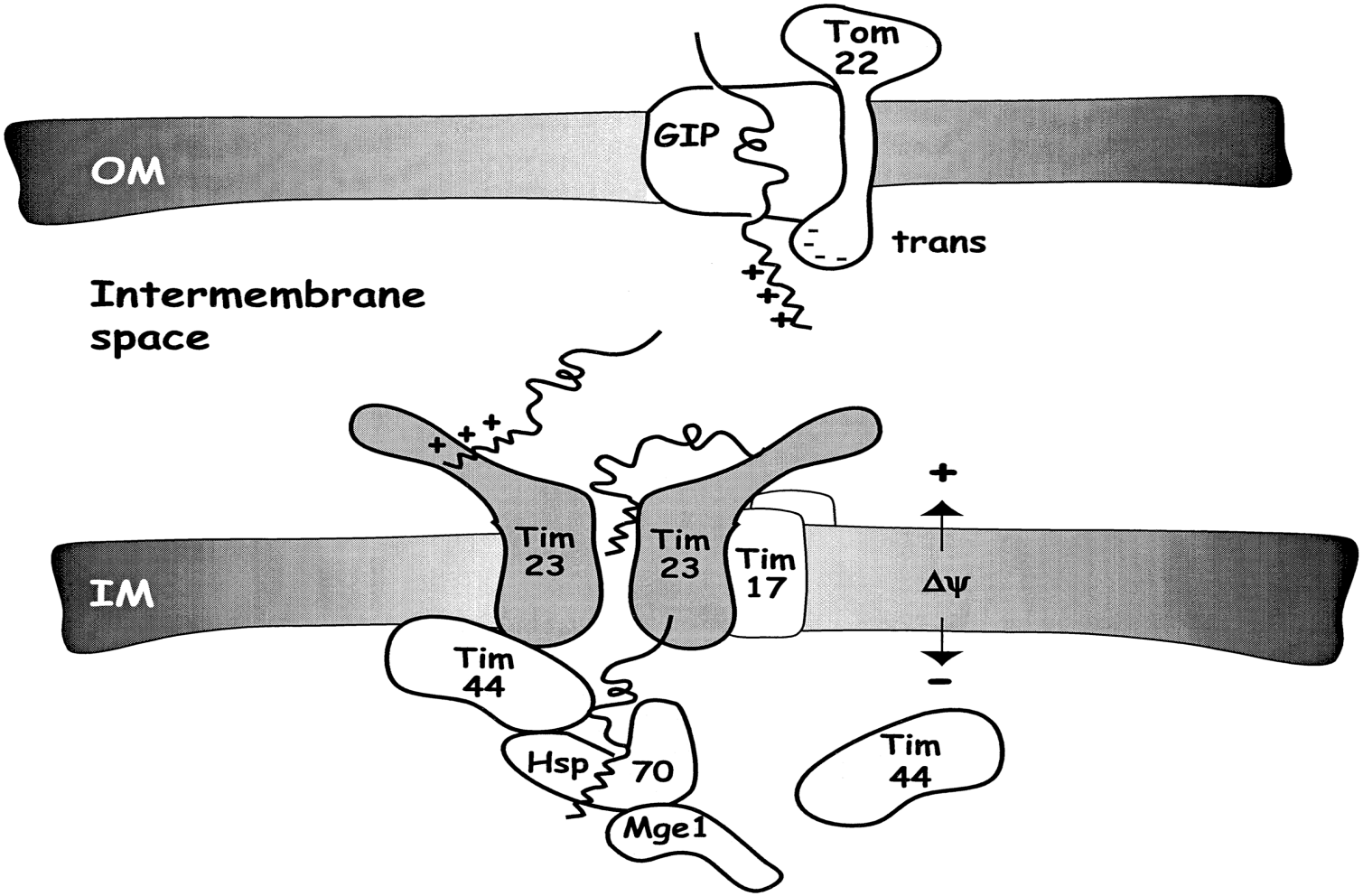

FIGURE 4. The TIM23 complex. Proteins destined for the intermembrane space or matrix, which contain amino-terminal presequences, associate at the *trans* side of the pore with the intermembrane space domain of Tom22. An acidic patch of amino acids in the intermembrane space domain of Tim23 subsequently serves as a receptor for the incoming presequence. Tim23 and Tim17 may form the channel through which proteins pass into the matrix. The membrane potential ($\Delta\psi$) is required for the insertion of the preprotein into the channel and the initial translocation of the presequence. The preprotein is recognized on the inner side of the mitochondrial membrane by mtHsp70. Together with Tim44 and the nucleotide exchange factor Mge1, mtHsp70 facilitates the completion of preprotein transfer into the matrix. OM, outer membrane; IMS, intermembrane space; IM, inner membrane

It is the current view that Tim23 and Tim17 form the 90-kDa complex in a 1:1 ratio.[109,111] However, at this point one cannot exclude the possibility that other proteins also participate in the TIM23 complex. Indeed, studies indicate the existence of additional proteins that are associated with the TIM23 complex, although none of them have been identified. In one case proteins of 14 and 33 kDa were found.[111] In a separate study another two proteins with apparent molecular masses of 20 and 55 kDa, respectively, have been described.[112] Therefore, it is possible that new components of the TIM23 complex await identification. Another interesting observation in this respect is the existence of low amounts of a 140- and 240-kDa form of the TIM23 complex.[83] Both complexes were identified using blue native PAGE analyses of digitonin solubilized mitochondria. As both complexes seem to be labile, a detailed analysis of their constituents has not yet been accomplished.

Besides the involvement of Tim23 and Tim17 in protein import, their localization to the inner membrane and their participation in a common complex, not much is known about the function of these proteins. In contrast to Tim17, Tim23 possesses an amino-terminal extension of about 100 amino acids that is essential for its function.[110] It is believed to serve as a preprotein binding site for incoming preproteins as it is accessible in the intermembrane space and binds to presequences *in vitro* (Figure 4).[66,113] Interestingly, there are indications that Tim23 forms homodimers via its amino-terminal segment.[110,113] Moreover, the dimerization of Tim23 seems to be positively regulated by the mitochondrial membrane potential *in vivo*. Dissociation is triggered by binding of a presequence to Tim23.[113] A model was devised based on these experiments that suggests Tim23 together with Tim17 forms a *bona fide* protein-conducting channel in the inner mitochondrial membrane. When preproteins are absent, the channel is closed due to association of the Tim23 amino-termini mediated by the membrane potential across the mitochondrial membrane. Accordingly, this membrane potential dependent dimerization and preprotein induced dissociation regulates the gating of the pore and the impermeability of the inner membrane to small molecules and protons. The association of an incoming preprotein with Tim23 triggers its dissociation and induces an opening of the translocation channel. Even though this represents an attractive hypothesis, the molecular nature of the channel through the inner membrane is yet to be defined. Studies on OEP16, an amino acid transporter of chloroplast outer envelopes that has significant sequence similarity to Tim23 and Tim17, indicate that this protein alone is sufficient to form pores.[114-116] Accordingly, we must take into consideration the possibility that Tim23 or Tim17, respectively, might be sufficient to form channels in the inner membrane. Lohret et al.[117] utilized electrophysiological measurements on mitochondrial membranes to characterize the so-called multiple conductance channel (MCC). The MCC supposedly represents the protein conducting pore. In these studies evidence is presented that indicates that antibodies directed against Tim23 inhibit the MCC activity. Moreover, they find that the activity of the MCC is altered in a *tim23* mutant, while its sensitivity to mitochondrial presequences is not significantly affected.[117] In conclusion, the authors suggest that the MCC is the pore through which precursor proteins are imported, while Tim23 contributes to its regulation.

In summary, the component(s) that form the translocation pore of the presequence translocase are not definitively known, although, in our opinion, there is strong evidence that Tim23 is a participant.

B. The 600-kDa TOM/TIM Supercomplex

Biochemical studies have demonstrated that the inner compartments of mitochondria (inner membrane and matrix) alone have all the prerequisites for the translocation of cleavable precursor proteins. For example, a number of precursor proteins can be directly transported across the inner membrane of mitoplasts. Mitoplasts are generated by selective disruption of the outer mitochondrial membrane under low osmotic conditions.[118,119] In addition, a preprotein that is arrested in transit across the inner membrane in mitoplasts leads to a molecular weight shift of the 90-kDa TIM23 core complex to a 130-kDa complex.[83]

As described previously, the number of TIM23 complexes is significantly lower than that of TOM complexes. When an excess of pure preprotein destined for the mitochondrial matrix is imported into mitochondria *in vitro*, but arrested in transit by a tightly folded carboxy-terminal domain, a 600-kDa supercomplex can be detected following separation by blue native gel electrophoresis.[83] The supercomplex contains all of Tim23 and Tim17, the precursor protein as well as a significant portion of Tom components. Under these conditions the preprotein spans both outer and inner mitochondrial membrane and thus connects TIM and TOM complexes in a stable manner. Work by Rassow et al.[120] showed that approximately 50 amino acids are sufficient to span the TOM/TIM machinery, suggesting that the polypeptide in transit is in an extended conformation. The fact that this TOM/TIM complex can only be generated after preprotein import suggests that stable associations between TIM and TOM complexes do not exist *in vivo*. There is ample evidence indicating that inner and outer membranes are in close proximity during protein translocation but do not form a continuous channel.[119,121,122] Donzeau et al.[123] reported recently that the amino-terminus of Tim23 also spans the outer mitochondrial membrane. Based on protease protection experiments they suggested that the first ~20 amino acids of Tim23 are exposed to the cytosol. Thus, Tim23 could potentially form a physical connection between outer and inner membranes. However, an association of Tim23 with the TOM complex could not be demonstrated.[123] We conclude that Tim23 most likely does not recruit the TOM complex actively into contact sites.

C. Protein Sorting to the Intermembrane Space

Preproteins with amino-terminal targeting signals destined for the mitochondrial matrix, inner membrane, and intermembrane space utilize the TIM23 complex for sorting. The sorting of soluble intermembrane space proteins such as cytochrome b_2 and inner membrane proteins like cytochrome c_1 have drawn much attention. Both proteins contain a bipartite cleavable presequence (Figure 1) that is composed of an amino-terminal matrix targeting signal, which directs them to the TIM23 complex, and an adjacent hydrophobic sorting signal.

Two different models have been put forward to describe the sorting process of these proteins.

The "conservative sorting" model suggests that the preprotein is completely translocated into the matrix and subsequently exported from the matrix into the membrane or intermembrane space.[19,124-129]

The alternative model is termed the "Stop transfer model" and proposes that the sorting signal leads to an arrest in the translocation process across the inner membrane. The membrane protein subsequently diffuses

laterally out of the pore and soluble intermembrane space bound segments are cleaved off by a specialized protease.[20,130-132]

A variation of this scheme that also takes aspects of the conservative sorting mechanism into consideration was suggested by Gärtner et al.[133] for the sorting of cytochrome b_2. Cytochrome b_2 is initially directed by its matrix targeting signal into the channel of the inner membrane. Here the sorting pathway separates from that of matrix proteins. The mature part of the protein subsequently forms a loop structure that also inserts into the sorting apparatus that could potentially be involved in the completion of translocation of the mature part across the outer membrane. Afterward the matrix processing peptidase cleaves off the sorting signal. The mature part is subsequently released into the intermembrane space.

At this point it is generally believed that both mechanisms could apply. While it is unlikely that cytochrome b_2 and c_2 are completely transported across the inner membrane and thus represent examples of a stop-transfer-like process, subunit 9 of the F_0ATPase is sorted by a conservative mechanism. In this regard the function of the Oxa1 protein is of special interest. Oxa1 is a nuclear-encoded protein that spans the inner mitochondrial membrane five times.[134] Recent analyses of the molecular function of Oxa1 have shown that it is involved in the export of amino-termini of imported proteins to the intermembrane space. After processing of subunit 9 of the F_0ATPase by the mitochondrial processing peptidase the amino-terminus undergoes an Oxa1 dependent export across the inner membrane.[134,135] Moreover, Oxa1 is required for its own import into the inner membrane of mitochondria[135] and as shown recently mediates membrane insertion of mitochondrially encoded inner membrane proteins.[136] Homologues of Oxa1 have been identified in chloroplasts[137-139] and in prokaryotes and seem to fulfill similar functions. YidC was shown to mediate the membrane insertion of Sec-independent proteins like the M13 virus procoat protein. Furthermore, the membrane insertion of Sec-dependent membrane proteins seems to be affected after depletion of YidC, suggesting some kind of relationship between the Sec pathway and YidC.[140-142]

D. The Membrane Potential ($\Delta\psi$)

The membrane potential across the inner membrane is critical for import of preproteins into the matrix, the inner membrane and for those intermembrane space proteins that utilize the TIM23 complex for sorting. Initial studies utilizing ionophores or inhibitors of the respiratory chain demonstrated that the membrane potential (negative on the matrix side) is crucial for import but not for the association of precursor proteins with the receptors.[143,144] Taking the net positive charge of mitochondrial presequences into consideration, it was suggested that the membrane potential generates an electrophoretic force on preproteins that results in their insertion into the pore and partial translocation until they emerge on the matrix side of the membrane. It is the electrical component of the electrochemical proton gradient across the inner membrane that generates the required force for protein import.[144] Work by Martin et al.[145] demonstrated that presequences with a different net positive charge have distinct requirements for the membrane potential, supporting the model of an electrophoretic effect. As already described (Section IX.A), $\Delta\psi$ additionally influences the oligomerization of Tim23 and thus the gating of the channel.[6,113]

Recent experiments indicate that the influence of $\Delta\psi$ on protein transport might

be more complex than originally anticipated. Using the bipartite presequence of cytochrome b_2 (Figure 1), Geissler et al.[146] showed that the $\Delta\psi$-dependence of protein import was not only influenced by the matrix-targeting signal, but also the sorting sequence. The presence of the sorting sequence lowered the $\Delta\psi$-dependence in an unexpected manner, not influenced by the charges in the sorting sequence or the actual sorting function. An amino acid alteration in the sorting sequence that enhanced the conformational flexibility of this segment stimulated import of the mutant preprotein. We propose that the sorting sequence forms a loop in the inner membrane and thereby facilitates translocation. Thus, it is likely that the $\Delta\psi$ requirement of mitochondrial protein import cannot be explained by a single mechanism alone.

E. Tim44 and Hsp70 Act Together in Protein Translocation

As mentioned above, the essential protein Tim44 was the first component of the import machinery identified in a genetic screen.[98] In addition, a biochemical approach aimed at identifying translocation components on the inner face of mitochondrial membranes led to the detection of a 45-kDa protein that turned out to be Tim44.[105,147,148] Tim44 comes into contact with presequences[105,106] but is not a component of the import channel itself.[149] During import only, minute amounts of Tim44 are found in association with the 600-kDa TOM-TIM complex.[83]

Tim44 is peripherally associated with the inner face of the translocation machinery (Figure 4) and is therefore carbonate extractable.[106,150] Using co-immunoprecipitation, Bömer et al.[151] demonstrated an association between Tim23 and Tim44. Subsequent work indicated that under low-salt conditions a complex of Tim23, Tim17, and Tim44 could be found.[152]

What is the role of Tim44 in protein translocation? It acts as a membrane anchor for the mitochondrial heat shock protein Hsp70 (mHsp70/ Ssc1) (Figure 4).[107,148,150] MtHsp70 is essential for cell viability and represents the only clear energy-requiring protein of the translocation machinery.[108,153,154] MtHsp70 binds to unfolded preproteins as they emerge from the translocation channel on the matrix side of the inner membrane. Following initial binding, cycles of ATP hydrolysis by mtHsp70 are required to complete the translocation of the polypeptide.[155] Recent analyses by Krimmer et al.[156] show that the physical interaction between Tim44 and mtHsp70 occurs via the ATPase domain of mtHsp70. A segment of Tim44 with low sequence similarity to so-called J domains has been implicated as a mtHsp70 binding site.[150,157] It is generally believed that mtHsp70 binds to Tim44 in the ATP-bound state and that ATP is subsequently hydrolyzed.[158-161] Precursor-associated mtHsp70 then binds its co-chaperone Mge1, which promotes the reaction cycle of Hsp70 and subsequently the release of ADP.[51,161-165] Thus, the essential protein Mge1 acts as a nucleotide exchange factor for mtHsp70. An additional binding site for mtHsp70 on the TIM23 core complex has also been reported.[151] It was suggested that Tim17 or a closely associated as yet unidentified protein contributed to this binding site. Nevertheless, a function for this association has not yet been found.

In order to explain how mtHsp70 participates in the translocation process, two models have been proposed. However, despite a large number of experiments that have been performed in order to support or exclude one or the other model a generally accepted definite proof for either is still

missing. The models are called the "Brownian ratchet" or "trapping" model and the "import motor" or "pulling" model. Both models have been reviewed extensively in recent years,[7,19,114,166,167] and therefore we only introduce the basic ideas behind the models.

According to the "Brownian ratchet" or "trapping" model the preprotein can move forward and backward in the TOM and TIM channels driven by Brownian motion. If mtHsp70 binds to the incoming polypeptide, it will abrogate the backward movement of the protein. This trapping increases the likelihood of forward movement of the precursor. If the preprotein moves further forward into the matrix another mtHsp70 can bind to it and also prevent backsliding.[168,169] In this scenario Tim44 serves as a means to position mtHsp70 close to the matrix entry site. Mge1 is required to regenerate mtHsp70 for new rounds of preprotein association.

The "import motor" or "pulling model" suggests that mtHsp70 plays a more active role in transport. The mtHsp70 that is bound to Tim44 binds to the incoming preprotein.[159] ATP hydrolysis by mtHsp70 leads to a conformational change within the molecule such that a force is generated that pulls the preprotein into the matrix.[158] According to this view Tim44 serves as a membrane anchor to support force generation by mtHsp70. There is experimental evidence indicating that the Tim44 – mtHsp70 association is critical for import.[157,170] The most important aspect of the pulling model is the generation of a force that can help to unfold protein domains on the opposite side of the membrane, because the preprotein has to be unfolded in order to pass through the pore.

At this point it seems likely that both mechanisms, pulling as well as trapping, occur *in vivo*,[170,171] and that the combination of both is involved in efficient preprotein translocation.

F. The Carrier Translocase

There is ample evidence indicating that the requirements for the import of preproteins with internal targeting signals differs from that of preproteins with presequences. As described above, preproteins with internal signals utilize Tom70 as their primary receptor on the outer face of mitochondria (Figure 2).[55,61-63,67,172] Despite differential receptor requirements, both transport pathways subsequently merge at the level of the GIP and then diverge again following passage of the preprotein through the translocation pore. This later point is indicated by the fact that the intermembrane space domain of Tom22 is expendable for the import of AAC.[58,173] In addition, under conditions where saturating amounts of precursor protein are arrested in transit through the TOM and TIM23 machinery the import of AAC remains unaffected.[83] Accordingly, AAC does not utilize the TIM23 complex for its transport, and therefore an independent mechanism must exist.

1. The TIM22 Complex

Sequence analysis of the yeast genome led to the identification of Tim22 because it displayed significant sequence similarity to Tim23 and Tim17.[174] Like these two known proteins, Tim22 is predicted to form multiple membrane spans and also behaves as an integral membrane protein in biochemical analyses (Table 3). The disruption of *TIM22* is lethal to yeast cells, and in mitochondria with reduced levels of Tim22 the import of preproteins with internal targeting signals like AAC and the phosphate carrier PiC is significantly affected.[174] Further support for a direct role in transport of these proteins comes from the finding that AAC could be chemically

TABLE 3
The Components of the TIM22 Complex, Carrier Translocase of the Inner Membrane

Protein	Function	Properties	Ref.
Tim8	Transport factor for preproteins	Nonessential, soluble intermembrane space, zinc finger, 70-kDa complex with Tim13	185,195,196
Tim9	Transport factor for carrier family	Essential, soluble intermembrane space, zinc finger, 70-kDa complex with Tim10, associates with Tim12	176,181,183
Tim10	Transport factor for carrier family	Essential, soluble intermembrane space, zinc finger 70-kDa complex with Tim9, associates with Tim12	175,176,181,184
Tim12	Acceptor of Tim9/10 and preprotein	Essential, component of 300-kDa complex, peripheral inner membrane	175,176,181,184
Tim13	Transport factor for preproteins	Nonessential, soluble intermembrane space, zinc finger, 70-kDa complex with Tim8	195,185,196
Tim18	Unknown, potentially involved in complex assembly or organization	Nonessential, component of 300-kDa complex, C_{out} topology, 3 potential membrane spans	178,179
Tim22	Unknown, potential cooperation with Tim54 in protein insertion, putative channel	Essential, component of 300-kDa complex	174
Tim54	Unknown, potential cooperation with Tim22 in protein insertion	Essential, component of 300-kDa complex, N_{in}-C_{out} topology	177,178,198

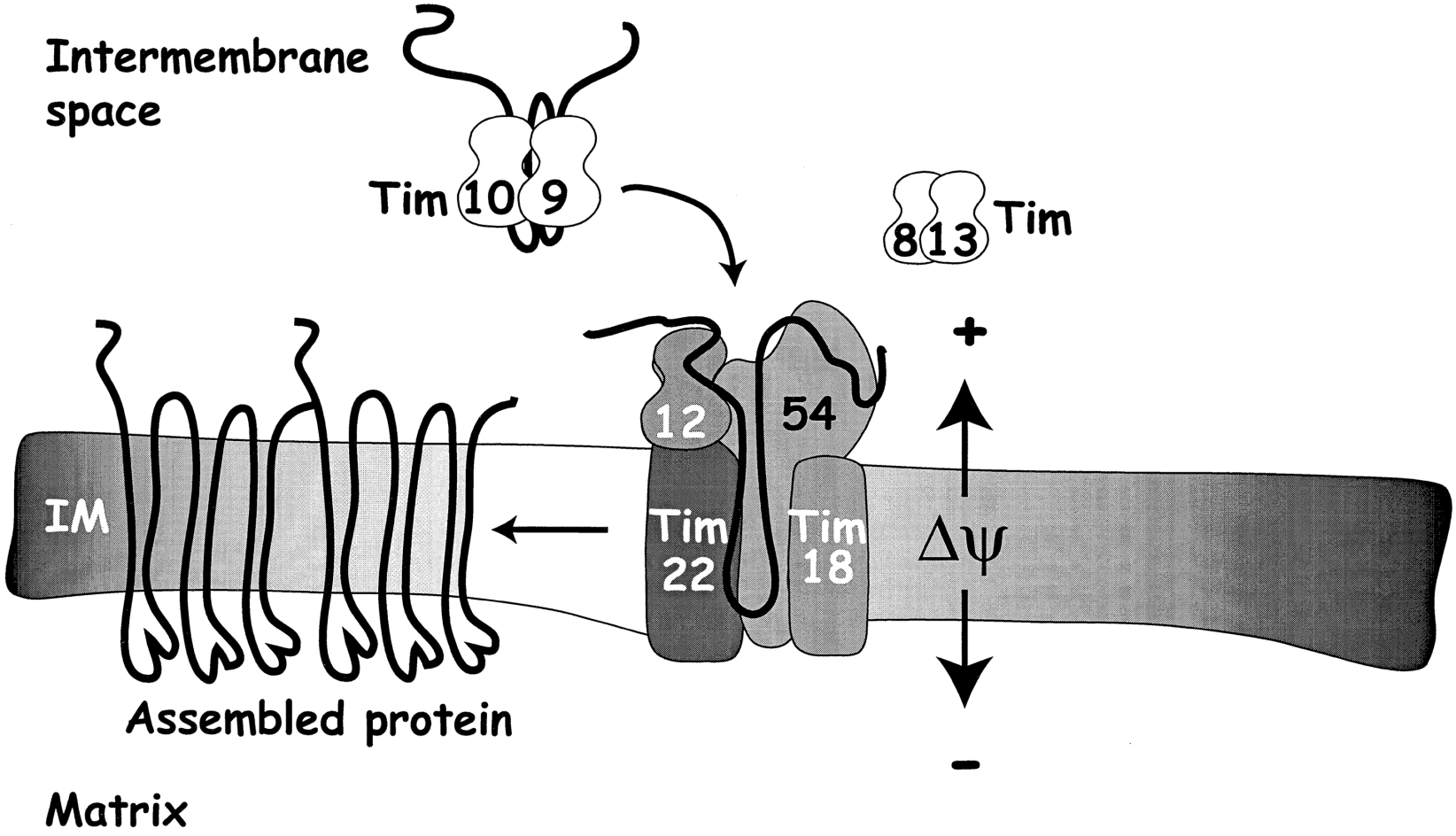

FIGURE 5. The carrier translocase. Components of the intemembrane space and the TIM22 complex of the inner mitochondrial membrane, which is also termed the carrier translocase, mediate the transport of preproteins with internal targeting signals. Once the preprotein emerges from the pore through the outer mitochondrial membrane, the tiny Tim proteins associate and assist in transporting the hydrophobic preprotein across the intermembrane space. Tim22, Tim18, Tim54, and Tim12 form a 300-kDa protein complex in the inner mitochondrial membrane. The association of the tiny Tims Tim9 and Tim10 with Tim12 initiates an as yet unknown mechanism that results in membrane integration of the preprotein. Similar to transport through the TIM23 complex, passage via the TIM22 complex also requires the membrane potential as a force for the transport of the preprotein. After release of the preprotein into the inner mitochondrial membrane, it can oligomerize into its active form.

crosslinked to Tim22. Tim22 is found in a high-molecular-weight complex that migrates at approximately 300 kDa on both blue native gel electrophoresis and gel filtration (Figure 5).[175,176] Additional constituents of this complex were subsequently identified (Table 3). Tim54 was recognized in a two-hybrid screen for interacting partners of the outer mitochondrial membrane protein Mmm1 which is implicated in mitochondrial inheritance.[177] Currently, there is no information regarding the significance of this potential *in vivo* interaction. However, Tim54 is an essential integral inner mitochondrial membrane protein. *In vitro* import studies indicate that like Tim22 it is required for the import of inner mitochondrial membrane preproteins with internal targeting signals. Coimmunoprecipitation experiments indicate that all of Tim22 associates with Tim54 while there is an excess of Tim54, which is free of Tim22.[177] However, using blue native gel electrophoresis virtually all of Tim54 is found in the 300-kDa complex.[178,179] In addition, physical association between Tim22 and Tim54 is supported by genetic experiments in which overexpression of Tim22 suppresses a temperature-sensitive growth defect of *tim54-1*.[177]

In addition to Tim54 and Tim22, two other proteins Tim12 and Tim18 (Table 3) are found in the 300-kDa complex (Figure 5). Tim12 was identified as a suppressor of mitochondrial splicing mutants.[180] A detailed analysis of the *tim12-1* temperature conditional allele[181] or strains with reduced amounts of Tim12[175] indicated an involvement in carrier protein transport. Moreover, Tim12 could be crosslinked to preproteins.[175] Tim12 localizes to the intermembrane space side of the inner mitochondrial membrane and associates with Tim22 as well as Tim10 (see below). Like Tim22 and Tim54, the function of Tim12 is essential for cell viability.

Tim18 is the only known component of the carrier translocase that is dispensable.[178,179] Two independent approaches led to the identification of Tim18. In a biochemical experiment in which Tim54 was immunoprecipitated, Tim18 was found as a coprecipitating factor.[179] Moreover, a genetic screen for suppressors of the *tim54-1* mutant led to the identification of an open reading frame encoding Tim18.[178] Tim18 is an integral membrane protein that potentially spans the inner mitochondrial membrane several times. Topology analyses indicate that the carboxy-terminus of Tim18 localizes to the intermembrane space. After solubilization with mild detergents, Tim18 was found in association with the 300-kDa carrier complex, while the deletion of Tim18 shifts the molecular mass of the complex to approximately 250 kDa.[178,182] Furthermore, the deletion of *TIM18* was found to be synthetically lethal in combination with temperature conditional mutations in Tim54, Tim9, and Tim10.[178]

Surprisingly, very little is known about the molecular function that can be attributed to these components. The structural similarity of Tim22 with Tim23 or Tim17 can be interpreted as an indication that it contributes to the formation of a translocation pore in the carrier translocase complex.[114] Binding of preproteins to Tim12 suggests that it is involved in the process of protein recognition and/or insertion. However, the function of Tim54 and Tim18 remains unclear.

2. The Tiny Tims

In addition to the membrane-associated components that form the 300-kDa TIM22 complex of the inner membrane, four soluble factors have been found that assist carrier protein transport. These proteins form two 70-kDa oligomers in the intermembrane space, one of which is formed by Tim9 and

Tim10 and the other by Tim8 and Tim13 (Table 3).

Tim9 was identified by two independent approaches. Koehler et al.[176] found it as an extragenic suppressor of the temperature-sensitive *tim10-1* allele, while Adam et al.[183] found that it co-precipitated together with Tim10 and Tim12. Tim10, the partner of Tim9, was previously identified, like Tim12, as a factor that suppressed defects in RNA splicing.[184] Analysis of the *tim10-1* mutant indicated, however, that it was actually involved in protein transport.[175,181] Both Tim9 and Tim10 are essential for yeast cells and together form a heterooligomer that is most likely built from three subunits of each of the two proteins.[181,183] A common feature of Tim9, Tim10, and Tim12 is that each subunit contains four cysteine residues that most likely participate in the formation of a metal binding site.[175] Zinc has been extracted from Tim10 and Tim12.[175] Only small amounts of Tim9 and Tim10 are found in association with Tim12 on the membrane, while the majority of the oligomer remains soluble (Figure 5).

Tim8 and Tim13 display features similar to the Tim9/Tim10 complex. Subunits of Tim8 and Tim13 associate to form a 70-kDa heterooligomer in the intermembrane space and both contain the cysteine residues required for metal binding.[185] In contrast to the other small Tim proteins, both Tim8 and Tim13 are nonessential for cell viability. However, they are synthetically lethal together with temperature-sensitive mutations in *TIM10,* and biochemically they associate with minute amounts of Tim9. One especially interesting aspect of Tim8 and Tim13 is the fact that they were identified due to sequence similarity with Tim9, 10, and 12. Moreover, they are homologous to the human deafness dystonia syndrome proteins DDP1 and DDP2.[185] DDP1 has been implicated in the rare X-linked human disease called Mohr-Tranebjaerg syndrome.

A more detailed description of the function of the small Tims is given below.

3. Biogenesis of Carrier Proteins

The carrier proteins are a large family of abundant polytopic mitochondrial inner membrane proteins. They have a tripartite structure comprised of three homologous modules of approximately 100 amino acids each. Each module contains two transmembrane segments connected by a matrix facing loop. Consequently, the amino- and carboxy-termini extend into the intermembrane space. In contrast to the import of precursor proteins with amino-terminal targeting sequences, the carrier proteins contain internal targeting information, and they are imported by different import pathways.

The import of carrier proteins was analyzed in detail over a decade ago and dissected into five different operational stages, mainly based on energetic requirements (Figure 6). After synthesis on cytosolic polysomes, the highly hydrophobic and thus aggregation prone precursors are bound by cytosolic factors maintaining them in an import competent state (termed stage I, discussed in Section II). The carrier precursors are targeted to the outer membrane and can be arrested on outer membrane receptors in the absence of ATP (stage II).[186,187] In the presence of ATP the precursor is partially translocated across the outer mitochondrial membrane to the intermembrane space (stage III) (Figure 6). This stage has been defined biochemically as an intermediate in the transport pathway at which the carrier protein is not accessible to proteases added from the outer side of mitochondria. It was suggested that this situation would represent a stage where most of the carrier protein is located within the channel across the outer membrane. Only in the presence of a membrane

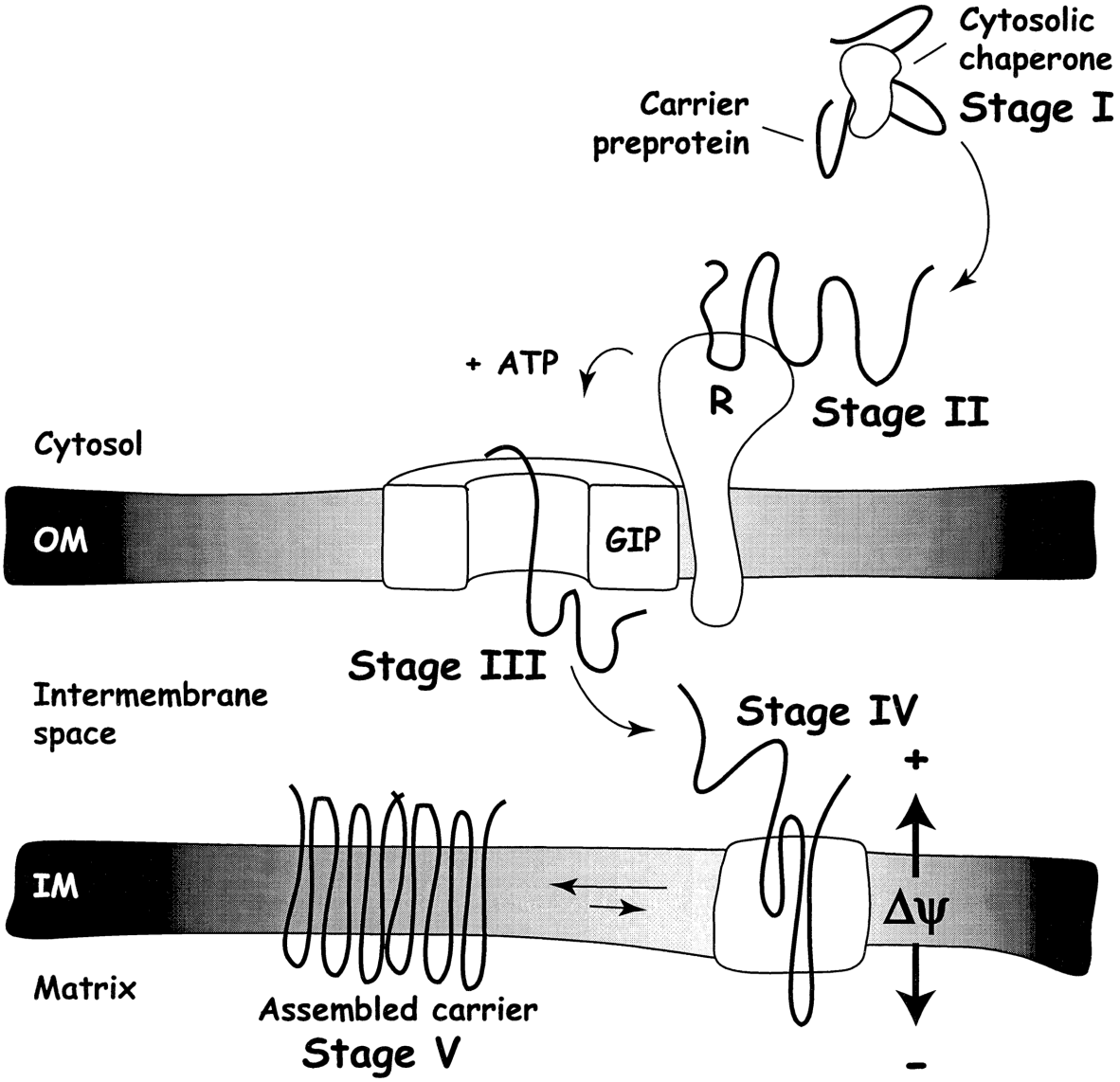

FIGURE 6. Protein transport of a carrier protein. Biochemically, the transport of the AAC (ADP/ATP carrier) has been divided into distinct steps.[186,187] After synthesis the preprotein associates with cytosolic chaperones and subsequently binds to proteinaceous components at the outer surface of mitochondria (Stage I and II). ATP hydrolysis is required for the subsequent transfer of AAC into the outer membrane pore (Stage III). At this stage most of the protein is transferred to the *trans* site of the membrane, and thus is no longer sensitive to externally added proteases. The membrane potential ($\Delta\psi$) is required for the exit of the preprotein out of Stage III and into Stage IV. Stage IV is defined as being the membrane insertion step, although only a kinetic intermediate has been found. The final stage (Stage V) is the assembly into the active dimer in the inner membrane. IM, inner membrane. OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

potential across the inner membrane is the carrier protein inserted into the inner membrane (stage IV),[144] where it subsequently dimerizes to its functional form (stage V).[188]

Only in the last few years were the components of the import apparatus for carrier proteins identified and more recently molecular details of the import of carrier proteins determined. There are conflicting reports describing the targeting signals of carrier proteins, but taken together mitochondrial targeting signals have been identified in all modules of the carrier proteins.[38,39,189-191] Peptide binding assays using the receptor domain of Tom70 revealed that carrier proteins contain multiple binding sites in individual modules.[40] The carrier proteins are recognized mainly by the receptor Tom70,[62,63] however, they are still imported in its absence but with strongly reduced efficiency.[61] In the absence of ATP, every module of a carrier protein can be crosslinked exclusively to a Tom70 dimer[69] (Figure 7). In the presence of ATP, the precursor is transferred module by module to the GIP.[69] Tom5 seems to be very important for the transfer of carrier proteins from Tom70 to the GIP, as indicated by a strong import defect in the deletion strain, whereas deletion of Tom20 or Tom22 independently cause only mild import defects.[45,48,52,79] The import of carrier proteins can be blocked at the GIP if they are fused to dihydrofolate reductase (DHFR), a tightly folded protein domain. In the presence of the competitive inhibitor methotrexate (MTX), unfolding of DHFR is blocked, therefore inhibiting complete import.[55,192] Under these conditions, the carrier proteins are partially translocated through the GIP and into the intermembrane space, as demonstrated by crosslinking to the intermembrane space Tim9/Tim10 complex. Interestingly, this translocation intermediate was detected for carrier proteins blocked with DHFR at either terminus individually or even when both ends were blocked together.[69] Accordingly, carrier proteins seem to initiate translocation across the outer membrane with an internal segment, for example, an internal loop. However, whether one, two, or even three loops of the carrier protein span the Tom40 channel[77] at one time and if these loops utilize one or more of the potential three channels seen within the TOM complex[87] is currently not known and will be a challenging issue to address in the future. With regard to the physical association of the carrier with the TOM complex Wiedemann et al.[69] were able to demonstrate that the stability of the arrested precursor complex at the general import pore depends on the number of carrier modules. In the presence of ATP the carrier precursors are partially translocated into the hydrophilic intermembrane space. The translocation of carrier proteins across the outer membrane does not depend on the IMS domain of Tom22,[58] in contrast to the import of proteins with amino-terminal presequences. Carrier proteins utilize only the soluble Tim9/Tim10 complex. Furthermore, each module of the carrier interacts specifically with the Tim9/Tim10 complex.[190] It seems that at this stage carrier proteins have already been sorted to the TIM22 insertion complex, as they can be crosslinked to Tim12, a peripheral component of the inner membrane TIM22 complex.[175] However, they are not inserted into the membrane in the absence of a membrane potential. In the presence of $\Delta\psi$ the carrier proteins are inserted into the inner membrane. This process is mediated by the TIM22 complex, although we do not know any details about the specific function of the individual complex components. Crosslinking indicates that the carrier protein interacts with Tim22 during import.[174] Finally, carrier proteins assemble with preexisting subunits and form functional dimers.[55,188] Full integrity of all three carrier modules is a prerequisite for dimerization,

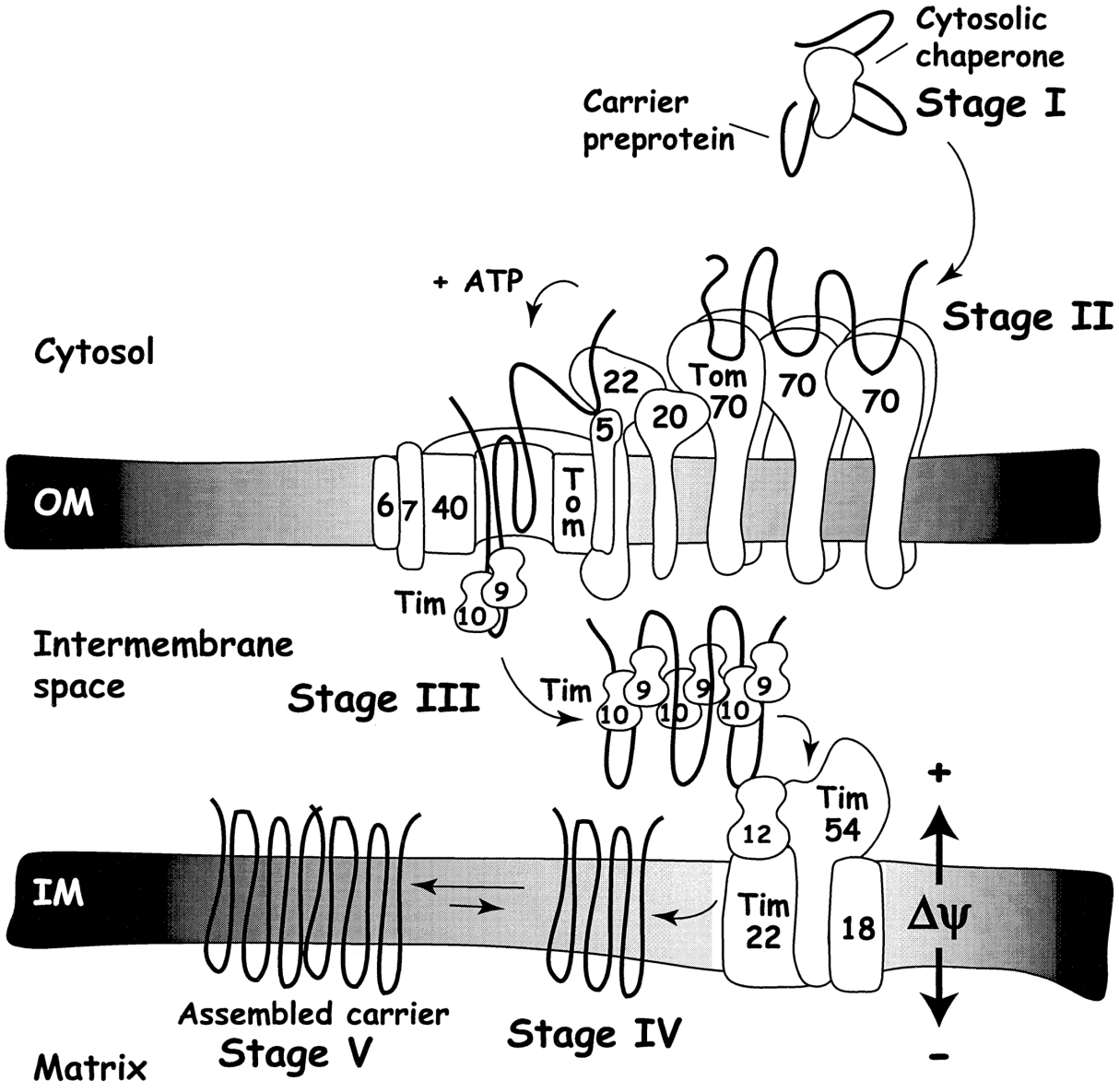

FIGURE 7. Protein transport of a carrier protein and its components. The chaperone associated AAC preprotein (Stage I) is bound by Tom70 on the outer mitochondrial membrane (Stage II) . Each module of AAC associates with a dimer of Tom70 — six Tom70 molecules per AAC. The AAC preprotein is transported via Tom22 to the GIP and inserts into the pore in a loop structure. Tim9 and Tim10 associate with the regions of the loop that appear in the intermembrane space (Stage III). After completion of the translocation process, the tiny Tim bound AAC is transported to the inner membrane. Tim9 and Tim10 bind together with their cargo to the TIM22 complex, which mediates the insertion of the preprotein into the inner membrane (Stage IV). Eventually, the released AAC assembles into a dimer (Stage V). OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

only the termini extending into the intermembrane space can be deleted.[69]

In summary, we believe that multiple interactions of hydrophobic inner membrane proteins with components of the import apparatus are essential to keep them in an import competent form. Precursor proteins must be transferred from their cytosolic chaperones to the general import pore. Tom70 acts as a receptor for carrier targeting signals and by binding to all individual carrier modules it may in addition simultaneously function as a chaperone, preventing precursor aggregation during transfer to the general import pore. The precursor is then transferred from Tom70 to the general import pore in a stepwise manner, module by module. In the hydrophilic intermembrane space, the carrier precursors need to be protected from aggregation; however, this compartment does not contain classic molecular chaperones such as the Hsp60 and 70 families. The inner membrane precursor proteins are perhaps maintained in a soluble state by the individual interaction of each module with the Tim9/Tim10 complex. These sequential interactions along the import pathway allow the aggregation-prone precursors to pass through hydrophilic environments on their way to the TIM22 complex. Additionally, these interactions may be important for the precursors to obtain a specific structural orientation for their transfer and insertion into the general import pore (via an internal loop) and their subsequent insertion into the inner membrane with the matrix facing loops inserting first. This is in contrast to the amino-terminal linear translocation of preproteins with presequences.

X. BIOGENESIS OF THE TIM PROTEINS

All Tim proteins are encoded by nuclear genes and must be imported from the cytosol. It is clear from sequence analyses that the precursors of Tim17, 23, and 22 lack a presequence and therefore utilize internal targeting signals. Tim23 and Tim22 are transported with the aid of the carrier translocase (Figure 8). For Tim17 and Tim23 it has been shown that both contain internal targeting information.[193,194] The import of Tim23 is similar to the carrier proteins. In the absence of a membrane potential the Tim23 precursor forms an intermembrane space complex and Tim23 is inserted into the inner membrane with the aid of the TIM22 complex.[177] It was unclear if the multiple internal targeting signals exhibited equal functions during the import of polytopic inner membrane proteins. Recently, Davis et al.[195] could demonstrate that Tim23 contains separate binding sites for the Tim9/Tim10 complex and the components of the TIM22 translocase, respectively. Deletion of all positive charges within the matrix facing loops of Tim23 abolished crosslinking to Tim54, 22, and 12, whereas crosslinking to Tim9/Tim10 was not affected. Additionally, Tim23 contains a binding site for the Tim8/Tim13 complex in its amino-terminus,[195,196] which is suggested to trap Tim23 in the intermembrane space and to prevent retrograde movement of Tim23 out of the GIP.[197] However, there is controversy with respect to the presence of additional amino-terminal targeting information within Tim23. Káldi et al.[193] identified such a signal and suggested that it is involved in directing the amino-terminus of Tim23 into the outer membrane.[123] In contrast, Davis et al.[194] could not demonstrate a topogenic signal within this sequence. Further detailed analysis will be required to solve this issue.

Compared with Tim22 and Tim23, the pathway of Tim17 import is not clear and the data, which is currently available, suggest that either route is involved. Tim17 import is reduced in *tim23* and *tim22* temperature-conditional mutants as well as in strains where their steady state level is reduced.[193,196]

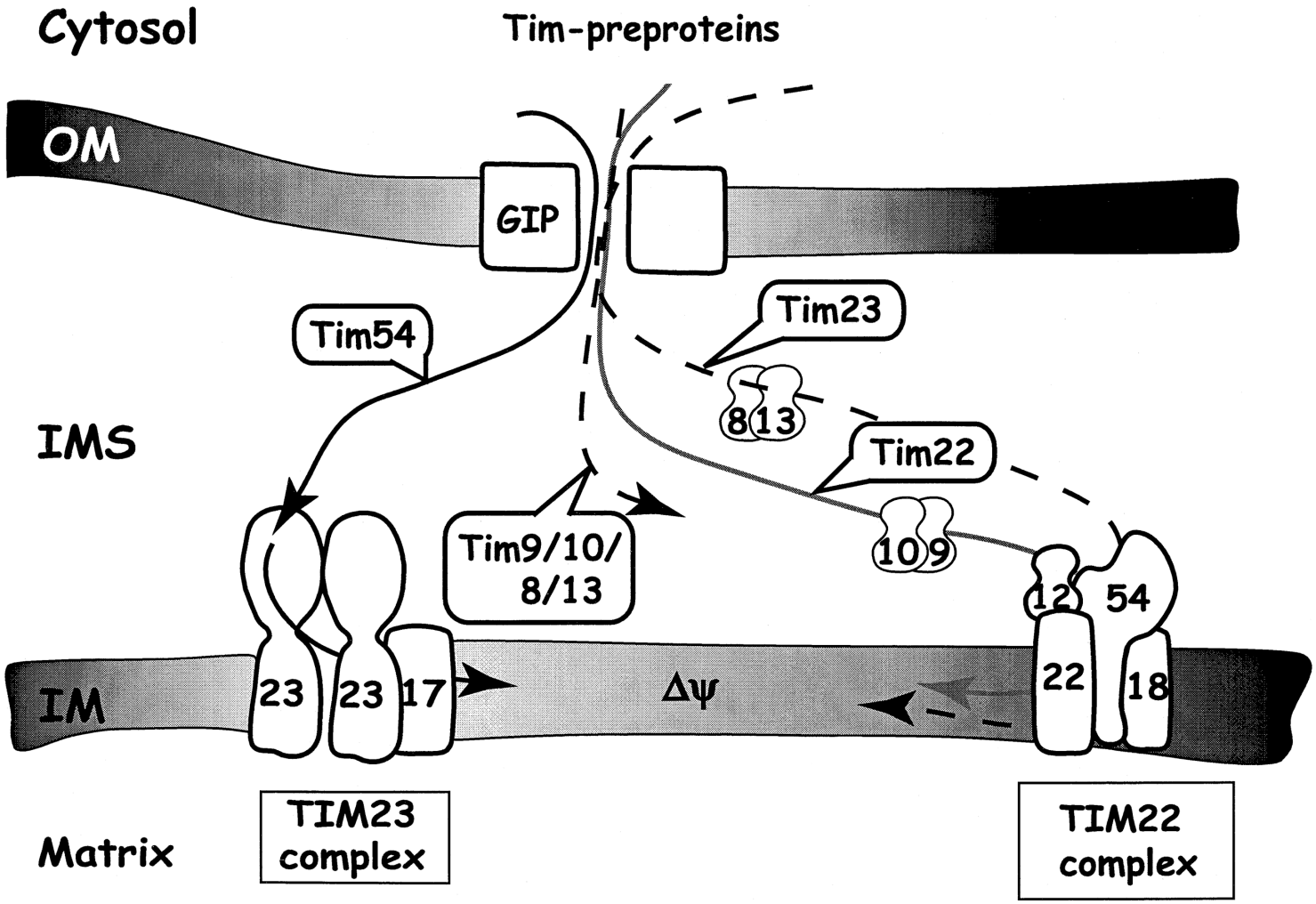

FIGURE 8. Biogenesis of Tim proteins. All Tim proteins utilize the GIP for import into mitochondria. Tim54 contains a noncleavable amino-terminal targeting signal and is transported via the TIM23 complex. Tim23, Tim17, and Tim22 utilize internal targeting information and are transported with the aid of the TIM22 complex. Import of the tiny Tims does not depend on any of the outer mitochondrial membrane receptors but they are transported through the general import pore. OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

Directed by an amino-terminal targeting signal, Tim54 and most likely Tim18 are transported with the aid of the TIM23 complex (Figure 8). Tim54 possesses a noncleavable presequence[198] and it can compete for import with preproteins that employ the TIM23 complex. In contrast, the import of Tim22 could not be competed by presequence-containing proteins. This result suggests that Tim22 is transported by a Tim23-independent mechanism, as predicted from the absence of a presequence within Tim22. Tim18 is synthesized as a 21-kDa precursor that is proteolytically cleaved after import into mitochondria.[178,182] Although there are no data to support it, it seems likely that Tim18 is transported via the TIM23 complex.

Interestingly, the import of the small Tim proteins does not depend on the protease accessible receptors Tom20, Tom22, and Tom70, but only on Tom5.[198] They are imported into the intermembrane space without the help of the TIM23 or TIM22 complex because their import is independent of the presence or absence of a membrane potential.[176]

Thus, the biogenesis of the various Tim proteins does not follow one common pathway, but involves several distinct import routes. This indicates a high versatility of the mitochondrial import machinery. We anticipate that the analysis of protein translocation into mitochondria will hold many more surprises for us.

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