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Two Modular Forms of the Mitochondrial Sorting and Assembly Machinery Are Involved in Biogenesis of α-Helical Outer Membrane Proteins

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The mitochondrial outer membrane contains two translocase machineries for precursor proteins—the translocase of the outer membrane (TOM complex) and the sorting and assembly machinery (SAM complex). The TOM complex functions as the main mitochondrial entry gate for nuclear-encoded proteins, whereas the SAM complex was identified according to its function in the biogenesis of β-barrel proteins of the outer membrane. The SAM complex is required for the assembly of precursors of the TOM complex, including not only the β-barrel protein Tom40 but also a subset of α-helical subunits. While the interaction of β-barrel proteins with the SAM complex has been studied in detail, little is known about the interaction between the SAM complex and α-helical precursor proteins. We report that the SAM is not static but that the SAM core complex can associate with different partner proteins to form two large SAM complexes with different functions in the biogenesis of α-helical Tom proteins. We found that a subcomplex of TOM, Tom5–Tom40, associates with the SAM core complex to form a new large SAM complex. This SAM–Tom5/Tom40 complex binds the α-helical precursor of Tom6 after the precursor has been inserted into the outer membrane in an Mim1 (mitochondrial import protein 1)-dependent manner. The second large SAM complex, SAM–Mdm10 (mitochondrial distribution and morphology protein), binds the α-helical precursor of Tom22 and promotes its membrane integration. We suggest that the modular composition of the SAM complex provides a flexible platform to integrate the sorting pathways of different precursor proteins and to promote their assembly into oligomeric complexes.

Keywords: Mdm10; protein sorting; *Saccharomyces cerevisiae*; SAM complex; TOM complex

Introduction

Mitochondria have to import ~1000 proteins from the cytosol.[1–6] The translocase of the outer membrane (TOM complex) forms the entry gate for most nuclear-encoded mitochondrial proteins. It consists of seven different subunits. The β-barrel protein Tom40 is the central channel-forming subunit.[7–10] The other six TOM subunits are anchored in the outer membrane by single α-helical transmembrane segments: the three receptors Tom20, Tom22 and Tom70 and three small Tom proteins (Tom5, Tom6 and Tom7) that affect the stability of the complex.[11–24]

All Tom proteins are encoded by nuclear genes and are thus synthesized on cytosolic ribosomes and have to be imported into mitochondria. Analysis of the biogenesis pathway of the precursor of Tom40 led to the identification of a second protein translocase in the mitochondrial outer membrane, the sorting and assembly machinery

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Abbreviations used: Mdm, mitochondrial distribution and morphology; Mim1, mitochondrial import protein 1; NTA, nitrilotriacetic acid; SAM complex, sorting and assembly machinery; TOM complex, translocase of the outer membrane.

(SAM complex).[25,26] The SAM complex is essential for the insertion of β-barrel proteins into the outer membrane. The biogenesis pathway of Tom40 has been studied in detail. The precursor is first translocated through the TOM complex to the intermembrane space side. Chaperone complexes of the intermembrane space then guide the precursor to the SAM complex.[27–29] The SAM_{core} complex, consisting of Sam35 (Tob38/Tom38), Sam37 and Sam50 (Tob55/Omp85), directly interacts with the precursor protein.[26,30–35] Sam35 and the central channel-forming protein Sam50 cooperate in precursor binding and insertion,[36] while Sam37 is involved in precursor release into the lipid phase.[37] A fraction of SAM_{core} complexes associate with the mitochondrial distribution and morphology protein Mdm10 to form a large SAM complex (SAM–Mdm10, SAM_{holo}) that promotes late stages of TOM assembly.[38–40] In addition, the outer membrane protein Mim1 (mitochondrial import protein 1) transiently interacts with the SAM complex[41] and promotes the maturation of Tom40.[34,41–43]

While it had been assumed that the SAM complex is dedicated to the biogenesis of β-barrel proteins, studies with yeast mutants unexpectedly suggested that the SAM is also involved in the biogenesis of a subset of α-helical Tom proteins.[41,44] Based on the location of the transmembrane segment, three groups of α-helical Tom proteins can be distinguished: (i) N-terminal membrane anchor (Tom20 and Tom70); (ii) internal membrane anchor (Tom22); and (iii) C-terminal membrane anchor (Tom5, Tom6 and Tom7).[15–17,19,21,22,45–49] Tom20 and Tom70 are inserted into the outer membrane in a SAM-independent but Mim1-dependent manner, indicating that a SAM-independent pool of Mim1 exists.[26,38,41,43,44,50,51] The biogenesis of Tom22 is inhibited in mutants of each of the SAM_{core} components and of Mdm10.[38,44] Since an interaction of the Tom22 precursor with SAM or Mdm10 has not been observed so far, it is unknown whether the SAM–Mdm10 complex is directly involved in the biogenesis of Tom22 or not. The biogenesis of small Tom proteins is impaired in mitochondria lacking Sam37, and the precursor of Tom6 was found in association with a large SAM form.[41,44] This large SAM form contained Sam35, yet its further composition has not been analyzed. Moreover, Tom22 and the small Tom proteins differ with regard to dependence on Mim1. Whereas the biogenesis of Tom22 is independent of Mim1, membrane insertion of Tom6 is impaired in mitochondria lacking Mim1.[41] Taken together, these studies suggest that the biogenesis of a subset of α-helical Tom precursors is influenced by SAM functions; however, it is unknown if and how the SAM complex plays a direct role in their import.

In this study, we performed a systematic analysis of the interaction of α-helical Tom precursors with SAM. We were able to show that mitochondria contain two large SAM complexes. The Mdm10-containing SAM complex directly interacts with the precursor of Tom22 and promotes its membrane insertion. We identified a second large SAM complex that surprisingly contains a module of endogenous Tom proteins, Tom5–Tom40, but not Mdm10. This SAM–Tom5/Tom40 complex binds the precursor of Tom6 and promotes its assembly. Our results suggest that the SAM complex does not function as a static entity but provides a modular assembly platform for different types of Tom precursor proteins.

Results and Discussion

Biogenesis of Tom6 involves a large SAM complex not containing Mdm10

The ^{35}S-labeled precursor of Tom6 was incubated with isolated yeast mitochondria. Upon lysis with the mild detergent digitonin, blue native electrophoresis resolved the mature TOM complex of ~450 kDa and, additionally, smaller intermediate forms (Fig. 1a, lane 1).[25,38,41] The largest of the intermediate forms, migrating at ~350 kDa, was shown to contain Sam35.[41] So far, two forms of the SAM complex have been described: the SAM_{core} complex of ~200 kDa, containing Sam35, Sam37 and Sam50, and a large SAM–Mdm10 complex of ~350 kDa, which additionally contains Mdm10.[38,39] To test whether the precursor of Tom6 was bound to SAM–Mdm10, we imported radiolabeled Tom6 into mitochondria that had been isolated from a yeast strain with His-tagged Mdm10 (Fig. 1a, lane 2). Using antibodies directed against the His tag, we performed an antibody-shift blue native electrophoresis, but the anti-His antibodies did not alter the gel mobility of the putative large SAM form (Fig. 1a, lane 4, SAM*). For comparison, when mitochondria containing His-tagged Sam35 were used, anti-His antibodies shifted the SAM* intermediate of the Tom6 precursor as reported (Fig. 1a, lane 8).[41] To exclude that the His tag at Mdm10 was not accessible to the anti-His antibodies, we analyzed Sam50 by Western blotting. Anti-His shifted Sam50 in $Mdm10_{His}$ mitochondria (Fig. 1a, lane 12, arrowhead), demonstrating that SAM–Mdm10 was accessible to anti-His. These results indicate that the precursor of Tom6 is not associated with SAM–Mdm10.

We thus asked which other components are present in the SAM* form that is associated with [^{35}S]Tom6. We imported Tom6 into mitochondria isolated from yeast strains with protein-A-tagged Sam37 or Sam50 (Fig. 1b). The protein A tag caused a size shift of SAM* in the cases of Sam37 and Sam50, demonstrating that both proteins are present in SAM* (Fig. 1b, lanes 2 and 4; the shifted bands migrate in the range of the TOM complex). It has been reported that the assembly of [^{35}S]Tom6 is delayed in mitochondria lacking Sam37,[44] but the SAM* intermediate has not been analyzed. We thus imported [^{35}S]Tom6 into *sam37*Δ mitochondria and observed that the SAM* intermediate was shifted to a band with smaller size and lower intensity (Fig. 1c,

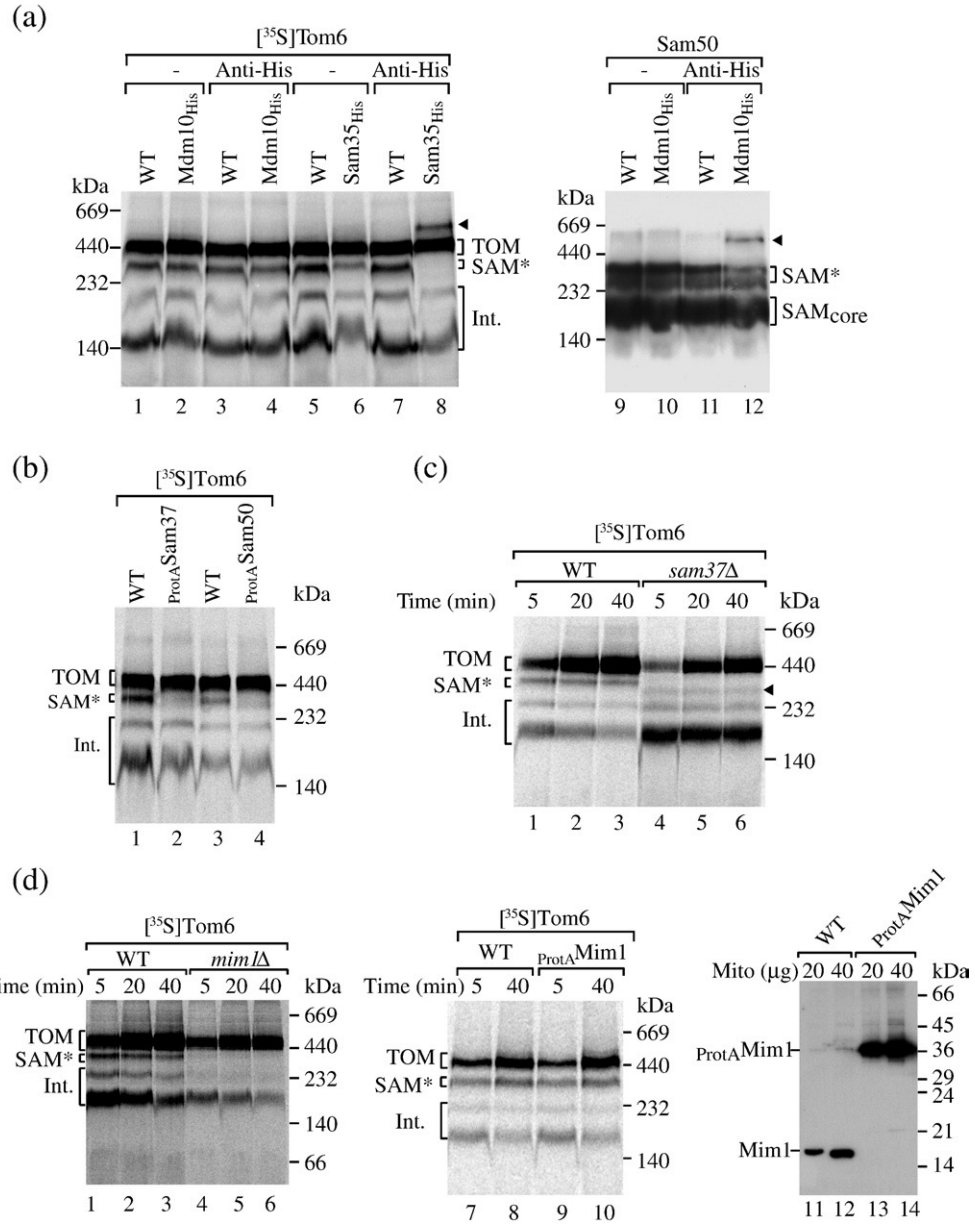

Fig. 1. The precursor of Tom6 interacts with a large SAM complex that contains neither Mdm10 nor Mim1. (a) Mitochondria isolated from wild-type yeast or yeast strains containing His-tagged Mdm10 or Sam35 were incubated with [^{35}S]Tom6 for 20 min at 25 °C (left panel) or left untreated (right panel). Subsequently, mitochondria were solubilized with digitonin and incubated with anti-His antibodies as indicated. Protein complexes were analyzed by blue native electrophoresis and detected by digital autoradiography (left panel) or immunoblotting with antibodies directed against Sam50 (right panel). Arrowheads indicate antibody-shifted complexes. (b) [^{35}S]Tom6 was imported into mitochondria containing protein-A-tagged Sam37 or Sam50. Protein complexes were analyzed by blue native electrophoresis. (c) [^{35}S]Tom6 was imported into isolated mitochondria for the indicated periods. The arrowhead indicates a shifted SAM* complex in *sam37*Δ mitochondria. (d) [^{35}S]Tom6 was imported into isolated mitochondria for the indicated periods, followed by blue native electrophoresis (left and middle panels). In the right panel, mitochondria were separated by SDS-PAGE and subjected to immunoblotting using antibodies directed against Mim1. WT indicates wild-type; SAM*, large SAM complex; Int., low-molecular-weight intermediates of [^{35}S]Tom6.

arrowhead). We conclude that Sam35, Sam37 and Sam50, but not Mdm10, are present in the 350-kDa SAM* form associated with [^{35}S]Tom6.

A fraction of Mim1 molecules have been shown to interact with the SAM complex in an Mdm10-independent manner.[41] The analysis was performed in the absence of precursor proteins and revealed that, under these conditions, Mim1 was not a stoichiometric SAM component. Moreover, it has been reported that mitochondria deficient of Mim1 do not form the SAM* intermediate of the Tom6 precursor[41] (comparison is shown in Fig. 1d, lanes 4–6). Thus, two possibilities were conceivable: (i) Mim1 is required to form the SAM* form of Tom6 but is not a component of the complex itself or (ii) Mim1 stably associates with SAM and becomes a

component of SAM* in the presence of the precursor protein. In order to distinguish between these possibilities, we imported radiolabeled Tom6 into mitochondria containing a protein-A-tagged Mim1 (Fig. 1d, lanes 9 and 10). SDS-PAGE analysis showed that the protein A tag was quantitatively present at the Mim1 molecules (Fig. 1d, lanes 13 and 14). However, the formation and mobility of [^{35}S]Tom6–SAM* were indistinguishable between wild-type mitochondria and mitochondria containing protein-A-tagged Mim1 (Fig. 1d, lanes 7–10). Thus, Mim1 is required to generate the large SAM* form associated with the precursor of Tom6 but is not a stoichiometric component of the complex. In conclusion, the precursor of Tom6 associates with a large SAM complex that contains all SAM_{core} subunits but neither Mdm10 nor Mim1 in stoichiometric amounts.

A large SAM complex containing Tom5/Tom40

In order to identify putative further components of a large SAM complex, we screened various mutant mitochondria deficient in outer membrane proteins by importing the radiolabeled precursor of Tom6. Unexpectedly, we observed that the lack of Tom5 affected the formation and mobility of the [^{35}S]Tom6–SAM* form. The form was present in lower amounts, and its size was shifted in *tom5*Δ mitochondria (Fig. 2a, lanes 4–6). For comparison, we used mitochondria lacking the other small Tom protein, Tom7, yet the mobility of [^{35}S]Tom6–SAM* was not altered in *tom7*Δ mitochondria (Fig. 2a, lanes 7–12).

Mim1 is required for membrane insertion of the Tom6 precursor as determined by resistance to alkaline extraction (carbonate treatment).[41,52,53] Soluble proteins and peripheral membrane proteins are extracted to the supernatant at alkaline pH, whereas membrane-integrated proteins remain in the pellet fraction. When performing carbonate extraction with radiolabeled precursor proteins, we observed that *in vitro* synthesized precursors of membrane proteins can be prone to aggregation upon dilution in the import buffer. Such unspecifically precipitated proteins may result in a false-positive detection in the membrane pellet after carbonate extraction. We searched for conditions to optimize the carbonate assay and found that unspecific precipitation was strongly reduced by preincubation of radiolabeled precursor proteins in import buffer followed by a clarifying spin (before addition of mitochondria). Utilizing this optimized procedure, we observed that membrane integration of Tom5 and Tom7 occurred with reduced efficiency in *mim1*Δ mitochondria (Fig. 2b, lanes 11–13), similarly to the import of Tom6 (Fig. 2b, upper panel, lanes 4–6).[41]

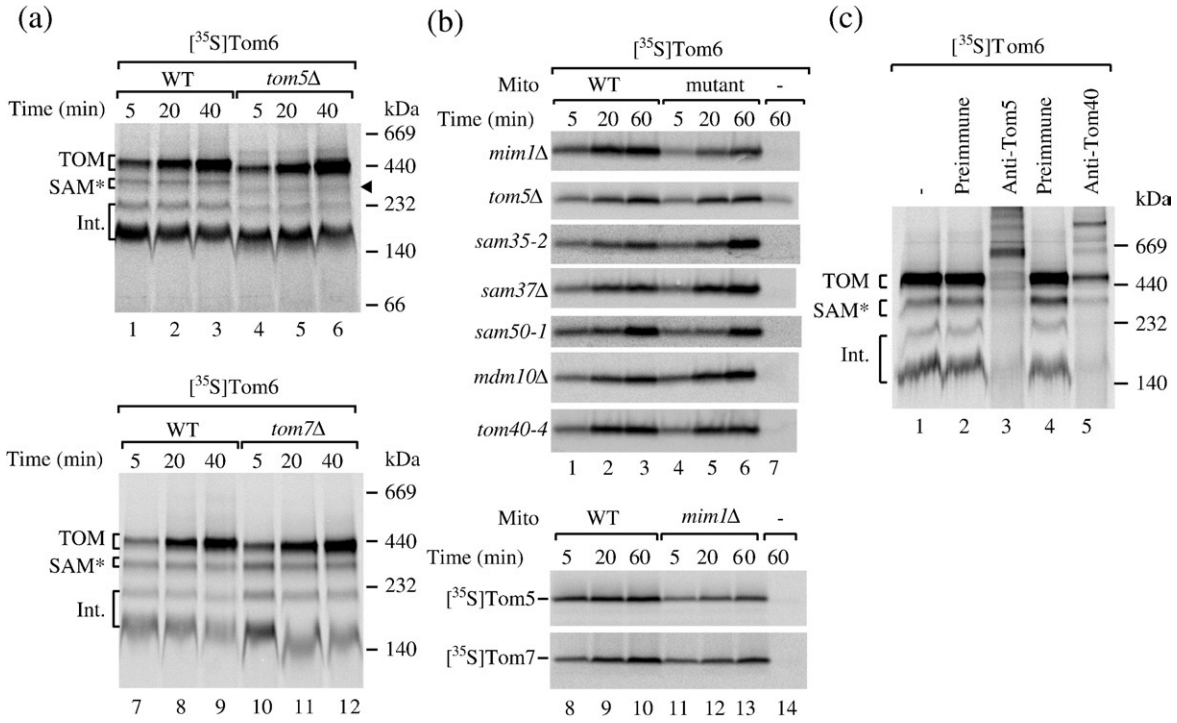

Fig. 2. The precursor of Tom6 associates with a large SAM complex containing Tom5 and Tom40. (a) [^{35}S]Tom6 was imported into mitochondria isolated from wild-type, *tom5*Δ or *tom7*Δ yeast strains for the indicated time points. Mitochondria were reisolated and analyzed by blue native electrophoresis and autoradiography. The arrowhead indicates a shifted SAM* complex in *tom5*Δ mitochondria. (b) [^{35}S]Tom6, [^{35}S]Tom5 and [^{35}S]Tom7 were imported into mitochondria (Mito) isolated from wild-type and mutant yeast strains as indicated. The mitochondria were subjected to carbonate extraction, and the membrane pellet was analyzed by SDS-PAGE and autoradiography. For samples 7 and 14, no mitochondria were added (mock control). (c) [^{35}S]Tom6 was imported into wild-type mitochondria for 20 min. The mitochondria were reisolated and incubated with the indicated antisera. Protein complexes were separated by blue native electrophoresis and analyzed by autoradiography.

Thus, Mim1 is involved in the membrane integration of all three small Tom proteins.

We then asked if Tom5 functioned at the same step of Tom6 import as Mim1. To address this issue, we used the optimized carbonate extraction procedure to analyze membrane integration of Tom6 in *tom5*Δ mitochondria. In contrast to *mim1*Δ mitochondria, the efficiency of membrane integration of the Tom6 precursor in *tom5*Δ mitochondria was indistinguishable from that of wild-type mitochondria (Fig. 2b, second panel, lanes 1–6). *tom5*Δ mitochondria thus behaved similar to mutant mitochondria of the SAM complex (*sam37*Δ and the conditional mutants *sam35-2* and *sam50-1*)[31,32] that inserted the precursor of Tom6 with wild-type efficiency (Fig. 2b).[44]

These findings indicated that Tom5 is not involved in the Mim1-dependent membrane insertion of Tom6 but acts at a later step after membrane integration of the precursor. Since Mim1 functions before SAM*, this raised the possibility that Tom5 may be a component of SAM*. To experimentally determine if Tom5 is present in [^{35}S]Tom6–SAM*, we used antibody-shift blue native electrophoresis.[26,54] Upon import of [^{35}S]Tom6, mitochondria were incubated with antibodies directed against Tom5. When antibodies bind to a complex in a stoichiometric manner, blue native electrophoresis shows the altered mobility (shift). Indeed, anti-Tom5 shifted not only the mature TOM complex in a quantitative manner but also the smaller [^{35}S]Tom6 forms (Fig. 2c, lane 3). Preimmune antibodies did not shift the complexes (Fig. 2c, lane 2). Thus, the [^{35}S]Tom6 complexes that are resolved by blue native electrophoresis, including SAM*, contain Tom5. The presence of the small protein Tom5 itself is not sufficient to explain the size difference of SAM_{core} and SAM*. Since Tom5 has been shown to stably associate with Tom40,[21,25,26,55] we asked if Tom40 was also present in SAM*. Antibodies directed against Tom40 shifted SAM* and the smaller forms as efficiently as they shifted the mature TOM complex, whereas the corresponding preimmune antibodies did not (Fig. 2c, lanes 4 and 5). We conclude that the large SAM* complex associated with [^{35}S]Tom6 contains Tom5 and Tom40. Mitochondria from a *tom40* mutant strain[56] were competent in membrane insertion of the precursor of Tom6, such as *tom5*, *sam35*, *sam37* and *sam50* mutant mitochondria (Fig. 2b), in agreement with the view that SAM, Tom5 and Tom40 function in the assembly pathway of Tom6 after its integration into the outer membrane.

Since only the precursor of Tom6 was radiolabeled in the import studies, the observed presence of Tom5 and Tom40 in SAM* suggested that endogenous Tom5 and Tom40 formed subunits of a large SAM complex. To test this, we analyzed isolated mitochondria by blue native electrophoresis and Western blotting without importing precursor proteins. In wild-type mitochondria, antibodies against Sam50 decorated the 200-kDa SAM_{core} complex and the 350-kDa large SAM complex (Fig. 3a, lane 1).[38,39,41]

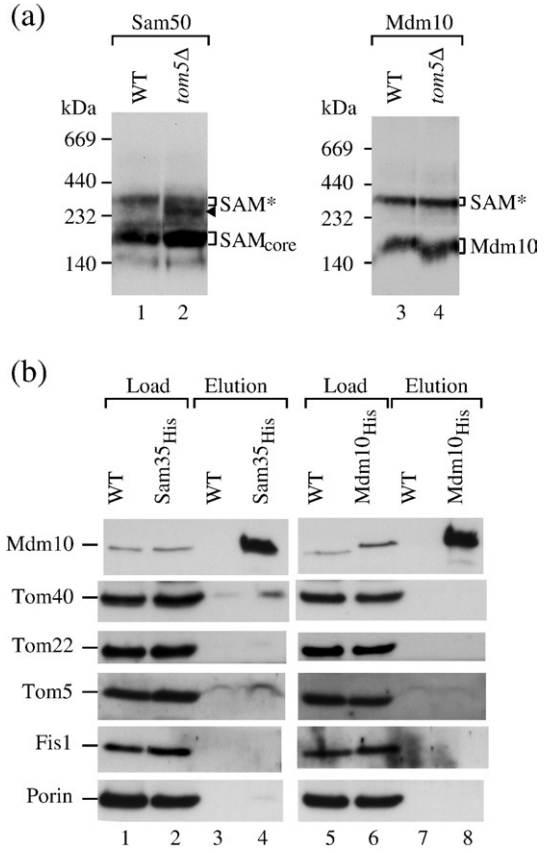

Fig. 3. A fraction of endogenous Tom5 and Tom40 are associated with SAM. (a) Wild-type and *tom5*Δ mitochondria were solubilized with digitonin and analyzed by blue native electrophoresis and immunoblotting with antisera against Sam50 or Mdm10. The arrowhead indicates a smaller form of SAM* in *tom5*Δ mitochondria. (b) Mitochondria (1 mg of protein) isolated from wild-type, $Sam35_{His}$ or $Mdm10_{His}$ yeast strains were solubilized with digitonin and incubated with Ni-NTA agarose. After extensive washing, bound proteins were eluted with digitonin buffer containing 250 mM imidazole and analyzed by SDS-PAGE and immunoblotting. Load, 2%; elution, 100%.

In *tom5*Δ mitochondria, an additional band migrating slightly faster than SAM* was decorated with anti-Sam50 (Fig. 3a, lane 2, arrowhead). In contrast, the large SAM complex detected with antibodies against Mdm10 was not altered in *tom5*Δ mitochondria (Fig. 3a, lanes 3 and 4), indicating that Tom5 and Mdm10 are present in different large SAM complexes that, however, show similar blue native mobility under wild-type conditions.

We used affinity purification after lysis of mitochondria under mild conditions (low concentrations of digitonin and salt) as an independent approach to determine the presence of endogenous Tom proteins in SAM complexes. We compared mitochondria containing a His tag at either Sam35 or Mdm10.[32,38,40] $Sam35_{His}$ co-purified not only Mdm10, as expected, but also a fraction of Tom40 and Tom5 (Fig. 3b, lane 4) (the majority of Tom40

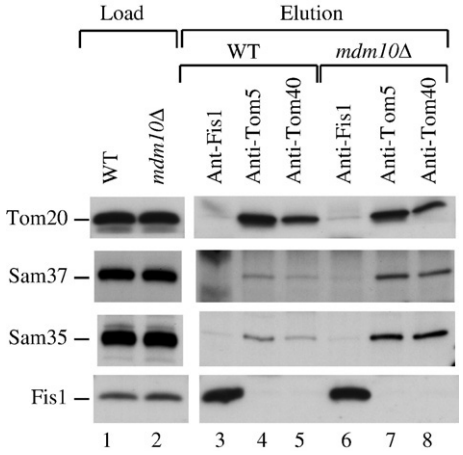

Fig. 4. Lack of Mdm10 enhances the association of Tom5 and Tom40 with the SAM complex. Wild-type and *mdm10*Δ mitochondria (1 mg of protein) were lysed with digitonin and incubated with antibodies coupled to protein A-Sepharose (anti-Fis1, anti-Tom5, anti-Tom40). After washing of the protein A-Sepharose beads with digitonin buffer, proteins were eluted with 0.1 M glycine, pH 2.5, loaded onto Tricine SDS-PAGE and detected by immunoblotting. Load, 2%; elution, 100%.

and Tom5 molecules are present in the mature TOM complex). Tom22 and other outer membrane proteins, such as Fis1 and porin, were not observed in the eluate (Fig. 3b, lane 4). $Mdm10_{His}$ co-purified neither Tom40 nor Tom5 (Fig. 3b, lane 8). Taken together, a fraction of endogenous Tom5 and Tom40, but not Tom22, are present in a large SAM complex that is different from the Mdm10-containing complex. We term this new large SAM complex the *SAM–Tom5/Tom40 complex*.

Lack of Mdm10 favors formation of the SAM–Tom5/Tom40 complex

To demonstrate the association of Tom5 and Tom40 with the SAM complex with authentic (non-tagged) proteins, we used co-immunoprecipitation. Antibodies against Tom5 and antibodies against Tom40 co-precipitated a fraction of Sam37 and Sam35 molecules (Fig. 4, lanes 4 and 5), whereas control antibodies against Fis1 did not (Fig. 4, lane 3). Fis1 was precipitated by antibodies against Fis1 but not by antibodies against Tom5 or Tom40 (Fig. 4, lower panel), confirming the specificity of the co-immunoprecipitation approach.

We asked how the lack of Mdm10 affected the interaction of Tom5/Tom40 with SAM and performed co-immunoprecipitation from *mdm10*Δ mitochondria. Remarkably, the fraction of Sam37/Sam35 that was co-purified with anti-Tom5 and anti-Tom40 was considerably increased when Mdm10 was absent (Fig. 4, lanes 7 and 8). For comparison, the yield of co-purification of Tom20 with anti-Tom5 or anti-Tom40 was not increased with *mdm10*Δ mitochondria (Fig. 4, upper panel). Thus, not only is Mdm10 dispensable for the association of Tom5/Tom40 with the SAM complex but also lack of Mdm10 enhances the formation of the SAM–Tom5/Tom40 complex. We conclude that two large SAM complexes exist: SAM–Tom5/Tom40 and SAM–Mdm10. When the latter complex is

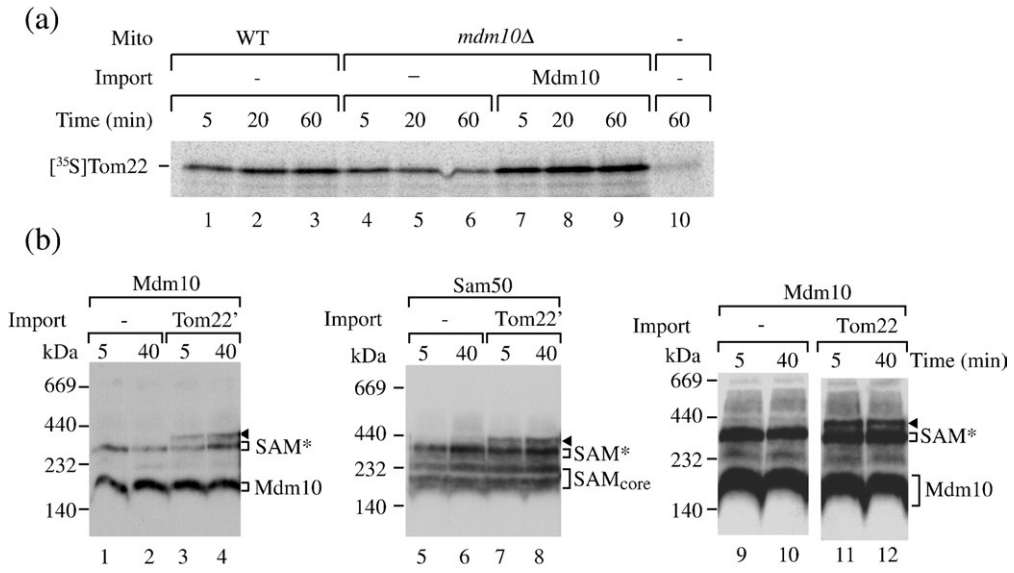

Fig. 5. The precursor of Tom22 interacts with the SAM–Mdm10 complex. (a) Wild-type or *mdm10*Δ mitochondria (Mito) were incubated with chemical amounts of Mdm10 as indicated. Subsequently, [^{35}S]Tom22 was imported for the indicated periods. Mitochondria were subjected to carbonate extraction, and the membrane pellet was analyzed by SDS-PAGE and autoradiography. For sample 10, no mitochondria were added. (b) Chemical amounts of Tom22 precursor were imported into wild-type mitochondria. Protein complexes were separated by blue native electrophoresis and detected by immunoblotting using antibodies directed against Mdm10 or Sam50. Arrowheads indicate a large form of SAM* with associated Tom22 precursor. Tom22′ indicates C-terminally truncated Tom22 (lacking the intermembrane space domain).

lacking due to deletion of Mdm10, more SAM–Tom5/Tom40 complexes are formed.

The precursor of Tom22 interacts with the SAM–Mdm10 complex

Mdm10 is present in two complexes, the SAM–Mdm10 complex and the MDM complex.[38–40,57] The MDM complex includes the morphology proteins Mdm12 and Mmm1 and has been shown to connect mitochondria to the endoplasmic reticulum, thus facilitating lipid transport between both organelles.[58,59] However, an interaction with substrate has been demonstrated neither for the MDM complex nor for the SAM–Mdm10 complex. We thus asked if substrate proteins interact with the SAM–Mdm10 complex in order to obtain evidence for a direct role of this complex in mitochondrial protein biogenesis. Two observations suggested that Tom22 may be a substrate protein that is imported via the SAM–Mdm10 complex: (i) the steady-state levels of Tom22 were reduced in mitochondria lacking Mdm10[38] and (ii) the biogenesis of Tom22 was not impaired in *mim1*Δ mitochondria in contrast to proteins connected to the SAM–Tom5/Tom40 complex, including small Tom proteins and Tom40 that are imported in an Mim1-dependent manner.[41] We first analyzed if Mdm10 was involved in membrane integration of the radiolabeled precursor of Tom22 by carbonate extraction. The yield of membrane inserted Tom22 was decreased in *mdm10*Δ mitochondria compared with wild-type mitochondria (Fig 5a, lanes 1–6). For comparison, membrane integration of the precursor of Tom6 was not affected in *mdm10*Δ mitochondria (Fig. 2b). In order to exclude the possibility that the lack of Mdm10 may only indirectly affect the membrane integration of Tom22 (putative pleiotropic effects in the deletion strain), we used chemical amounts of Mdm10 and imported them into isolated *mdm10*Δ mitochondria. Membrane integration of radiolabeled Tom22 was increased (Fig. 5a, lanes 7–9). With the observation that mutants of Sam35, Sam37 or Sam50 also impaired the membrane integration of Tom22,[44] we conclude that all subunits of the SAM–Mdm10 complex are involved in the insertion of Tom22 into the outer membrane, raising the possibility that the SAM–Mdm10 complex plays a direct role in the biogenesis of Tom22.

It will be crucial to demonstrate a physical interaction of SAM–Mdm10 with substrate to show a direct role of the complex. We thus imported chemical amounts of the Tom22 precursor and asked if the mobility of the SAM–Mdm10 complex was altered on blue native gels. We first used a truncated version of Tom22 lacking the intermembrane space domain.[60] This truncated Tom22′ was shown to be still imported and assembled into the TOM complex, but with slower kinetics.[14,61,62] Upon import of Tom22′, an additional band migrating above the large SAM complex was observed by blue native electrophoresis, with both antibodies against Mdm10 and antibodies against Sam50 (Fig. 5b, lanes 3, 4, 7 and 8). Thus, the imported Tom22′ shifted the mobility of a portion of SAM–Mdm10 complexes. The import of full-length Tom22 similarly shifted the SAM–Mdm10 complex decorated with anti-Mdm10 antibodies (Fig. 5b, lanes 11 and 12). We can exclude that endogenous (mature) Tom22 is a component of the SAM–Mdm10 complex since the protein was not co-purified with His-tagged Sam35 or Mdm10, in contrast to the co-purification of Tom5/Tom40 with $Sam35_{His}$ (Fig. 3b). These results demonstrate that the precursor of Tom22 interacts with the SAM–Mdm10 complex and is thus the first described substrate of this complex.

Conclusions

We report that the SAM complex of the mitochondrial outer membrane is not a static machinery but exists in three forms: SAM_{core}, SAM–Mdm10 and the newly identified SAM–Tom5/Tom40. The core subunits Sam50, Sam37 and Sam35 are present in all three SAM forms. Further modules, Mdm10 or Tom5/Tom40, associate with the core complex to form the different large SAM complexes. Whereas the SAM_{core} complex has been shown to interact with β-barrel precursors,[26,30,32,34–39,44] we show here that the large SAM complexes bind different types of α-helical Tom precursor proteins. The SAM–Mdm10 complex binds the precursor of Tom22 and mediates its membrane insertion, representing the first demonstration of substrate interaction with an Mdm10-containing complex. The second large SAM complex contains a module of the TOM complex, Tom5/Tom40. While the majority of Tom5/Tom40 is of course present in the mature TOM complex, we found that a fraction of this TOM module associates with the SAM complex to form SAM–Tom5/Tom40. This large SAM complex binds the α-helical precursor of Tom6 and thus promotes its association with Tom5/Tom40 at an early stage of TOM assembly.

Mim1 functions before the association of Tom6 with the SAM–Tom5/Tom40 complex. We observed that Mim1 promotes the membrane integration not only of the precursor of Tom6 but also of the precursors of Tom5 and Tom7. Moreover, Sam37 was shown to be required for proper assembly of all three small Tom proteins into the TOM complex.[44] It is thus conceivable that Tom5 and Tom7 may follow a similar import pathway as Tom6.

We propose that the SAM complex represents a modular assembly platform for the TOM complex. By the association of different partner proteins, Mdm10 or Tom5/Tom40, with the SAM_{core} complex, the central SAM function in sorting of β-barrel proteins is extended to α-helical proteins. Although SAM–Mdm10 and SAM–Tom5/Tom40 bind α-helical precursor proteins with non-overlapping specificity, the formation of the two large SAM complexes is interdependent. In mitochondria lacking Mdm10, the association of the Tom5/Tom40 module with SAM is enhanced. Since the large SAM complex contains either Mdm10 or Tom5/Tom40, it

is likely that these modules bind to SAM_{core} in an alternating manner and determine the specificity of interaction with α-helical precursors. In conclusion, at least three sorting pathways for Tom precursors converge at the SAM complex. The SAM system provides a flexible platform to assemble different α-helical precursors with a β-barrel-type protein.

Materials and Methods

Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains *mdm10*Δ, $MDM10_{His}$, *mim1*Δ, $_{ProtA}$MIM1, *sam35-2*, $SAM35_{His}$, *sam37*Δ, $_{ProtA}$SAM37, *sam50-1*, $_{ProtA}$SAM50, *tom5*Δ, *tom7*Δ and *tom40-4* and their corresponding wild-type strains were used.[21,23,26,31,32,36,38,41,56] Yeast strains were grown on YPD, YPG or YPS (1% [w/v] yeast extract, 2% [w/v] bactopeptone, 2% [w/v] glucose or sucrose or 3% [w/v] glycerol) at 19–24 °C. Mitochondria were isolated and stored according to published procedures.[52]

In vitro import, blue native electrophoresis and antibody-shift assays

^{35}S-labeled precursor proteins were synthesized using rabbit reticulocyte lysate (GE Healthcare or Novagen) and [^{35}S]methionine.[52] Precursor proteins were imported into isolated mitochondria at 25 °C as described previously.[52] Mitochondria were transferred to ice after the import reaction. After solubilization, protein intermediates were analyzed by blue native electrophoresis. Antibody-shift assays with antisera were performed as described previously.[26,54] Anti-His antibodies (Qiagen) were used to shift tagged protein complexes. Digitonin-lysed mitochondria were incubated for 1 h on ice with anti-His antibodies. After a clarifying spin, size shifts of protein complexes were detected by blue native electrophoresis.

Carbonate extraction

Carbonate extraction is used to separate integral membrane proteins from peripheral membrane proteins and soluble proteins.[52,53,63,64] ^{35}S-labeled precursor proteins were incubated in the import mix (lacking mitochondria) for 10 min at 25 °C. Precipitated proteins were removed by centrifugation for 10 min at 16,100*g* and 20 °C. The import reaction was started by the addition of mitochondria to the soluble import mix. In the cases of [^{35}S]Tom6, [^{35}S]Tom5 and [^{35}S]Tom7, the import mix was incubated for 5 min at 4 °C after the clarifying spin, followed by addition of mitochondria and import incubation at 4 °C. Mitochondria were reisolated, washed and resuspended in 0.1 M Na_2CO_3, pH 11.5. After 30 min of incubation on ice, the mitochondrial membrane pellet was collected by centrifugation (100,000*g*, 30 min, 4 °C). Mitochondrial membrane proteins were analyzed by SDS-PAGE.

Isolation of protein complexes and co-immunoprecipitation

Mitochondria with His-tagged Sam35 or Mdm10 were solubilized in 0.5% (w/v) digitonin, 20 mM Tris–HCl, pH 7.4, 0.1 mM ethylenediaminetetraacetic acid, 10 mM imidazole, 50 mM NaCl, 10% (w/v) glycerol and 1 mM PMSF at a protein concentration of 1 mg/ml and incubated with Ni-NTA (nitrilotriacetic acid) agarose for 1 h at 4 °C. Subsequently, the Ni-NTA agarose was washed with an excess of digitonin buffer containing 0.1% (w/v) digitonin and increasing concentrations of imidazole (up to 40 mM). Bound proteins were eluted with 250 mM imidazole. After separation by SDS-PAGE, bound proteins were detected via Western blotting.

For co-immunoprecipitation assays, mitochondria were solubilized in digitonin buffer containing 0.5% (w/v) digitonin at a final protein concentration of 1 mg/ml. After a clarifying spin, the supernatant was incubated for 2 h with protein A-Sepharose containing covalently coupled antibodies. Column material was washed with a large volume excess of digitonin buffer containing 0.2% (w/v) digitonin. Bound proteins were eluted with 0.1 M glycine, pH 2.5, and separated by Tricine (*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine) SDS-PAGE.

Miscellaneous

Standard techniques were used for Western transfer on polyvinylidene difluoride membranes and immunodecoration. ECL (GE Healthcare) was used for detection.

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