Biogenesis of the protein import channel Tom40 of the mitochondrial outer membrane: intermembrane space components are involved in an early stage of the assembly pathway

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Running title: Mitochondrial protein assembly
SUMMARY

Tom40 forms the central channel of the preprotein translocase of the mitochondrial outer membrane (TOM complex). The precursor of Tom40 is encoded in the nucleus, synthesized in the cytosol and imported into mitochondria via a multi-step assembly pathway that involves the mature TOM complex and the sorting and assembly machinery of the outer membrane (SAM complex). We report that opening of the mitochondrial intermembrane space by swelling blocks the assembly pathway of the β-barrel protein Tom40. Mitochondria with defects in small Tim proteins of the intermembrane space are impaired in the Tom40 assembly pathway. Swelling as well as defects in the small Tim proteins inhibit an early stage of the Tom40 import pathway that is needed for formation of a Tom40-SAM intermediate. We propose that the biogenesis pathway of β-barrel proteins of the outer mitochondrial membrane not only requires TOM and SAM components, but also involves components of the intermembrane space.
INTRODUCTION

Mitochondria contain about 800 to 1,500 different proteins (1-4). 99% of these proteins are encoded in the nucleus and synthesized as precursors on cytosolic ribosomes (5-9). The precursor proteins are recognized by receptors of the mitochondrial outer membrane and are translocated by the general import pore (GIP) across the membrane. The receptors and the GIP assemble to form a high molecular weight complex, termed the translocase of the outer mitochondrial membrane (TOM complex) (10-14). The three receptors Tom20, Tom22, and Tom70 show a distinct, yet overlapping specificity for different classes of mitochondrial precursor proteins. With the help of the small Tom protein, Tom5, the precursors are transferred to the core of the TOM complex, the GIP formed by the essential protein Tom40 (15, 16). Two further small Tom proteins, Tom6 and Tom7, are involved in the stability and assembly of the TOM complex. Tom40, the three small Tom proteins and the receptors Tom22 and Tom20 are associated in a complex of about 450 kDa, while the receptor Tom70 is only loosely associated with the other Tom proteins (17-22).

After passing the TOM complex, the precursor proteins are transferred to the other mitochondrial compartments, the intermembrane space, inner membrane, and matrix. Two main classes of precursor proteins can be distinguished (5-8, 13). Preproteins with amino-terminal cleavable targeting sequences (presequences) are transferred from the TOM complex to the presequence translocase (TIM23 complex) of the inner membrane and its associated protein import motor PAM. Non-cleavable precursor proteins with multiple internal targeting signals, like the hydrophobic metabolite carriers of the inner membrane, are transferred from the TOM complex to small Tim proteins of the intermembrane space that guide them through this aqueous compartment. The hydrophobic proteins are then delivered to the protein insertion machinery (carrier translocase, TIM22 complex) of the inner membrane (5, 6, 23, 24). Two soluble complexes of small Tim proteins are known in the
intermembrane space, the essential Tim9-Tim10 complex and the Tim8-Tim13 complex (25-33). The Tim9-Tim10 complex is involved in transfer of many hydrophobic proteins through the intermembrane space, while the Tim8-Tim13 complex plays a special role for a few precursor proteins, like the precursor of the inner membrane protein Tim23 (34-38).

All mitochondrial outer membrane proteins are encoded by nuclear genes, synthesized in the cytosol and imported via the TOM complex. Thus the precursors of Tom proteins require the pre-existing mature TOM complex for entry into mitochondria (22, 39-45). The precursor of the GIP-forming β-barrel protein Tom40 is imported and assembled into the TOM complex via a multi-step pathway. The Tom40 precursor is recognized by Tom receptors (22, 41, 42) and transported by the GIP, i.e. pre-existing mature Tom40 (46). Subsequently, the precursor of Tom40 leaves the TOM complex and undergoes two maturation steps, termed assembly intermediate I and assembly intermediate II. The intermediates can be monitored by blue native electrophoresis of lysed mitochondria as 250 kDa and 100 kDa complexes, respectively (22). The assembly intermediate I is formed by the sorting and assembly machinery (SAM complex) (46, 47). The SAM complex contains the subunits Mas37 and Sam50 (Tob55/Omp85) (46, 48-50). Subsequently, Tom40 stably associates with Tom5, forming the assembly intermediate II (46). Then Tom6, Tom7, Tom22 and finally Tom20 associate to form the mature TOM complex (21, 22, 39, 46).

Here we report a surprising connection between two mitochondrial protein biogenesis pathways that have been assumed to represent separate entities so far, the SAM assembly pathway for Tom40 and the system of small Tim proteins that transfer the precursors of hydrophobic inner membrane proteins across the intermembrane space. The results presented here suggest that the intermembrane space is involved in the insertion of Tom40 into the outer membrane.
EXPERIMENTAL PROCEDURES

_Yeast Strains, Media, Mitochondrial Isolation and Swelling - The Saccharomyces cerevisiae_ strains used in this study are the wild-type strain YPH499 (51) and the corresponding mutant strains _tim8Δ tim13Δ_ (PRY34) and _tim10-2_ (GB102) (33). Yeast cells were grown on YPG medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 3% (v/v) glycerol) at 24°C or 30°C. Mitochondria were isolated by differential centrifugation as described (52, 53) and aliquots were stored at –80°C in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2). For swelling of mitochondria, the pelleted mitochondria were resuspended in 1 vol SEM buffer, the suspension was diluted with 9 vol of swelling buffer (1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) and incubated for 30 min on ice.

_Import of Preproteins –_ Radiolabeled precursor proteins were obtained by _in vitro_ transcription from pGEM-4Z (or genomic PCR products containing an SP6 Promotor) using SP6 RNA polymerase (Stratagene) (54). _In vitro_ translation was performed with rabbit reticulocyte lysate in the presence of [³⁵S]methionine/cysteine (Amersham Pharmacia). Import assays were carried out with 25 - 50 µg mitochondria (protein amount) in 100 - 200 µl import buffer (3% (w/v) fatty acid free bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS-KOH, pH 7.2, 2-5 mM ATP, and in case of matrix precursor proteins 2 mM NADH) with 2.5 – 10% (v/v) reticulocyte lysate. Import of precursor proteins was performed at 25°C except for the precursor of Tom20 where 10°C were used (at 25°C, import and assembly of Tom20 are too fast to be resolved in a kinetic manner). Where indicated, mitochondria were treated with 20 - 50 µg/ml proteinase K or 20 µg/ml trypsin for 15 min on ice. The proteases were inactivated by the addition of 1% (v/v) 200 mM phenylmethlysulfonyl fluoride/ethanol or 200 µg/ml soybean trypsin inhibitor, respectively. After 5 min incubation on ice, the mitochondria were reisolated by centrifugation and washed with SEM buffer. Mitochondrial proteins were separated by glycine-SDS-PAGE, tris-tricine-SDS-PAGE or blue native PAGE (17, 55).
Miscellaneous – For affinity purification of anti-Tim9 antibodies, *S. cerevisiae* Tim9 was expressed in *E. coli* strain BL21-Codon Plus (DE3)-RIL (Stratagene) from the plasmid pET10N to produce an N-terminal His10-tagged protein. Cultures were grown at 37°C for 4 hrs following induction of expression with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Tim9 was purified from isolated inclusion bodies under denaturing conditions via NiNTA-agarose chromatography essentially as described for Tim23 (56), then dialysed against coupling buffer (30 mM Hepes-NaOH, pH 7.2, 2.5% (w/v) SDS) overnight at room temperature. Tim9 was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia) as described by the manufacturer. To purify anti-Tim9 antibodies raised against an N-terminal region (peptide ALNSKEQQEFQKVVE), serum was diluted 1:1 with 1 x TBS (10 mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl) then poured over the Tim9-Sepharose column. The column was washed extensively with 1 x TBS prior to elution of specific antibodies with 100 mM glycine-HCl, pH 2.5. Samples were immediately neutralised with Tris buffer.

Standard protocols were used for Western blotting and detection of immune complexes by enhanced chemiluminescence (Amersham Pharmacia). Non-relevant gel lanes were excised digitally. Radiolabeled proteins were analyzed by digital autoradiography using the Phosphor Image technology (Amersham Pharmacia).
RESULTS

Opening of the mitochondrial intermembrane space blocks the biogenesis pathway of Tom40, but not Tom20 – The precursor of yeast Tom40 was synthesized in rabbit reticulocyte lysate in the presence of [35S]methionine/cysteine. The precursor was incubated with isolated yeast mitochondria and the assembly complexes were monitored by lysis of the mitochondria with digitonin and separation by blue native electrophoresis. In a time-dependent manner, the assembly intermediate I was formed, followed by the assembly intermediate II and the mature TOM complex as described (22, 46, 48) (Fig. 1A, lanes 1-3). To address a possible role of the intermembrane space in the biogenesis of Tom40, isolated mitochondria were subjected to swelling under conditions that led to an opening of the intermembrane space, while the inner membrane and matrix remained intact (22, 57). The swollen mitochondria (mitoplasts) were then incubated with the radiolabeled precursor of Tom40. The assembly pathway of Tom40 was dramatically affected by swelling since the formation of the assembly intermediate I was already blocked (Fig. 1A, lanes 4-6).

Under these swelling conditions, neither the mature TOM complex nor the SAM complex were disturbed (22, 46). The ruptured outer membrane remained attached to the mitoplasts, as shown by a Western blot analysis of endogenous Tom40 and Tom22 in comparison to Tim22 of the inner membrane (Fig. 1B). To directly analyze the intactness of the TOM complex we imported the precursor of Tom20 that associates with the mature TOM complex. The assembly of Tom20 was not affected by the swelling of mitochondria (Fig. 1C). As an additional control, we show that the import of the matrix-targeted precursor of F1-ATPase subunit β was even moderately enhanced in mitoplasts (58) (Fig. 1D, lanes 5-7) compared to intact mitochondria (Fig. 1D, lanes 1-3). We conclude that opening of the intermembrane space blocks the assembly pathway of the Tom40 precursor, while other mitochondrial precursor proteins are still efficiently imported.
Mutant mitochondria of small Tim proteins are impaired in the biogenesis pathway of Tom40 – We used a yeast strain where both TIM8 and TIM13 were deleted (33, 59). The assembly of Tom40 still took place, however, with a reduced efficiency. In particular, the assembly intermediate I was reduced (Fig. 2A, lanes 7 and 8 vs. lanes 2 and 3) and the formation of the mature TOM complex was delayed (Fig. 2A, lanes 9 and 10 vs. lanes 4 and 5). To exclude indirect effects of the tim8Δ tim13Δ mitochondria, we analyzed the steady-state levels of various marker proteins. Except for the lack of the Tim8-Tim13 complex, all other proteins studied were present in roughly wild-type amounts, including the receptor Tom22, Tim9, Tim23 of the inner membrane, and the matrix heat shock protein 70 (mtHsp70) (Fig. 2B). Blue native electrophoresis showed that the pre-existing TOM complex as well as the SAM complex were intact in tim8Δ tim13Δ mitochondria (Fig. 2C). Radiolabeled Tom20 assembled into the TOM complex of tim8Δ tim13Δ mitochondria with an efficiency close to that of wild-type mitochondria (Fig. 2D). Thus the delayed assembly of Tom40 cannot be attributed to a lack of TOM complex or SAM complex, supporting the conclusion that it was specifically due to the lack of the Tim8-Tim13 complex.

Yeast cells lacking Tim8-Tim13 are viable (29, 36). As Tom40 is essential for cell viability (60), the Tim8-Tim13 complex can only play a supportive (accelerating) role in the assembly pathway of Tom40. Since the Tim9-Tim10 complex is essential for viability of yeast (25-28), we asked if this complex may also be required for the biogenesis of Tom40. We used the yeast mutant tim10-2 that carries point mutations in the TIM10 gene, causing a temperature-sensitive growth phenotype (33, 59). When the cells were grown at the permissive temperature of 24°C, the isolated mitochondria contained wild-type amounts of the marker proteins analyzed, including Tom22, Tim13, Tim23 and mtHsp70. Only the amount of Tim9 was reduced by about half (Fig. 3A). Moreover, the TOM complex as well as the SAM complex were present in wild-type amounts under steady-state conditions (Fig. 3B). tim10-2 mitochondria are selectively impaired in the function of the Tim9-Tim10 complex (33, 59). We imported the 35S-labeled precursor of Tom40 into isolated tim10-
The Tom40 assembly pathway was significantly impaired at the level of the assembly intermediate I (Fig. 3C, lanes 6 and 7 vs. lanes 1 and 2). To control for the specificity of the assembly defect, we used the precursor of Tom20 and found an efficient assembly reaction into the TOM complex (Fig. 3D).

We conclude that both complexes of small Tim proteins of the intermembrane space, the essential Tim9-Tim10 complex and the non-essential Tim8-Tim13 complex, are involved in the assembly pathway of Tom40. In mutant mitochondria of each complex, the formation of the assembly intermediate I complex is partially inhibited.

The TIM complexes of the intermembrane space are involved in an early stage of the assembly pathway of Tom40 – Characterization of the role of small Tim proteins in the import pathway of hydrophobic inner membrane proteins indicated that these intermembrane space complexes were involved in an efficient translocation of the precursor proteins through the TOM complex to the intermembrane space (25, 26, 28, 33, 36). Tom40 inserted into the outer membrane is largely protected against externally added trypsin except of a small fragment that can be cleaved off by the protease from about half of the Tom40 molecules (16, 46). When the Tom40 precursor has reached the SAM complex, i.e. assembly intermediate I, it already shows this characteristic protease protection (22, 46). We used this trypsin assay to study if defects in the small Tim proteins affected an early stage of Tom40 biogenesis before formation of the assembly intermediate I. The formation of trypsin-protected Tom40 and the characteristic Tom40 fragment were indeed delayed in both tim8Δ tim13Δ mitochondria and tim10-2 mitochondria (Fig. 4A and B). Under the same import conditions, the import and protease-protection of the matrix-targeted model preprotein Su9-DHFR, consisting of the presequence of F₀-ATPase subunit 9 and the passenger dihydrofolate reductase (61), were not impaired (Fig. 4C and D). We conclude that the transfer of Tom40 precursor to a protease-protected location is delayed when small Tim proteins of the intermembrane space are impaired, indicating that small Tim proteins are involved in the assembly pathway of Tom40 before the assembly intermediate I.
We asked if intermembrane space components were also required for later stages of the Tom40 assembly pathway. To selectively deplete intermembrane space components, we used the swelling assay shown in Fig. 1 at distinct import stages. Swelling led to a release of the bulk of the Tim9-Tim10 and Tim8-Tim13 complexes, as shown by Western blot analysis for Tim9 and Tim13 (Fig. 5A, lane 4), while proteins of the outer membrane (Tom40), inner membrane (Tim22), and matrix (citrate synthase and the cochaperone Mge1) remained completely in the mitoplast fraction (Fig. 5A, lane 3). The 35S-labeled precursor of Tom40 was accumulated at different intermediate stages and then chased to the next step of the assembly pathway (Fig. 5B) (46). While the formation of assembly intermediate I was blocked when the mitochondria were swollen before the import reaction (Fig. 5C, lane 2; Fig. 1A), the subsequent steps were not inhibited by a swelling of mitochondria. When Tom40 precursor was first accumulated at the intermediate stage I (Fig. 5C, lane 3) and the mitochondria were then subjected to swelling, the chase to intermediate II still occurred with good efficiency (Fig. 5C, lane 5). Similarly, Tom40 accumulated at intermediate stage II (Fig. 5C, lane 6) was chased into the mature TOM complex with comparable yield in non-swollen and swollen mitochondria (Fig. 5C, lanes 7 and 8). These results indicate that a depletion of the intermembrane space selectively blocks an early stage of Tom40 assembly required for formation of the assembly intermediate I.
DISCUSSION

We report that the mitochondrial intermembrane space is involved in the biogenesis pathway of the central component of the TOM complex, the protein import channel Tom40. Depletion of the intermembrane space selectively blocks the assembly pathway of Tom40 at an early stage that is required to form the assembly intermediate I (Fig. 6). Once the assembly intermediate I has been formed, i.e. the Tom40 precursor is accumulated at the SAM complex, the subsequent assembly steps are not affected by a depletion of intermembrane space components, including formation of the assembly intermediate II and the association of small Tom proteins as well as Tom receptors with Tom40 (22, 46).

These findings have important implications for the sorting pathway of the Tom40 precursor. The Tom40 precursor accumulated at the SAM complex has been shown to be largely protected against protease added to the outside of the mitochondria, but accessible to protease upon opening of the intermembrane space by swelling (22). Moreover, the recently identified essential subunit of the SAM complex, Sam50, contains a β-barrel domain conserved from bacteria to man (48-50). The bacterial homolog Omp85 is possibly involved in protein export to the bacterial outer membrane (62). Based on these observations it had been suggested that the precursor of Tom40, after passing through the TOM complex, may follow a conserved SAM-pathway of insertion into the outer membrane from the intermembrane space side (22, 46-50, 63). However, experimental evidence for an involvement of the intermembrane space in the assembly pathway has been lacking so far. The findings reported here indeed demonstrate that the intermembrane space plays a critical role in the assembly pathway of Tom40. The role of intermembrane space components is specific and cannot be explained by a general effect on the stability of the TOM complex since the assembly of the precursor of Tom20 into the TOM complex is not affected by depletion of intermembrane space components.
We found that at least two protein complexes of the intermembrane space are involved in the assembly pathway of Tom40. Partial inactivation of the essential Tim9-Tim10 complex as well as deletion of the related, but non-essential Tim8-Tim13 complex already delayed the biogenesis pathway of Tom40 at the level of assembly intermediate I formation. The Tim9-Tim10 complex has been known so far for its role in the import of hydrophobic inner membrane carrier proteins, while the Tim8-Tim13 complex has been found to show a preference for the precursor of Tim23 (34-38). These soluble TIM complexes interact with hydrophobic segments and some additional regions of the inner membrane precursor proteins and are assumed to possess chaperone-like properties in guiding the hydrophobic precursors through the aqueous intermembrane space (32, 35, 36, 38, 64). It is thus conceivable that the complexes of these small Tim proteins assist in guiding hydrophobic segments of Tom40 exposed to the intermembrane space. Apparently, the two soluble TIM complexes can in part substitute for each other in the biogenesis pathway of Tom40, explaining why the Tom40 assembly pathway is only delayed, but not blocked when either complex is inactivated in yeast mutants. Thus the steady-state level of the TOM complex remains at a wild-type level in these mutants, excluding the concern that the observed defects in Tom40 assembly would simply be caused by a defective pre-existing TOM complex. During the import of carrier proteins and Tim23, the soluble TIM complexes promote the transfer of the precursor proteins through the TOM complex (25, 26, 28, 33, 36). Similarly, we found that these TIM complexes are required for an efficient translocation of the Tom40 precursor to a protease-protected location. As recognition and translocation by the TOM complex represents the initial stage of the Tom40 import pathway (22, 41, 42, 46), we propose that the small Tim proteins and possibly further intermembrane space components are involved in the efficient transfer of the Tom40 precursor through the TOM complex to the intermembrane space side and its transfer to the SAM complex (Fig. 6).

Tom40 is a β-barrel protein like the most abundant outer membrane protein porin (VDAC) (65, 66) and the morphology protein Mdm10 of the outer membrane
The currently available evidence supports the view that the sorting and assembly pathway established for Tom40 may serve as a paradigm for the biogenesis pathways of other β-barrel proteins. The precursor of porin requires the TOM complex for the initial stages of its assembly pathway (68) and rupturing of the outer membrane impairs its assembly (data not shown) (69). For both porin and Mdm10 it has been shown that the SAM complex containing the highly conserved Sam50 is required for their biogenesis pathway as for Tom40 (46, 48-50). We propose that β-barrel proteins of the mitochondrial outer membrane follow a conserved biogenesis pathway which involves intermembrane space components for transfer to the SAM complex.
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1 The abbreviations used are: GIP, general import pore; SAM, sorting and assembly machinery; TIM, translocase of inner mitochondrial membrane; TOM, translocase of outer mitochondrial membrane.
FIGURE LEGENDS

FIG. 1. **Swelling of mitochondria blocks the assembly pathway of Tom40, but not Tom20.**

A, yeast mitochondria were preincubated in isotonic buffer or hypotonic (swelling) buffer for 30 min on ice. The mitochondria/mitoplasts were reisolated and incubated with the $^{35}$S-labeled precursor of Tom40 for the indicated times. Import and assembly of Tom40 was analyzed by blue native electrophoresis and digital autoradiography. B, isolated yeast mitochondria ($\mu$g of protein) were incubated in isotonic buffer or swelling buffer. The mitochondria/mitoplasts were reisolated and protein levels were analyzed by SDS-PAGE and Western blotting. IM, inner membrane; OM, outer membrane. C, preincubation of mitochondria and import of Tom20 were performed as described in the legend to A and Experimental Procedures. D, mitochondria were pretreated as described in the legend to A. After import of the precursor of F$_1$-ATPase subunit $\beta$ (F$_1$$\beta$) at 25°C, the samples were treated with proteinase K and analyzed by SDS-PAGE. Where indicated, the membrane potential ($\Delta\psi$) was dissipated. $p$, precursor; $m$, mature.

FIG. 2. **Mitochondria lacking Tim8-Tim13 are partially impaired in the assembly pathway of Tom40.**

A, wild-type (WT) and $\text{tim8}^\Delta \text{tim13}^\Delta$ mitochondria were incubated with $^{35}$S-labeled Tom40 for the indicated times. Tom40 assembly was analyzed by blue native electrophoresis and digital autoradiography. B, protein levels of WT mitochondria and $\text{tim8}^\Delta \text{tim13}^\Delta$ mitochondria ($\mu$g of protein) were analyzed by SDS-PAGE and Western blotting. IM, inner membrane; IMS, intermembrane space; OM, outer membrane. C, TOM and SAM complexes were analyzed by blue native electrophoresis and Western blotting with antibodies against Tom40 or Mas37, respectively. The SAM complex migrates in two bands of about 200 kDa and 350 kDa (46). D, the precursor of Tom20 was imported as described in the legend to A and Experimental Procedures.
FIG. 3. Mutant mitochondria of Tim10 are partially impaired in the assembly pathway of Tom40. A, protein levels of isolated wild-type (WT) mitochondria and tim10-2 mitochondria (µg of mitochondrial protein used). B, TOM complex and SAM complex analyzed by blue native electrophoresis and Western blotting. C, D, WT and tim10-2 mitochondria were incubated with 35S-labeled Tom40 or Tom20 at 25°C or 10°C, respectively, and analyzed by blue native electrophoresis and digital autoradiography.

FIG. 4. Transport of the Tom40 precursor to a protease-protected location is diminished in mutant mitochondria of small Tim proteins. A, radiolabeled Tom40 was incubated with WT mitochondria and tim8Δ tim13Δ mitochondria for the indicated times. The mitochondria were treated with trypsin and analyzed by SDS-PAGE and digital autoradiography. The amounts of protease-protected Tom40 and Tom40' were quantified with Image Quant. The amount of protected Tom40/Tom40' after an import time of 30 min into wild-type mitochondria was set to 100% (control). Tom40', fragment of Tom40 (16, 46). B, the experiment was performed as described for A except that tim10-2 mitochondria were used. C, D, Su9-DHFR was imported as described in the legend to A with the indicated mitochondria. Where indicated, the membrane potential (∆ψ) was dissipated. The mature-sized form, mSu9-DHFR, was quantified. The amount of mSu9-DHFR in wild-type mitochondria after an import time of 18 min was set to 100% (control).

FIG. 5. Swelling of mitochondria selectively inhibits an early stage of Tom40 biogenesis. A, isolated wild-type yeast mitochondria were incubated in isotonic buffer or hypotonic buffer (swelling) for 30 min on ice. After centrifugation, the protein levels were analyzed in the mitochondria/mitoplasts (Pellet) and the supernatant (Sup.) by SDS-PAGE and Western blotting. Cit1, citrate synthase; Mge1, mitochondrial GrpE. B, experimental scheme of the import-chase-assembly assay for the precursor of Tom40. C, lanes 1 and 2, wild-type yeast mitochondria were
preincubated in isotonic buffer or swelling buffer for 30 min on ice. The mitochondria/mitoplasts were reisolated and incubated with $^{35}$S-labeled Tom40 for 5 min at 25°C. The generation of the assembly intermediate I of Tom40 was analyzed by blue native electrophoresis and digital autoradiography. Lanes 3-5, mitochondria were incubated with radiolabeled Tom40 for 5 min at 25°C. The mitochondria were reisolated and either kept on ice (control) or incubated in isotonic vs. swelling buffer for 30 min on ice, followed by a chase for 10 min at 25°C. Lanes 6-8, mitochondria were incubated with Tom40 precursor at 25°C for 5 min. The mitochondria were reisolated and chased for 10 min at 25°C. One sample of the mitochondria was kept on ice (control), the other samples were incubated in isotonic buffer or swelling buffer for 30 min on ice, followed by a second chase for 45 min at 25°C.

FIG. 6. Hypothetical model of the mitochondrial import and assembly pathway of the precursor of Tom40. The model is based on results presented here and in previous studies (22, 41, 42, 46, 48-50). See text for details.
A. [35S]Tom40 for WT and tim8Δ/13Δ.

B. Western blot analysis for WT and tim8Δ/13Δ with proteins Tom22, Tim9, Tim13, Tim23, and mtHsp70 in OM, IMS, IM, and Matrix.

C. Western blot analysis for TOM complex and SAM complex.

D. [35S]Tom20 for WT and tim8Δ/13Δ.

Wiedemann et al., Fig. 2
A

WT  tim10-2

μg

25 50 25 50

Tom22

Tim9

Tim13

Tim23

mtHsp70

1 2 3 4

OM

IMS

IM

Matrix

B

WT  tim10-2

kDa

TOM complex

SAM complex

C

[35S]Tom40

WT  tim10-2

Time (min)

5 10 20 40 60 5 10 20 40 60

TOM complex

Assembly I

Assembly II

kDa

D

[35S]Tom20

WT  tim10-2

Time (min)

1 4 20 1 4 20

TOM complex

kDa

Wiedemann et al., Fig. 3
Wiedemann et al., Fig. 4
Wiedemann et al., Fig. 5

A

Swelling

Tom40 -

Tim13 -

Tim 9 -

Tim22 -

Cit1 -

Mge1 -

OM

IMS

IM

Matrix

1 2 3 4

B

[\textsuperscript{35}S]Tom40 Reisolation of mitochondria

Mitochondria

Import

5 min 25°C

10 min 25°C

Assembly I

Assembly II

Chase 1

Chase 2

Assembly I

Assembly II

TOM complex

C

Import

Swelling

Control (Assembly I)

Chase 1

Chase 2

Control (Assembly II)

TOM complex

Assembly I

Assembly II

kDa

669

440

232

140

67

1

2

3 4 5

6 7 8
Tom40 precursor

**Targeting** via
- Tom receptors
- GIP (*mature Tom40*)

**Intermembrane space** (*small Tims*)

**Assembly intermediate I** (250 kDa):
*SAM complex with Sam50 and Mas37*

**Assembly intermediate II** (100 kDa):
*Association with Tom5*

**Association of**
- Tom6, Tom7
- Tom22
- Tom20

**Mature TOM complex** (450 kDa)

Wiedemann et al., Fig. 6
Biogenesis of the protein import channel Tom40 of the mitochondrial outer membrane: Intermembrane space components are involved in an early stage of the assembly pathway
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