The mitochondrial import protein Mim1 promotes biogenesis of multispanning outer membrane proteins

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The mitochondrial outer membrane contains translocase complexes for the import of precursor proteins. The translocase of the outer membrane complex functions as a general preprotein entry gate, whereas the sorting and assembly machinery complex mediates membrane insertion of β-barrel proteins of the outer membrane. Several α-helical outer membrane proteins are known to carry multiple transmembrane segments; however, only limited information is available on the biogenesis of these proteins. We report that mitochondria lacking the mitochondrial import protein 1 (Mim1) are impaired in the biogenesis of multispanning outer membrane proteins, whereas overexpression of Mim1 stimulates their import. The Mim1 complex cooperates with the receptor Tom70 in binding of precursor proteins and promotes their insertion and assembly into the outer membrane. We conclude that the Mim1 complex plays a central role in the import of α-helical outer membrane proteins with multiple transmembrane segments.

Introduction

The mitochondrial outer membrane contains proteins of two distinct architectures: β-barrel proteins and proteins with α-helical transmembrane segments. All of these outer membrane proteins are encoded by nuclear genes, are synthesized as precursors on cytosolic ribosomes, and are imported into mitochondria. The precursors of β-barrel proteins are transported via the translocase of the outer membrane (TOM) complex to the intermembrane space. Chaperone complexes formed by small translocase of the inner membrane proteins transfer these precursors to the sorting and assembly machinery (SAM; also termed the topogenesis of mitochondrial outer membrane β-barrel protein complex) complex that promotes insertion into the outer membrane (Matouschek and Glick, 2001; Mihara, 2003; Johnson and Jensen, 2004; Ryan, 2004; Dolezal et al., 2006; Neupert and Herrmann, 2007; Chacinska et al., 2009; Endo and Yamano, 2009; Walther and Rapaport, 2009; Schleiff and Becker, 2011). In contrast, the biogenesis of outer membrane proteins with α-helical transmembrane segments is only partly understood.

α-Helical outer membrane proteins can be divided into proteins with a single transmembrane segment and proteins with multiple transmembrane segments. Different views have been reported on the import pathways of single-spanning outer membrane proteins. Depending on the precursor protein and system used, the findings ranged from a spontaneous insertion into the lipid phase to the involvement of TOM and/or SAM subunits (Keil and Pfanner, 1993; Motz et al., 2002; Ahting et al., 2005; Setoguchi et al., 2006; Bellot et al., 2007; Ott et al., 2007; Sanjuán Szklarz et al., 2007; Stojanovski et al., 2007a; Kemper et al., 2008; Meineke et al., 2008; Thornton et al., 2010; Becker et al., 2011). For several precursors of single-spanning

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Abbreviations used in this paper: MBS, maleimidobenzoyl-*N*-hydroxysuccinimide ester; NTA, nitrilotriacetic acid; SAM, sorting and assembly machinery; TOM, translocase of the outer membrane.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 194 No. 3 387–395
www.jcb.org/cgi/doi/10.1083/jcb.201102044

TOM subunits, an involvement of the mitochondrial import protein 1 (Mim1) was reported: mitochondria lacking Mim1 are impaired in membrane insertion of the precursors of Tom20, Tom70, and small Tom proteins (Becker et al., 2008, 2010; Hulett et al., 2008; Popov-Celeketić et al., 2008; Lueder and Lithgow, 2009; Thornton et al., 2010). Mitochondria defective in Mim1 are also impaired in the biogenesis of the β-barrel protein Tom40 (Ishikawa et al., 2004; Waizenegger et al., 2005; Becker et al., 2008; Lueder and Lithgow, 2009); a recent study showed that Mim1 does not directly promote the biogenesis of Tom40 but functions via the import of small Tom proteins that are needed for Tom40 assembly (Becker et al., 2010). Importantly, no direct interaction of Mim1 with precursor proteins has been reported so far, and, thus, it also remains open whether Mim1 plays a direct role in the import of α-helical outer membrane proteins.

Little is known about the biogenesis of mitochondrial outer membrane proteins with multiple α-helical membrane spans. The precursor of the human peripheral benzodiazepine receptor, which contains five transmembrane segments, binds to the receptor Tom70 but does not require other TOM or SAM subunits for insertion into the outer membrane (Otera et al., 2007; Yamano et al., 2008). Import of the precursor of Ugo1, a protein of the mitochondrial fusion machinery with three transmembrane segments, occurs independently of SAM components, yet further characteristics have not been analyzed (Wiedemann et al., 2003; Stojanovski et al., 2007a). Thus, it is unknown whether multispanning proteins are inserted by a proteinaceous machinery or whether the proteins are directly inserted into the lipid bilayer of the outer membrane.

For this paper, we studied the biogenesis of multispanning outer membrane proteins in the model organism *Saccharomyces cerevisiae*. The precursor proteins interact with the TOM complex via Tom70, pointing to a general role of the receptor Tom70 for multispanning mitochondrial proteins. The critical component for the subsequent import into the outer membrane is Mim1, and the precursor proteins directly interact with Mim1. Our results indicate that the Mim1 complex cooperates with the receptor Tom70 and forms a central machinery for protein insertion into the mitochondrial outer membrane.

Results and discussion

Role of the TOM machinery in the import of multispanning outer membrane proteins

Ugo1 is a 58-kD subunit of the fusion machinery of the mitochondrial outer membrane and contains at least three transmembrane segments (Fig. 1 A; Sesaki and Jensen, 2001, 2004; Wong et al., 2003; Coonrod et al., 2007; Hoppins et al., 2009). The precursor of Ugo1 was synthesized in reticulocyte lysate in the presence of [^{35}S]methionine and imported into isolated yeast mitochondria. To study whether the precursor of Ugo1 interacts with the TOM complex, we generated a yeast strain in which the central TOM subunit Tom40 was expressed with a C-terminal HA tag. Upon import of Ugo1, mitochondria were lysed with the nonionic detergent digitonin, and Tom40-associated proteins were copurified by affinity chromatography (Fig. 1 A). Subunits of the TOM machinery, such as the receptors Tom70 and Tom22, were copurified with Tom40 as expected, whereas the abundant outer membrane protein porin was not coeluted with Tom40, demonstrating the specificity of the copurification approach. Ugo1 was found in the elution fraction when tagged Tom40 was used but not when wild-type mitochondria were used (Fig. 1 A). Additionally, we synthesized the precursor of Ugo1 carrying a His tag in chemical amounts using a wheat germ–based translation system (Becker et al., 2010, 2011). $Ugo1_{His}$ was imported into isolated mitochondria, which were then lysed and subjected to Ni^{2+}–nitrilotriacetic acid (NTA) affinity chromatography. Upon separation by blue native electrophoresis, the TOM complex migrates at ∼450 kD (Fig. 1 B; Dekker et al., 1998; Ishikawa et al., 2004; Popov-Celeketić et al., 2008; Dukanovic et al., 2009; Becker et al., 2010; Yamano et al., 2010). Tagged Ugo1 pulled down a fraction of TOM complexes (Fig. 1 B). We conclude that the precursor of Ugo1 interacts with the TOM machinery.

Mitochondria have three surface receptors for precursor proteins: Tom20, Tom22, and Tom70 (Brix et al., 1997, 1999; Abe et al., 2000; Wu and Sha, 2006; Neupert and Herrmann, 2007; Yamano et al., 2008; Yamamoto et al., 2009). We used mitochondria from yeast mutant strains that lacked either one of the receptors. Ugo1 import into mitochondria was assessed by formation of an ∼140-kD complex resembling the endogenous Ugo1 dimer and by generation of protease-protected fragments (Wiedemann et al., 2003; Stojanovski et al., 2007a; Hoppins et al., 2009). The import of Ugo1 was decreased in mitochondria lacking Tom70 but not in mitochondria lacking Tom20 (Figs. 1 C and S1 A). Import of Ugo1 into *tom22Δ* mitochondria was even enhanced, whereas the control import of a major β-barrel protein, porin, was impaired (Figs. 1 D and S1 B; Krimmer et al., 2001). In *tom22Δ* mitochondria, the TOM complex dissociates into smaller core units containing Tom40 and small Tom proteins (van Wilpe et al., 1999; Wiedemann et al., 2003). The Tom40 core units were able to interact with Ugo1 also in the absence of Tom22 (Fig. S1 C). To study whether the TOM core components Tom40 and Tom5 were crucial for import of Ugo1, we used mitochondria from a temperature-sensitive *tom40* mutant as well as *tom5*Δ mitochondria. Import of Ugo1 into both mutant mitochondria was not or only mildly affected, whereas the import of porin was strongly inhibited (Figs. 1 [E and F] and S1 D). We conclude that the import of Ugo1 involves Tom70 but does not depend on an intact TOM complex.

As a second substrate, we used the 20-kD outer membrane protein Scm4 (suppressor of *cdc4* mutation; Smith et al., 1992; Zahedi et al., 2006). Scm4 is predicted to contain four α-helical transmembrane segments. Upon analysis by blue native electrophoresis, imported Scm4 assembled into an ∼120-kD complex (Fig. S1 E). The import dependence of Scm4 agreed with that of Ugo1: import of Scm4 was reduced in mitochondria lacking Tom70 (Fig. S1 E), and His-tagged Scm4 pulled down a fraction of TOM complexes (Fig. S1 F).

Together with the findings by Otera et al. (2007), these results suggest that Tom70 functions as a general receptor for multispanning outer membrane proteins. The precursor proteins

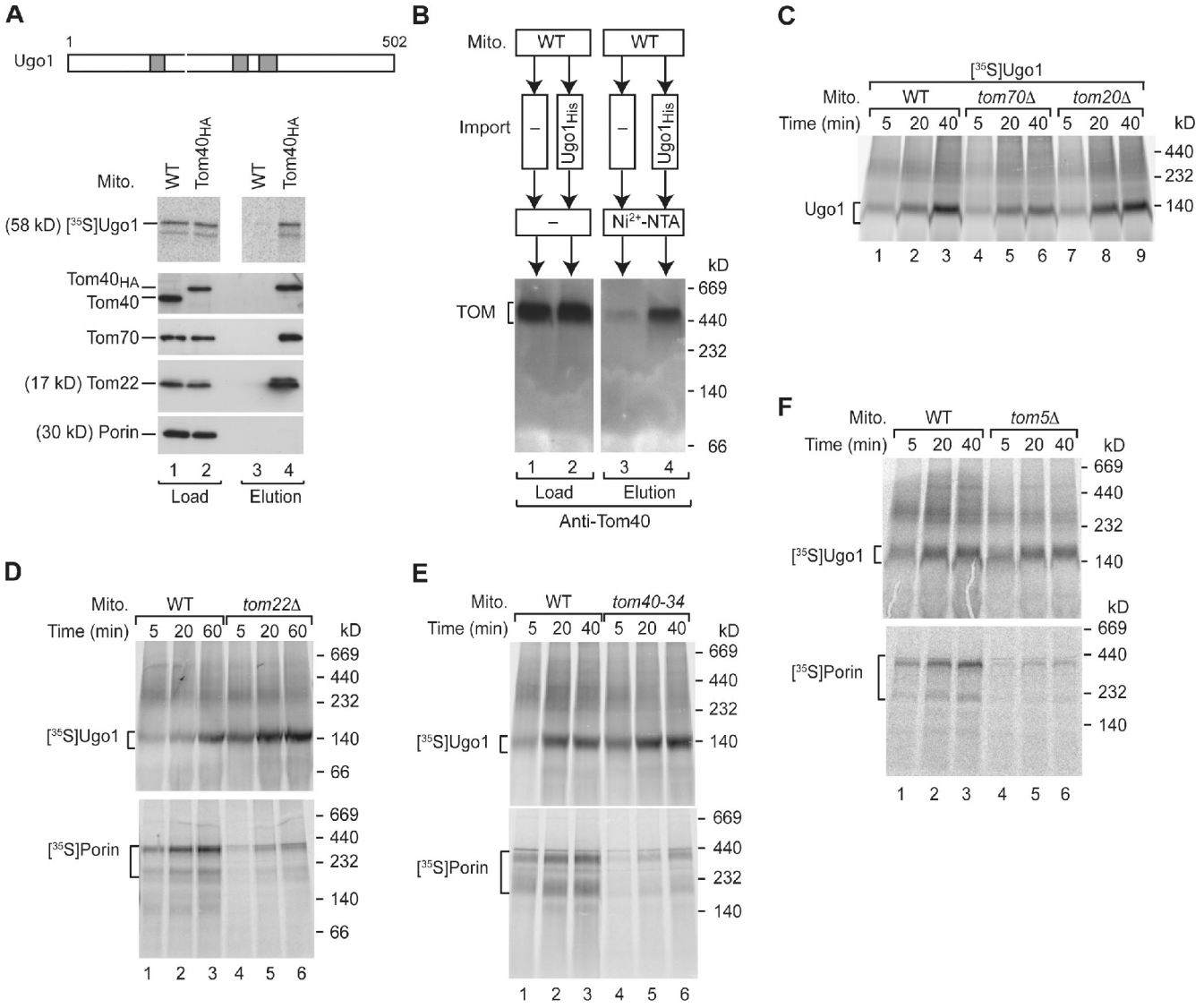

Figure 1. **Import of Ugo1 into mitochondria involves Tom70.** (A, top) A schematic representation of Ugo1. Transmembrane α helices are marked in gray. (middle) ^{35}S-Ugo1 was imported into wild-type (WT) and Tom40$_{HA}$ mitochondria (Mito.) for 5 min at 25°C. Mitochondria were lysed with digitonin and subjected to coprecipitation with HA-specific antibodies followed by SDS-PAGE and autoradiography. Load, 2%; elution, 100%. (bottom) Wild-type and Tom40$_{HA}$ mitochondria were treated as described above and analyzed by SDS-PAGE and Western blotting. Load, 5%; elution, 100%. (B) Chemical amounts of Ugo1$_{His}$ were imported into wild-type mitochondria for 10 min at 25°C. Mitochondria were lysed with digitonin and subjected to Ni^{2+}-NTA agarose purification, blue native electrophoresis, and Western blotting. Load, 5%; elution, 100%. (C–F) ^{35}S-Ugo1 or porin was imported into isolated mitochondria and analyzed by blue native electrophoresis and autoradiography.

can associate with the TOM complex and Tom40 core units; however, an intact TOM complex is not required for the import of these precursors, but Tom70 plays the major role.

Mim1 is required for the import of multispanning outer membrane proteins

Upon recognition by Tom70, two main possibilities are conceivable for the further import of multispanning outer membrane proteins: direct insertion into the lipid phase or insertion mediated by a proteinaceous machinery. An interesting candidate is Mim1, which is involved in membrane insertion of several single-spanning Tom proteins (Becker et al., 2008; Hulett et al., 2008; Popov-Celeketić et al., 2008; Thornton et al., 2010).

We analyzed the steady-state levels of mitochondrial proteins in a Mim1-deficient yeast strain and observed a strong reduction of the levels of Ugo1 and Scm4 (Fig. 2 A). The level of Tom20 was reduced (Becker et al., 2008), whereas control proteins, Mia40 of the intermembrane space assembly machinery and Tim23 of the inner membrane translocase, were not affected by the lack of Mim1. The level of Tom70 was only moderately reduced in the *mim1Δ* strain (Fig. 2 A), excluding that the strong reduction of Ugo1 and Scm4 levels was caused by a lack of Tom70.

To determine whether Mim1 was required for the import of Ugo1 and Scm4, we incubated the ^{35}S-labeled precursor proteins with isolated mitochondria and analyzed their import by blue native electrophoresis. Mim1-deficient mitochondria were

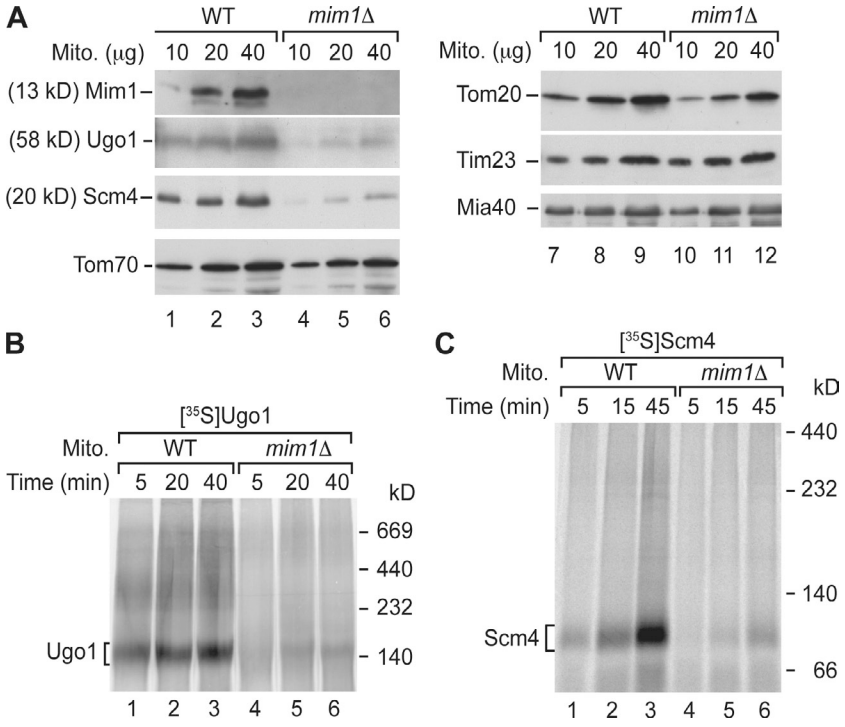

Figure 2. **Assembly of multispanning outer membrane proteins is inhibited in the absence of Mim1.** (A) Mitochondria (Mito.) were analyzed by SDS-PAGE and Western blotting. WT, wild type. (B and C) ^{35}S-Ugo1 or Scm4 was imported into wild-type and *mim1Δ* mitochondria. Mitochondria were lysed with digitonin and analyzed by blue native electrophoresis.

strongly inhibited in the assembly of both Ugo1 and Scm4 (Fig. 2, B and C). Further assays, alkaline extraction, and generation of protease-protected fragments support the conclusion that Mim1 promotes membrane insertion of Ugo1 (Fig. S2, A–C). To directly demonstrate that Mim1 is the critical component for the import of Ugo1, we overexpressed Mim1 in a *mim1Δ* yeast strain under an inducible promoter. Overexpression of Mim1 stimulated Ugo1 import over that of wild-type mitochondria (Fig. 3). Thus, in vivo protein levels and in organello import experiments indicate that Mim1 is crucial for the biogenesis of multispanning outer membrane proteins.

The precursors of multispanning proteins bind to the Mim1 complex

To determine whether Mim1 plays a direct role in protein import, we asked whether it interacts with the precursor of Ugo1. We imported chemical amounts of Ugo1 carrying a His tag into mitochondria. After lysis of mitochondria with nonionic detergent, Mim1 was copurified with Ugo1, whereas porin and Sam50 (Tob55) were not enriched in the eluate (Fig. 4 A). The yield of copurification was higher for Mim1 than for Tom subunits (Fig. S3 A).

Mim1 was reported to form oligomeric complexes (Ishikawa et al., 2004; Waizenegger et al., 2005; Hulett et al., 2008). Using blue native electrophoresis, we observed a main Mim1 complex of ∼200 kD (Fig. 4 B, lane 1). We analyzed the eluate of the Ni^{2+}-NTA affinity chromatography by blue native electrophoresis. His-tagged Ugo1 precipitated a Mim1-containing complex of ∼250 kD (Fig. 4 B, lanes 4 and 6), indicating that the Mim1 precursor complex can be directly visualized by blue native electrophoresis. Similarly, His-tagged Scm4 precipitated a fraction of the Mim1 complex (Fig. S3 B). Additionally, we studied the import of Om14, a mitochondrial outer membrane protein of 14.6 kD with three predicted α-helical transmembrane segments (Burri et al., 2006). Upon import into mitochondria, Om14 assembled into a complex of ∼180 kD; its import was strongly inhibited in *mim1Δ* mitochondria (Fig. S3 C). His-tagged Om14 coprecipitated the Mim1 complex (Fig. S3 D).

To determine whether Mim1 also interacts with single-spanning outer membrane proteins, we imported the precursor of Tom20 and, for comparison, the precursor of the β-barrel protein Tom40. ^{35}S-Tom20 was coprecipitated with anti-Mim1, and the coprecipitation was strongly increased upon overexpression of Mim1 (Fig. 4 C). In contrast, only background amounts of the ^{35}S-Tom40 precursor were precipitated, independent of the import time and levels of Mim1 (Fig. 4 C). Thus, Mim1 can interact with multispanning and single-spanning outer membrane precursor proteins.

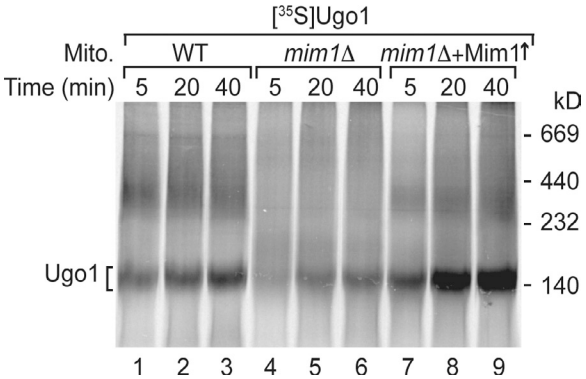

Figure 3. **Mim1 promotes the import of Ugo1.** ^{35}S-Ugo1 was imported into mitochondria (Mito.) isolated from wild-type (WT) yeast, *mim1Δ* yeast, and a *mim1Δ* strain overexpressing Mim1. Mitochondria were lysed with digitonin and analyzed by blue native electrophoresis.

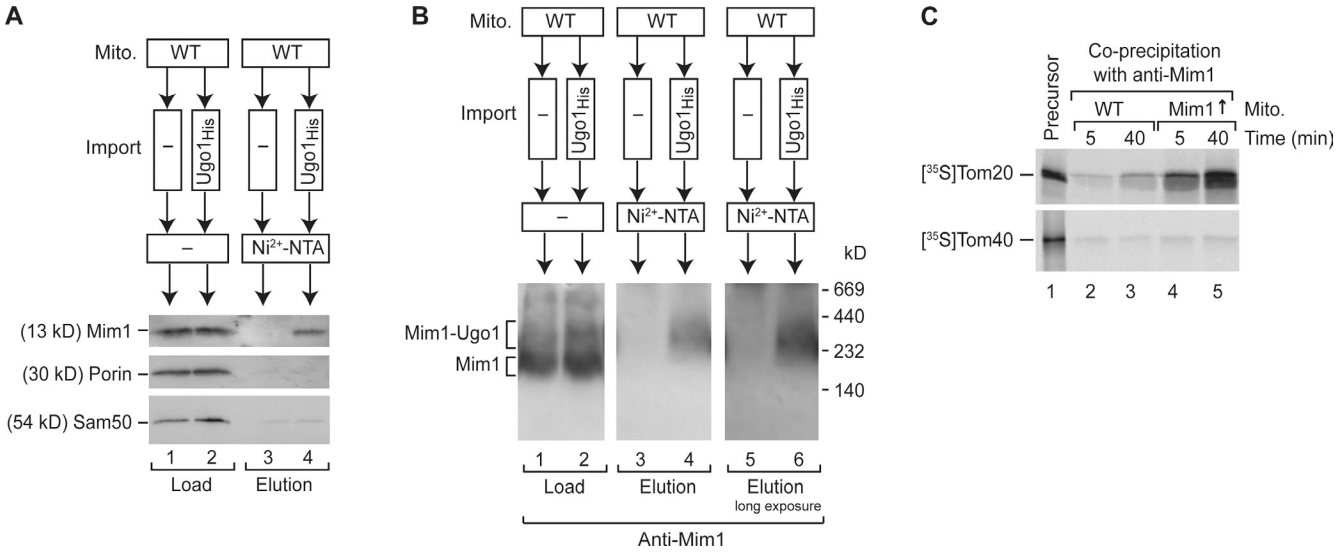

Figure 4. **Ugo 1 binds to the Mim1 complex.** (A) Chemical amounts of $Ugo1_{His}$ were imported into wild-type (WT) mitochondria (Mito.) for 10 min at 25°C. Mitochondria were lysed with digitonin and subjected to Ni-NTA agarose purification, SDS-PAGE, and immunodecoration. Load, 1%; elution, 100%. (B) Import and purification of $Ugo1_{His}$ were performed as described in A followed by blue native electrophoresis and Western blotting. Load, 5%; elution, 100%. (C) ^{35}S-Tom20 or Tom40 was imported into wild-type mitochondria and mitochondria from a Mim1-overexpressing strain. Mitochondria were lysed with digitonin and subjected to coprecipitation with Mim1-specific antiserum and SDS-PAGE. Precursor, 5% of reticulocyte lysate.

To characterize the role of Tom70 in the import of multispanning proteins, we performed several assays. The precursor of Ugo1 was bound by the purified cytosolic receptor domain of Tom70 but not Tom20 (Fig. 5 A), indicating a direct role of Tom70 in precursor recognition. In mitochondria lacking Tom70, binding of the Ugo1 precursor to the TOM complex, as well as to Mim1, was diminished (Fig. 5, B and C). As the steady-state level of Mim1 was comparable between wild-type and *tom70Δ* mitochondria (Fig. S3 E), these results suggest that Tom70 is involved in precursor binding to Mim1. To probe whether Tom70 interacts with Mim1, we generated a yeast strain expressing His-tagged Tom70. A fraction of Mim1, but not porin, was copurified with Tom70 (Fig. 5 D). Using the heterobifunctional cross-linking reagent maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), a covalent Tom70-Mim1 product was generated in mitochondria (Fig. 5 E), indicating a close proximity of Tom70 and Mim1 in organello. Finally, we asked why the import of Ugo1 was increased in *tom22Δ* mitochondria (Fig. 1 D). The central receptor Tom22 recruits Tom70 and Tom20 to the TOM core complex (van Wilpe et al., 1999; Yamano et al., 2008); dissociation of the TOM complex in *tom22Δ* mitochondria leads to the release of the receptors, and, thus, more Tom70 may be available for interaction with Mim1. With *tom22Δ* mitochondria, the fraction of Tom70 coprecipitated with Mim1 was indeed increased (Fig. S3 F). Collectively, we conclude that Tom70 and Mim1 cooperate in the import of Ugo1.

Conclusions

We report that Mim1 is a central component of the mitochondrial import pathway for multispanning α-helical outer membrane proteins. Mim1 cooperates with the receptor Tom70 in the import of the precursors into the outer membrane. Thus, Tom70 functions as a general receptor for multispanning mitochondrial proteins both of the outer membrane (Otera et al., 2007; this study) and the inner membrane (Brix et al., 1999; Wiedemann et al., 2001; Suzuki et al., 2002; Young et al., 2003; Wu and Sha, 2006). Mitochondria defective in Mim1 are impaired in biogenesis of the β-barrel protein Tom40 (Ishikawa et al., 2004; Waizenegger et al., 2005; Becker et al., 2008; Lueder and Lithgow, 2009), of several single-spanning outer membrane proteins (Becker et al., 2008; Hulett et al., 2008; Popov-Celeketić et al., 2008; Thornton et al., 2010), and of multispanning outer membrane proteins (this study). We show that Mim1 specifically interacts with single-spanning and multispanning precursors, identifying Mim1 as a genuine component of an outer membrane import machinery for α-helical proteins. It was recently shown that Mim1 indirectly promotes the biogenesis of Tom40 (Becker et al., 2010), and we indeed did not observe any specific interaction of Mim1 with the Tom40 precursor. It will be interesting to see whether further single-spanning outer membrane proteins (such as C tail–anchored proteins), for which a spontaneous membrane insertion has been discussed (Kemper et al., 2008), are imported in a Mim1-dependent manner. A critical step will be to develop specific import and assembly assays for these outer membrane proteins.

So far, four mitochondrial protein import pathways have been established: the presequence pathway that directs preproteins to the matrix, inner membrane, or intermembrane space; the carrier pathway for noncleavable multispanning inner membrane proteins; the redox-regulated mitochondrial intermembrane space assembly pathway; and the β-barrel SAM pathway to the outer membrane (Hoogenraad et al., 2002; Koehler, 2004; Dolezal et al., 2006; Neupert and Herrmann, 2007; Endo and Yamano, 2009; Schmidt et al., 2010; Sideris and Tokatlidis, 2010). We conclude that Mim1 forms the core of an α-helical import machinery of the outer membrane, constituting a fifth mitochondrial protein import pathway that transports multispanning outer

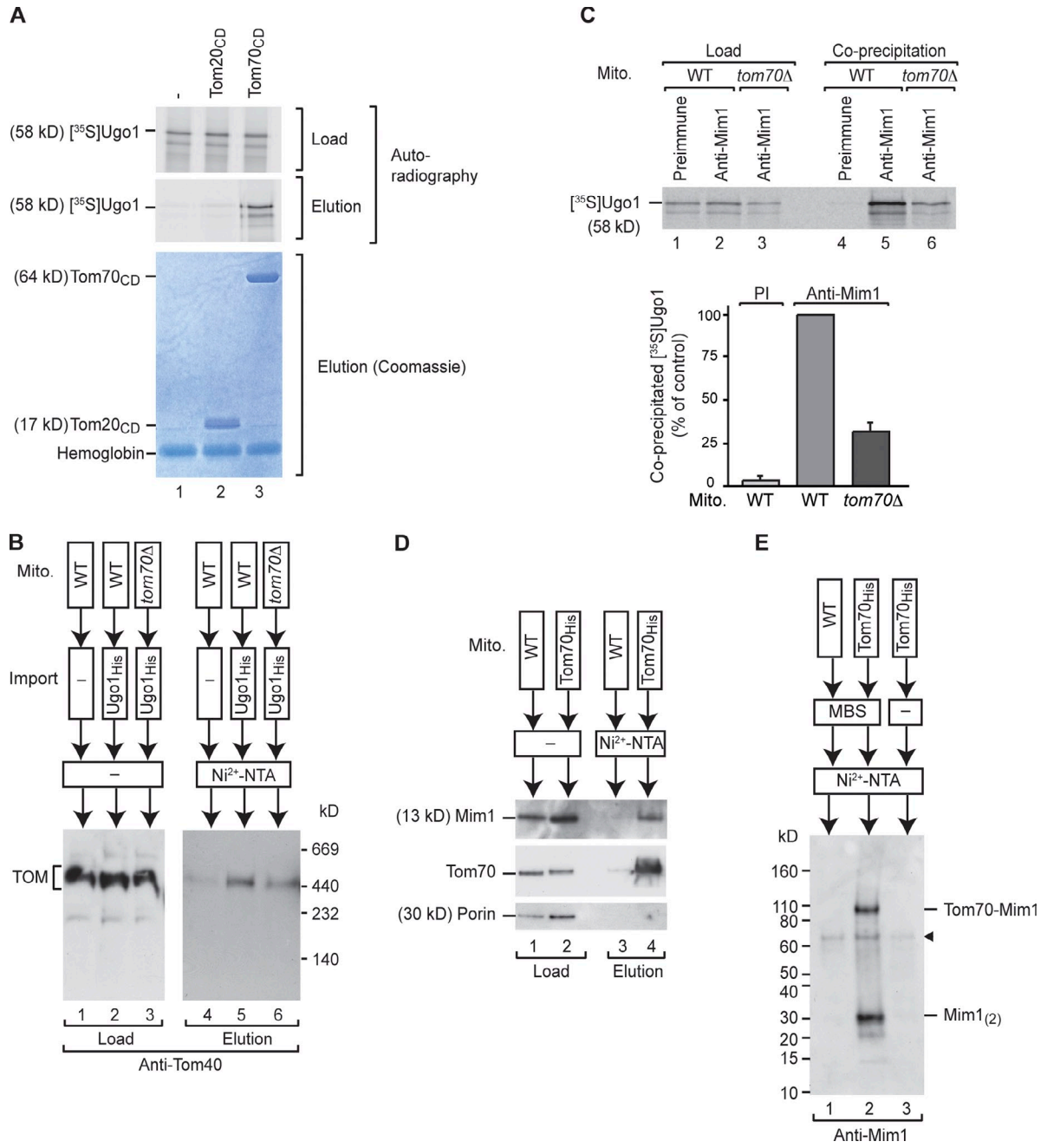

Figure 5. **Tom70 and Mim1 cooperate in the biogenesis of Ugo1.** (A) ^{35}S-Ugo1 was incubated with $Tom70_{CD}$ and $Tom20_{CD}$ coupled to Ni-NTA. Bound proteins were eluted and analyzed by SDS-PAGE. Load, 5%; elution, 100%. (B) Chemical amounts of $Ugo1_{His}$ were imported into mitochondria (Mito.) for 10 min at 25°C. Mitochondria were lysed with digitonin and subjected to Ni-NTA agarose purification, blue native electrophoresis, and Western blotting. Load, 2%; elution, 100%. WT, wild type. (C, top) ^{35}S-Ugo1 was imported into mitochondria for 10 min at 25°C. Mitochondria were lysed with digitonin, and immunoprecipitation with the indicated antisera was performed, followed by SDS-PAGE and autoradiography. Load, 3%; elution, 100%. (bottom) Quantification of three independent experiments with standard error of the means. Coprecipitation of ^{35}S-Ugo1 with anti-Mim1 in wild type was set to 100% (control). PI, preimmune. (D) Mitochondria were solubilized with digitonin and incubated with Ni-NTA agarose. Bound proteins were eluted and subjected to SDS-PAGE and immunodecoration with the indicated antisera. Load, 0.5%; elution, 100%. (E) Mitochondria were subjected to cross-linking with MBS, solubilized with SDS, and incubated with Ni-NTA agarose. Bound proteins were eluted and analyzed by SDS-PAGE and immunodecoration. Arrowhead, unspecific band.

membrane proteins and at least some single-spanning outer membrane proteins. In agreement with our findings, a Mim1-dependent import of Ugo1 is reported in a parallel study (see Papić et. al. in this issue). The Mim1 pathway can use TOM and/or SAM components but does not use the entire TOM complex (Ahting et al., 2005; Becker et al., 2008; Kemper et al., 2008; Thornton et al., 2010; this study). Mim1 contains a single transmembrane segment; however, it does not function as a 13-kD monomer but forms an oligomeric complex of ~200 kD (Ishikawa et al., 2004; Waizenegger et al., 2005; Hulett et al., 2008) that binds the incoming precursor proteins. The N- and C-terminal domains of Mim1 are dispensable, whereas its transmembrane

segment is critical for oligomerization and function (Popov-Celeketić et al., 2008). The transmembrane segment of Mim1 is thus likely involved in binding the transmembrane segments of outer membrane precursor proteins. The oligomeric structure of the Mim1 complex may provide an appropriate environment for the insertion of precursor proteins into the lipid phase of the outer membrane.

Materials and methods

Yeast strains and growth conditions

The *S. cerevisiae* strains *mim1Δ*, *tom70Δ*, and *tom20Δ* and the corresponding wild-type strains have been previously described (Moczko et al., 1994; Hönlinger et al., 1995; Becker et al., 2008). *TOM22* was deleted in the YPH499 background via plasmid shuffling. YPH499 was transformed with a Yep532 plasmid encoding Tom22 under the control of the *MET25* promoter and the *CYC1* terminator. The chromosomal gene *TOM22* was disrupted with a *HIS3* cassette. Subsequently, the plasmid was lost by growth on medium containing 5-fluoroorotic acid. The $Tom70_{His}$ strain was generated by chromosomal integration of a coding region for a deca-His tag at the C terminus of Tom70 using a *HIS3* cassette (Meisinger et al., 2001). The coding region of Tom40 fused to a triple HA tag was introduced into the centromeric pFL39 plasmid under the control of the endogenous promoter of *TOM40* and the terminator of *MIA40*. The construct was used for the transformation of YPH499. The chromosomal *TOM40* was deleted by an *ADE2* marker. The strains were grown on YPG or YPS medium (1% yeast extract, 2% peptone, and 3% glycerol or 2% sucrose). The plasmid pRS415 encoding Tom40-HA was introduced into *tom22Δ* (YPH499) and the corresponding wild type (rho^0; $Tom40_{HA}$ was coexpressed with wild-type Tom40); the control wild-type (rho^0) strain received the plasmid pRS415 without insert. The strains were grown on selective medium under fermentative conditions. The Mim1-overexpressing strain was generated by introducing the ORF of *MIM1* into a pYES2 vector containing a *URA3* marker under the control of a galactose-inducible promoter. Yeast cells were grown on selective medium with 2% glucose at 24°C. Overexpression was induced by growth on selective medium containing 2% galactose overnight at 24°C. Immunodecoration with Mim1-specific antiserum confirmed the overproduction of Mim1.

Protein import into mitochondria

Mitochondria were isolated from yeast cells by differential centrifugation and were stored at −80°C at a protein concentration of 10 mg/ml in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS/KOH, pH 7.2; Stojanovski et al., 2007b). For in vitro transcription, PCR products containing an SP6 promoter were generated using yeast genomic DNA as a template. In the case of Om14, the cDNA was cloned into the vector SP73 (Om14 is one of the rare yeast proteins that are encoded by an intron-containing gene; Burri et al., 2006); a nucleotide sequence encoding four additional methionines was inserted into the *OM14* ORF directly before the stop codon. In vitro transcription was performed according to the manufacturer's recommendation (mMESSAGE mMACHINE SP6 kit; Invitrogen). Subsequently, RNA was purified (MEGAclear kit; Invitrogen) and used for in vitro translation (TNT kit; Promega). The efficiency of the translation reaction was controlled by SDS-PAGE and autoradiography. In a standard import reaction, 10% (volume/volume) reticulocyte lysate was incubated with 50 µg mitochondria (protein amount) at 25°C in the presence of 2 mM NADH, 2 mM ATP, 2.5 mM methionine, 5 mM creatine phosphate, and 100 µg/ml creatine kinase in import buffer (3% [weight/volume] BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS/KOH, pH 7.2, and 2 mM KH_2PO_4). Transfer on ice stopped the import reaction. The mitochondrial pellet was washed with SEM buffer and was subsequently lysed in lysis buffer (20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, and 10% [volume/volume] glycerol) containing 1% (weight/volume) digitonin for 15 min on ice. After a clarifying spin, the mitochondrial extract was subjected to blue native electrophoresis, and the imported proteins were visualized by autoradiography. For protease treatment, import samples were incubated with 50 µg/ml proteinase K for 15 min at 4°C. The activity of the protease was stopped by addition of PMSF and incubation for 10 min at 4°C. After washing, mitochondria were analyzed by SDS-PAGE. Carbonate extraction was performed following a previously described improved method (Thornton et al., 2010). Imported proteins were detected by SDS-PAGE or blue native electrophoresis and analyzed by digital autoradiography (Storm 820 imaging system; GE Healthcare) and ImageQuant software (version 5.2; GE Healthcare).

Affinity purification

For coimmunoprecipitation, antibodies were covalently coupled to protein A–Sepharose (GE Healthcare) with dimethylpimelimidate. Mitochondria were resuspended in lysis buffer containing 1% digitonin at a protein concentration of 1 mg/ml for 20 min on ice. After a clarifying spin, the supernatant was incubated with the indicated matrix under constant rotation for 1 h at 4°C. After excessive washing with lysis buffer containing 0.1–0.3% digitonin, the proteins were eluted and subjected to SDS-PAGE, Western blotting, and immunodecoration with the indicated antisera. For coprecipitation of ^{35}S-labeled precursor proteins, the precursors were imported into isolated mitochondria for 5–40 min (two- to sixfold import reaction). After the import reaction, mitochondria were washed and lysed in lysis buffer. Binding to the anti–HA matrix and washing steps were performed as described above. Samples were subjected to SDS-PAGE, and ^{35}S-labeled proteins were analyzed by digital autoradiography. Affinity purification under native conditions using Ni-NTA agarose was performed as previously described (Thornton et al., 2010).

Cross-linking

Wild-type and $Tom70_{His}$ mitochondria were treated with the cross-linking reagent MBS for 30 min at 4°C in SEM buffer. The cross-linking reaction was stopped by an addition of 100 mM DTT and 100 mM Tris/HCl, pH 7.4, and by incubation for 15 min at 4°C. For affinity purification of the cross-linked products, mitochondria were solubilized with 1% SDS in lysis buffer for 15 min at room temperature. After a clarifying spin, the mitochondrial lysate was diluted 1:10 in lysis buffer containing 0.2% Triton X-100 and 10 mM imidazole and incubated with Ni-NTA agarose. After excessive washing, bound proteins were eluted with 250 mM imidazole and 0.1% SDS in lysis buffer. Samples were subjected to SDS-PAGE.

Purification of imported His-tagged precursor proteins

The assay was performed essentially as previously described (Becker et al., 2011). In brief, chemical amounts of His-tagged precursor proteins were synthesized by in vitro transcription/translation (RTS 100 Wheat Germ CECF kit; 5Prime). Import of His-tagged proteins into isolated mitochondria was performed in a sixfold import reaction for 10 min. After washing and reisolation, mitochondria were lysed in lysis buffer containing 1% digitonin and 10 mM imidazole and incubated on ice for 20 min before a clarifying spin. The supernatant was mixed with the preequilibrated Ni-NTA agarose and incubated under constant rotation for 1 h at 4°C. After excessive washing with lysis buffer containing 0.1% digitonin and 20 mM imidazole, proteins were eluted by 250 mM imidazole in lysis buffer and analyzed by SDS-PAGE or blue native electrophoresis and Western blotting.

Binding assay with cytosolic receptor domains

The cytosolic domains of Tom70 and Tom20 ($Tom70_{CD}$ and $Tom20_{CD}$) were recombinantly expressed and purified as previously described (Brix et al., 1997). For binding studies, $Tom70_{CD}$ and $Tom20_{CD}$ were bound to Ni-NTA agarose and incubated with ^{35}S-Ugo1 precursor in import buffer containing 0.2% BSA for 40 min at 4°C. After excessive washing, bound proteins were eluted with 500 mM imidazole in lysis buffer and subjected to SDS-PAGE. Bound proteins were detected by Coomassie staining and autoradiography.

Miscellaneous

Western blotting signals were detected using the ECL kit (GE Healthcare). Scanning of x-ray films was performed using ScanMaker 1000XL and SilverFast SDKXRay 6.6.2r1 software (Microtek). Quantification was performed using ImageQuant 5.2 software. Nonrelevant lanes were excised digitally (indicated by separating white lines).

Online supplemental material

Fig. S1 shows that mitochondrial import of the multispanning proteins Ugo1 and Scm4 requires Tom70. Fig. S2 provides additional evidence for a role of Mim1 in the import of Ugo1 by using alkaline extraction and protease protection. Fig. S3 shows additional evidence for the interaction of Mim1 with precursor proteins and Tom70. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201102044/DC1.

We thank Dr. Nils Wiedemann for discussion.

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 746, Baden-Württemberg Stiftung, Excellence Initiative of the German federal and state governments (Biological Signalling Studies [EXC 294] and Spemann Graduate School of Biology and Medicine [GSC-4]), Bundesministerium für Bildung und Forschung (Dynamo), Landesforschungspreis Baden-Württemberg, and the Gottfried Wilhelm Leibniz Program.

Submitted: 8 February 2011
Accepted: 11 July 2011

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