The morphology proteins Mdm12/Mmm1 function in the major β -barrel assembly pathway of mitochondria

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The β -barrel proteins of mitochondria are synthesized on cytosolic ribosomes. The proteins are imported by the translocase of the outer membrane (TOM) and the sorting and assembly machinery (SAM). It has been assumed that the SAM_{core} complex with the subunits Sam35, Sam37 and Sam50 represents the last import stage common to all β -barrel proteins, followed by splitting in a Tom40-specific route and a route for other β -barrel proteins. We have identified new components of the β -barrel assembly machinery and show that the major β -barrel pathway extends beyond SAM_{core}. Mdm12/Mmm1 function after SAM_{core} yet before splitting of the major pathway. Mdm12/Mmm1 have been known for their role in maintenance of mitochondrial morphology but we reveal assembly of β -barrel proteins as their primary function. Moreover, Mdm10, which functions in the Tom40-specific route, can associate with SAM_{core} as well as Mdm12/Mmm1 to form distinct assembly complexes, indicating a dynamic exchange between the machineries governing mitochondrial β -barrel assembly. We conclude that assembly of mitochondrial β -barrel proteins represents a major function of the morphology proteins Mdm12/Mmm1.

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Introduction

All nuclear-encoded mitochondrial precursor proteins have to be transported into or across the mitochondrial outer mem-

brane. This membrane contains the central entry gate of mitochondria, the translocase of the outer membrane (TOM) complex (Neupert, 1997; Endo *et al*, 2003; Young *et al*, 2003; Koehler, 2004; Pfanner *et al*, 2004; Wiedemann *et al*, 2004a; Rapaport, 2005; Dolezal *et al*, 2006). The TOM complex consists of receptor proteins that recognize the precursor proteins and a core complex that includes the central pore-forming subunit, Tom40. The TOM complex is able to translocate hundreds of different precursor proteins across the outer membrane to internal mitochondrial compartments; however, the TOM complex is not sufficient to insert a major class of proteins into the outer membrane. Several integral outer membrane proteins possess a β -barrel structure, including Tom40 and the most abundant outer membrane protein, porin. Wiedemann *et al* (2003) identified a specific machinery in the outer membrane that is responsible for sorting and assembly of β -barrel proteins (SAM complex). The precursors of β -barrel proteins are first translocated via the TOM complex to the *trans* side of the outer membrane (Model *et al*, 2001) and are transferred to the SAM complex with the help of chaperone complexes in the intermembrane space, formed by small Tim proteins (Hoppins and Nargang, 2004; Wiedemann *et al*, 2004b, 2006a).

The SAM complex contains a stable core formed by Sam35 (Tob38/Tom38) (Ishikawa *et al*, 2004; Milenkovic *et al*, 2004; Waizenegger *et al*, 2004), Sam37 (Mas37) (Wiedemann *et al*, 2003) and Sam50 (Tob55/Omp85) (Kozjak *et al*, 2003; Paschen *et al*, 2003; Gentle *et al*, 2004). Sam50 is an integral β -barrel protein of the outer membrane, conserved from bacteria (Omp85) to man (Voulhoux *et al*, 2003; Ryan, 2004; Dolezal *et al*, 2006). Sam35 and Sam37 are peripheral membrane proteins exposed to the mitochondrial surface. The three subunits of the SAM_{core} complex are required for the biogenesis of all outer membrane β -barrel proteins analyzed.

The mitochondrial distribution and morphology protein Mdm10 was found to associate with the SAM_{core} complex, forming a SAM_{holo} complex, which plays a specific role in the late steps of assembly of the TOM complex, whereas the assembly pathway of other outer membrane β -barrel proteins does not require Mdm10 (Meisinger *et al*, 2004). Mdm10 was identified by Sogo and Yaffe (1994) for its role in maintaining mitochondrial morphology. Mdm10 is predicted to form a β -barrel in the outer membrane (Paschen *et al*, 2003; Wiedemann *et al*, 2003). The presence of Mdm10 in the SAM complex led to a controversial situation since Boldogh *et al* (2003) had reported that Mdm10 was associated with two other outer membrane proteins, Mdm12 (Berger *et al*, 1997) and Mmm1 (Burgess *et al*, 1994). Mdm12 and Mmm1 were shown to be required for mitochondrial morphology and distribution like Mdm10. Cells lacking Mmm1, Mdm10 or Mdm12 display a similar morphological defect with condensed giant mitochondria (Burgess *et al*, 1994; Sogo and Yaffe, 1994; Berger *et al*, 1997; Prokisch *et al*, 2000; Hobbs

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et al, 2001; Kondo-Okamoto *et al*, 2003), supporting the view of a close connection of the three proteins (Boldogh *et al*, 1998, 2003, 2005). The discussion whether Mdm10 is a subunit of the SAM complex or the Mdm12/Mmm1 complex is controversial (Boldogh *et al*, 2005; Jensen, 2005; Stojanovski *et al*, 2006).

Here, we used affinity purification under mild conditions to determine interaction partners of Mdm10. Surprisingly, we found that Mdm10 shows a dual localization in both the SAM complex and the Mdm12/Mmm1 complex, thereby clarifying the controversial debate on its localization. We then tested the hypothesis that also Mdm12/Mmm1 may perform a function in protein assembly. Indeed, mitochondria defective in Mdm12 or Mmm1 were strongly impaired in outer membrane protein assembly. Mdm12/Mmm1 are required after the SAM_{core} complex but before separation of the Tom40-specific route and thus before Mdm10. Mdm12/Mmm1 are the first mitochondrial morphology proteins that function in the major β -barrel assembly pathway of mitochondria.

Results

Dual localization of Mdm10

To analyze if Mdm10 was present in the SAM complex or the Mdm12/Mmm1 complex, we used yeast cells that expressed chromosomally integrated tagged forms of distinct components of these complexes. Mitochondria were isolated from the cells, lysed with the mild detergent digitonin and subjected to affinity chromatography. Tagged Mdm10 (Meisinger *et al*, 2004) pulled out the three subunits of the SAM_{core} complex (Sam50, Sam37, Sam35), as well as Mmm1 and Mdm12 (Figure 1A, lane 2). Control proteins, such as Tom proteins, the mitochondrial intermembrane space assembly protein Mia40 (Chacinska *et al*, 2004) and the subunit Tim23 of the translocase of the inner membrane, were not copurified with Mdm10 (Figure 1A, lane 2).

A different picture, however, was observed when mitochondria containing tagged Mmm1 were used. Only Mdm12

and Mdm10 were copurified whereas none of the three subunits of the SAM_{core} complex was found in the eluates (Figure 1B, lane 2). Thus, although Mmm1 was copurified with tagged Mdm10, as well as Mdm10 was copurified with tagged Mmm1, the eluates significantly differed with respect to other proteins. Mdm12 was the only additional protein found in both eluates. To further analyze this unusual pull-down behavior, we used mitochondria containing tagged Mdm12. Mmm1 and Mdm10 were copurified but none of the subunits of the SAM_{core} complex (Figure 1C, lane 2).

These results indicated that Mdm12 and Mmm1 are associated with Mdm10 but not with the SAM_{core} complex, whereas Mdm10 is associated with Mdm12/Mmm1 as well as the three subunits of the SAM_{core} complex. A direct explanation for these findings would be the presence of Mdm10 in different outer membrane complexes, the SAM complex and the Mdm12/Mmm1 complex. To obtain further evidence, we used mitochondria carrying tagged Sam35. Indeed, Sam50, Sam37 and Mdm10 were copurified but neither Mdm12 nor Mmm1 (Figure 1D, lane 2).

We conclude that the mitochondrial outer membrane possesses distinct complexes containing Mdm10: the SAM complex with Sam50, Sam37 and Sam35; and the Mdm12/Mmm1 complex. Thus, the apparently controversial observations on the localization of Mdm10 (Boldogh *et al*, 2003; Meisinger *et al*, 2004) can now be explained by a dual localization of this protein.

Morphology proteins Mdm12/Mmm1 are required for β -barrel assembly

The interaction of Mdm10 with both SAM and Mdm12/Mmm1 raised the hypothesis that also the morphology proteins Mdm12/Mmm1 may play a role in mitochondrial protein assembly. To test this hypothesis, we analyzed yeast mutants lacking either the *MDM12* gene or the *MMM1* gene. Mitochondria were isolated from *mdm12* Δ and *mmm1* Δ yeast and the corresponding wild-type strains. The steady-state levels of proteins were analyzed by immunodecoration

Figure 1 Mdm10 is present in different protein complexes, SAM and Mdm12/Mmm1. Mitochondria from *S. cerevisiae* strains containing tagged Mdm10 (A), Mmm1 (B), Mdm12 (C) and Sam35 (D) were isolated and lysed with 1% digitonin as described in Materials and methods. After affinity chromatography, protein complexes were eluted and separated by SDS-PAGE. Proteins were analyzed by Western blotting with the indicated antibodies. Load, 16%; elution, 100%.

(Figure 2A and B). Mmm1 was largely absent in *mdm12Δ* mitochondria and the levels of Mdm12 were strongly reduced in *mmm1Δ* mitochondria in agreement with previous observations on the close interaction of both proteins (Boldogh *et al*, 2003). In addition, the levels of further outer membrane proteins like Tom40, Mdm10 and SAM_{core} subunits were moderately reduced in both mutant mitochondria whereas the levels of the inner membrane proteins Tim23 and ADP/ATP carrier were same as those of wild-type mitochondria (Figure 2A and B). For most of the outer membrane proteins analyzed here, a β -barrel fold has been reported (Paschen *et al*, 2003; Wiedemann *et al*, 2003; Pfanner *et al*, 2004), suggesting a possible relation of Mdm12/Mmm1 with the steady-state levels of mitochondrial β -barrel proteins. We thus lysed isolated mitochondria with digitonin and analyzed the TOM complex by blue native electrophoresis. Upon immunodecoration for Tom40, we observed the mature TOM complex of 450 kDa in both *mdm12Δ* mitochondria and *mmm1Δ* mitochondria but in reduced amounts compared to wild-type mitochondria (Figure 2C and D). In addition, small amounts of a 100 kDa TOM form were observed in the mutant mitochondria, that is, Tom40 that was not fully assembled into the mature 450 kDa complex. Previous studies had shown that appearance of the small

TOM form under steady-state conditions indicated an impaired assembly of the TOM complex (Dekker *et al*, 1998; Meisinger *et al*, 2004; Waizenegger *et al*, 2004) (it should be noted that the 100 kDa form contains one dimer of Tom40 whereas the 450 kDa complex contains three dimers (Dekker *et al*, 1998; Model *et al*, 2002) and thus in an immunodecoration for Tom40 the molar ratio of 100 kDa form to 450 kDa form is underestimated by a factor of 3). When porin complexes of digitonin-lysed mitochondria were analyzed by blue native electrophoresis and immunodecoration (Krimmer *et al*, 2001), a significant reduction in both *mdm12Δ* and *mmm1Δ* mitochondria was observed (Figure 2E). The possibility that the extraction of porin from mitochondrial membranes by digitonin was less efficient from the mutant mitochondria than from wild-type mitochondria was of concern. We thus compared the digitonin extracts by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting and observed a similar extractability of porin from wild-type and mutant mitochondria (Figure 2F), supporting the view that the defect observed by blue native electrophoresis may be caused by an impaired assembly of porin complexes in mitochondria lacking Mdm12/Mmm1.

To directly probe for a role of Mdm12/Mmm1 in the assembly of mitochondrial β -barrel proteins, we studied

Figure 2 Steady-state protein levels in mitochondria lacking Mdm12 or Mmm1. (A) Mitochondria were isolated from wild-type (WT) and *mdm12Δ* yeast strains. Indicated amounts of mitochondrial protein (μ g) were subjected to SDS-PAGE and Western blot analysis. AAC, ADP/ATP carrier. (B) Western blot analysis of mitochondria from WT and *mmm1Δ* yeast strains after separation by SDS-PAGE. (C) Mitochondria from WT and *mdm12Δ* strains were lysed by digitonin-containing buffer and subjected to blue native electrophoresis. The TOM complex was detected by Western blotting and immunodecoration with affinity-purified Tom40 antibodies. (D) Western blot analysis of mitochondria from WT and *mmm1Δ* strains separated by blue native electrophoresis as described for panel C. (E) Porin complexes of mitochondria from WT, *mmm1Δ* or *mdm12Δ* yeast strains were analyzed by blue native electrophoresis and Western blotting after solubilization in 0.6% digitonin-containing buffer. (F) Analysis of solubilized mitochondria (0.4 or 1% digitonin) from WT, *mmm1Δ* or *mdm12Δ* yeast strains by SDS-PAGE and immunodecoration with porin antiserum.

TOM assembly in a kinetically linear range by importing the radiolabeled precursor of Tom40 into isolated mitochondria. Upon lysis with digitonin, the assembly steps were monitored by blue native electrophoresis. In wild-type mitochondria, assembly of Tom40 occurs via two intermediates of about 250 kDa (assembly I) and 100 kDa (assembly II) before the mature 450 kDa complex is formed (Figure 3A, lanes 4–6) (Model *et al*, 2001; Wiedemann *et al*, 2003; Meisinger *et al*,

2004). In *mdm12Δ* mitochondria as well as *mmm1Δ* mitochondria, formation of the 450 kDa TOM complex was strongly impaired (Figure 3A, lanes 2 and 3; and B, lanes 7 and 8), demonstrating that Mdm12/Mmm1 are required for assembly of the precursor into the mature TOM complex.

To determine the assembly stage that depended on Mdm12/Mmm1, we made use of the observation that the 250 kDa intermediate represents the interaction of the pre-

Figure 3 Mitochondria lacking Mdm12/Mmm1 are impaired in assembly of β -barrel proteins. (A, B) Assembly defect of Tom40. ^{35}S -labeled precursor of Tom40 was imported into mitochondria isolated from wild-type (WT), *mdm12Δ* and *mmm1Δ* yeast strains as described in Materials and methods. The incubation time for the import reaction is indicated (min). Assembly into complexes was analyzed by digitonin lysis, blue native electrophoresis and digital autoradiography. (C, D) Import of radiolabeled Tom40 precursor into mitochondria from WT, *mmm1Δ* or *mdm12Δ* yeast strains. Mutant mitochondria were adjusted to the same amount of TOM complexes and import was performed at 20°C. After treatment with proteinase K (10 $\mu\text{g}/\text{ml}$), the mitochondria were reisolated and analyzed by SDS-PAGE and digital autoradiography. (E, F) Assembly defect of porin. ^{35}S -labeled precursor of porin was imported into isolated yeast mitochondria. Assembly into complexes was analyzed as described for panels (A, B). (G) Assembly of radiolabeled Sam50 precursor in WT and *mdm12Δ* mitochondria was performed as described for panel A. (H) Assembly of radiolabeled Mdm10 in WT and *mdm12Δ* mitochondria was performed as described for panel (A).

cursor protein with the SAM_{core} complex (Mihara, 2003; Paschen *et al*, 2003; Wiedemann *et al*, 2003; Gentle *et al*, 2004; Ishikawa *et al*, 2004; Pfanner *et al*, 2004). Mitochondria deficient in any subunit of the SAM_{core} complex (Sam35, Sam37 or Sam50) are inhibited from forming of the 250 kDa intermediate (Kozjak *et al*, 2003; Paschen *et al*, 2003; Wiedemann *et al*, 2003; Gentle *et al*, 2004; Ishikawa *et al*, 2004; Milenkovic *et al*, 2004; Waizenegger *et al*, 2004). In contrast, mitochondria lacking Mdm10 efficiently form the 250 kDa intermediate but are impaired in formation of the mature TOM complex in agreement with the role of Mdm10 in late steps of TOM assembly that take place after the SAM_{core} complex (Meisinger *et al*, 2004, 2006b). Both *mdm12Δ* mitochondria and *mmm1Δ* mitochondria formed the 250 kDa assembly intermediate of Tom40 after a 5 min import, indicating that the SAM_{core} complex was functional in these mitochondria (Figure 3A, lane 1; and B, lane 5). Moreover, translocation of the Tom40 precursor to a protease-protected location occurred with similar efficiency in wild-type, *mdm12Δ* and *mmm1Δ* mitochondria (Figure 3C and D), indicating that the mutant mitochondria were competent in the early import step of precursor translocation through the TOM complex. (At longer import times, the amount of Tom40 precursor accumulated at the 250 kDa intermediate decreased in *mdm12Δ* and *mmm1Δ* mitochondria (Figure 3A and B); as the subsequent assembly steps of Tom40 are impaired in the mutant mitochondria, the turnover of the precursor at the 250 kDa stage is likely increased.) We conclude that Mdm12/Mmm1 are required for Tom40 assembly after the SAM_{core} complex.

We thus asked if Mdm12/Mmm1 performed a similar function like Mdm10 in outer membrane protein assembly. Mdm10 is selectively required for the biogenesis of Tom40 but not of other β -barrel proteins such as the most abundant outer membrane protein porin (Meisinger *et al*, 2004, 2006b). We analyzed the assembly of the radiolabeled precursor of porin into mature complexes by blue native electrophoresis (Krimmer *et al*, 2001; Wiedemann *et al*, 2003; Meisinger *et al*, 2004). Surprisingly, the assembly of porin was strongly inhibited in both *mdm12Δ* mitochondria and *mmm1Δ* mitochondria (Figure 3E and F). Thus, mitochondria lacking Mdm12 or Mmm1 show a β -barrel assembly defect that is different from mitochondria lacking Mdm10. Additionally, we studied the assembly of two further β -barrel proteins, Sam50 and Mdm10, and observed a significant inhibition in *mdm12Δ* mitochondria (Figure 3G and H). We conclude that Mdm12/Mmm1 are required for the function of the general β -barrel pathway that is used by all β -barrel proteins analyzed and thus Mdm12/Mmm1 act before the separation of the Tom40-specific route from the general β -barrel pathway.

Specificity of β -barrel assembly defect in mitochondria lacking Mdm12/Mmm1

We performed several control experiments to demonstrate that the defect of mitochondria lacking Mdm12 or Mmm1 is specific for the β -barrel pathway. First, we studied the import of preproteins destined for internal mitochondrial compartments in order to exclude a general defect of *mdm12Δ* or *mmm1Δ* mitochondria in protein import. We used the β -subunit of the F₁-ATPase (F₁ β) and a matrix-targeted model protein consisting of the presequence of F₀-ATPase subunit 9

and dihydrofolate reductase (Su9-DHFR) (Pfanner *et al*, 1987). Import of the proteins was determined by processing to the mature-sized form and the membrane potential ($\Delta\psi$)-dependent translocation to a protease-protected location (Wiedemann *et al*, 2006b). *mdm12Δ* and *mmm1Δ* mitochondria imported the preproteins like wild-type mitochondria (Figure 4A and B), indicating that the mutant mitochondria were competent in protein translocation across the outer membrane to internal compartments.

Second, the assembly of β -barrel proteins of the outer membrane has been shown to depend on an intact intermembrane space. Opening of the outer membrane, which leads to a release of intermembrane space proteins like the small Tim proteins, strongly inhibits assembly of Tom40 and porin (Smith *et al*, 1994; Wiedemann *et al*, 2004b). We thus tested the integrity of the outer membrane by treating isolated mitochondria with increasing concentrations of proteinase K. The outer membrane receptor Tom22 was degraded whereas Tom40 as well as Tim23, which is exposed to the intermembrane space, was protected against the protease in both *mdm12Δ* and *mmm1Δ* mitochondria (Figure 4C, lanes 2–4; Figure 4D, lanes 6–8). For comparison, we subjected mitochondria to swelling in order to open the outer membrane (Wiedemann *et al*, 2004b). Now both Tom40 and Tim23 were accessible to proteinase K (Figure 4C, lanes 10–12). We conclude that the outer membrane barrier of *mdm12Δ* and *mmm1Δ* mitochondria is intact.

Mitochondrial fusion and fission components are not required for β -barrel assembly

As mutants lacking Mdm10, Mdm12 or Mmm1 are defective in both mitochondrial morphology and assembly of outer membrane proteins, we asked whether alteration of mitochondrial morphology leads to defects in β -barrel assembly. We therefore analyzed deletion mutants of mitochondrial outer membrane proteins, which have been reported to be required for mitochondrial fission (Otsuga *et al*, 1998; Bleazard *et al*, 1999; Fekkes *et al*, 2000; Mozdy *et al*, 2000; Tieu and Nunnari, 2000; Cervený *et al*, 2001; Jofuku *et al*, 2005) or fusion (Hermann *et al*, 1998; Rapaport *et al*, 1998; Sesaki and Jensen, 2001; Ishihara *et al*, 2004; Meeusen *et al*, 2004). We used isolated mitochondria and the radiolabeled precursors of Tom40 and porin. As some of the fusion mutants have a tendency to lose mitochondrial DNA, wild-type mitochondria were isolated from both *rho*⁺ and *rho*[−] strains and the assembly reactions in mutant mitochondria were compared to both types of wild-type mitochondria. None of the mutants of the outer membrane components known to be required for mitochondrial fission (Fis1, Mdv1, Dnm1) or fusion (Fzo1, Ugo1) showed a significant defect in assembly of β -barrel proteins (Figure 4E and F). We conclude that alterations of mitochondrial fusion or fission do not lead to defects in protein assembly of the mitochondrial outer membrane.

Rapid induction of protein assembly defects in *mmm1-1* mutant yeast

As mutants of the SAM_{core} complex as well as mutants of *MDM12/MMM1* show defects in β -barrel assembly and in mitochondrial morphology, the primary function of these components is of major interest. In the case of components of the SAM_{core} complex, a primary function in protein

Figure 4 Mitochondrial fusion and fission components are not required for assembly of β -barrel proteins. (A) ^{35}S -labeled precursors of Su9-DHFR and F $_1\beta$ were imported into isolated mitochondria from wild-type (WT) and *mdm12Δ* yeast in the presence or absence of a membrane potential ($\Delta\psi$). The incubation time for the import reaction is indicated (min). After treatment with proteinase K, the samples were analyzed by SDS-PAGE and digital autoradiography. p, precursor; m, mature. (B) Import of ^{35}S -labeled precursor of Su9-DHFR into WT and *mmm1Δ* mitochondria. Proteinase K-treated samples were separated by SDS-PAGE. (C) *mdm12Δ* mitochondria possess an intact outer membrane. Mitochondria, isolated from WT and *mdm12Δ* strains, were subjected to swelling where indicated, treated with proteinase K and subjected to SDS-PAGE and Western blotting. (D) *mmm1Δ* mitochondria possess an intact outer membrane. Isolated mitochondria were treated with proteinase K and analyzed by SDS-PAGE and Western blotting. (E) ^{35}S -labeled precursors of Tom40 and porin were imported into mitochondria isolated from WT yeast and the indicated mitochondrial fission and fusion mutant strains (derived from the Euroscarf collection). Mitochondria were lysed by digitonin, protein complexes were separated by blue native electrophoresis and analyzed by digital autoradiography. (F) Quantification of assembled TOM complex in *fis1Δ* and *fzo1Δ* mitochondria using ImageQuant software (GE Healthcare). Import of the radiolabeled precursor of Tom40 was performed as described for panel (E). For each time point (20 and 60 min for *fis1Δ*, 30 and 60 min for *fzo1Δ*), the ratio of mutant to wild-type mitochondria is shown. Bars indicate s.e.m. from at least three independent experiments.

assembly is evident (Stojanovski *et al*, 2006). Mutants of SAM_{core} subunits grown at low temperature show kinetic defects in assembly of β -barrel proteins whereas mitochondrial morphology is not affected. Only upon shift of the cells to elevated temperature for longer time spans, defects in mitochondrial morphology are observed; under these conditions, the steady-state levels of outer membrane protein complexes like the TOM complex are significantly reduced (Wiedemann *et al*, 2003; Meisinger *et al*, 2004, 2006b). These findings show that the SAM_{core} complex plays a primary role in the assembly of outer membrane proteins such as the TOM complex, which is required for import of various morphology factors (Stojanovski *et al*, 2006).

In the case of Mdm12/Mmm1, the selective defect of deletion mutants in the β -barrel pathway at a post-SAM_{core} stage (Figure 3) and the normal β -barrel assembly in mutants of the various fusion and fission components (Figure 4E) similarly suggest a primary function of Mdm12/Mmm1 in mitochondrial protein assembly. To obtain further evidence, we used a temperature-conditional mutant of *MMM1* (Burgess *et al*, 1994) and monitored the time dependence of induction of phenotypes upon shift to nonpermissive

temperature. We observed that 33°C was a lethal temperature for the *mmm1-1* cells, whereas the growth was impaired at 30°C and was comparable to wild-type cells at 23°C (Figure 5A). Mitochondrial morphology was analyzed by confocal laser scanning microscopy. Upon growth of *mmm1-1* cells at 24°C, as well as after a shift to 33°C for 30 min, the cells preferentially showed the typical tubular mitochondrial morphology like wild-type cells (Figure 5B). After a 90 min shift to 33°C, *mmm1-1* cells contained condensed giant mitochondria whereas wild-type mitochondria still showed the tubular morphology (Figure 5B). A quantification revealed that after the 30 min shift, 5% of the *mmm1-1* cells displayed an altered mitochondrial morphology, whereas after the 90 min shift, 99% of *mmm1-1* cells contained altered mitochondria (Figure 5C). We conclude that after a 90 min shift to 33°C, the morphological phenotype of *mmm1-1* cells is expressed. We isolated mitochondria from the cell cultures used for the morphological analysis. The steady-state levels of the mutant protein Mmm1-1 as well as of Mdm12 decreased upon a 30 min shift to 33°C whereas the steady-state levels of other proteins analyzed, including outer membrane proteins and control proteins of internal mito-

Figure 5 Early induction of protein assembly defect in *mmm1-1* mutant cells. (A) Growth phenotype of the conditional *mmm1-1* yeast strain. WT and *mmm1-1* cells were spotted via serial dilution onto YPG agar plates and incubated at 23, 30 and 33°C. (B) Mitochondrial morphology defect of *mmm1-1* cells. Precultures (YPG, 24°C) of WT and *mmm1-1* cells were diluted with fresh medium of appropriate temperature to maintain 24°C or reach 33°C by mixing. The cultures were further incubated at 24 or 33°C. After 30 and 90 min, cells were immediately stained with DiOC₆ and analyzed by laser scanning confocal microscopy. DIC, differential interference contrast; scale bar, 2 μm. (C) Statistics of the mitochondrial morphology of WT and *mmm1-1* cells grown at 24°C or after shift to 33°C for 30 and 90 min. Number of cells analyzed for each condition >500. (D) Analysis of steady-state protein levels of WT and *mmm1-1* mitochondria isolated from yeast strains that were grown at 24°C and shifted to 33°C for 30 and 90 min where indicated as described for panel (B). (E) The endogenous TOM complex of mitochondria isolated as described for panel (D) was analyzed by blue native electrophoresis and immunodecoration with Tom40-specific antibodies. (F) ³⁵S-labeled precursor of Tom40 was imported into WT and *mmm1-1* mitochondria isolated as described for panel (D) for the indicated periods (min). TOM complex assembly was analyzed by blue native electrophoresis and digital autoradiography. (G) Quantification of assembled TOM complex in *mmm1-1* mitochondria using ImageQuant software (GE Healthcare). The experiments were performed as described for panel (F). Bars indicate s.e.m. from four independent experiments. (H) Import of the radiolabeled precursors of Sam50 and porin was performed and analyzed as described for panel (F).

chondrial compartments, were not affected (Figure 5D). After a shift of 90 min to 33°C, the steady-state level of Mdm10 was moderately reduced (5D). Upon analysis by blue native electrophoresis, it became evident that assembly of the mature 450 kDa TOM complex was significantly impaired after the 90 min shift to 33°C (Figure 5E).

We then studied the kinetics of TOM assembly in the mutant mitochondria by using the radiolabeled precursor of Tom40 and observed an efficient assembly process in mitochondria isolated from non-shifted *mmm1-1* cells (Figure 5F, lanes 4–6; and G). After the 30 min shift, a significant defect in formation of the mature TOM complex was observed compared to wild-type mitochondria (Figure 5F, lane 12 versus lane 9; and G). Similarly, after the 90 min shift, the formation of mature TOM complex was strongly inhibited

(Figure 5F, lane 18 versus lane 15; and G). In both cases, the formation of assembly intermediate I, which represents the SAM_{core} complex, was not impaired, thus confirming the results obtained with *mmm1Δ* mitochondria (Figure 3B). The assembly of further β-barrel proteins, like Sam50 and porin, was also impaired in the *mmm1-1* mutant after the 30 min shift to nonpermissive conditions (Figure 5H).

As the β-barrel assembly defect is observed very early (after a 30 min shift), it is obviously not a consequence of morphological alterations of the mutant mitochondria. The morphology defect is observed under conditions (90 min shift) where the steady-state levels of mature assembled TOM complex are significantly reduced. These findings support the view of a primary role of Mmm1 in β-barrel assembly.

Overexpression of Mmm1, Mdm10 or Mdm12 partially suppresses the growth defect of *sam37Δ* cells

To obtain additional *in vivo* evidence for a relation of Mmm1, Mdm12 and Mdm10 with the SAM_{core} complex, we used *sam37Δ* cells that cannot grow at 37°C on fermentable and non-fermentable media (Gratzer *et al*, 1995; Wiedemann *et al*, 2003). We asked if overexpression of these three so-called morphology components was able to suppress the growth defect of *sam37Δ* cells. As control, we overexpressed Sam37 as well as Sam35 and Sam50 in the deletion mutant. Whereas Sam37 rescued growth on both media as expected, the two other subunits of the SAM_{core} complex, Sam35 and Sam50, were only able to suppress the growth defect on fermentable medium (Figure 6). Transformation of the mutant cells with the control plasmid (no insert) did not lead to suppression. Each of the three morphology components partially suppressed the growth defect of *sam37Δ* cells on fermentable medium (Figure 6). Thus, Mdm10, Mmm1 and Mdm12 can partially substitute for Sam37, supporting the view of a functional relation of the Mdm/Mmm proteins to the SAM_{core} complex.

Discussion

We have identified two new components of the mitochondrial pathway for assembly of β -barrel proteins. Mdm12 and Mmm1 were originally identified in genetic screens for their involvement in maintenance of mitochondrial morphology and distribution (Burgess *et al*, 1994; Berger *et al*, 1997). Mdm12 and Mmm1 are closely connected, as the deletion of either one component leads to a nearly complete loss of mitochondrial localization of the other partner (Boldogh *et al*, 2003; this study). We found that Mdm12/Mmm1 function in the major β -barrel pathway that is responsible for biogenesis of outer membrane proteins like porin, Tom40, Sam50 and Mdm10. Mdm12/Mmm1 shift the branching point of the β -barrel pathway to a stage after the SAM_{core} complex.

To date, it had been assumed that splitting of β -barrel assembly into the Tom40-specific route and the general route for the other β -barrel proteins occurs upon exiting from the SAM_{core} complex. Mdm10 and Mim1 (Tom13) then selectively direct the final steps of assembly of Tom40 with the other subunits of the TOM complex (Ishikawa *et al*, 2004; Meisinger *et al*, 2004, 2006b; Waizenegger *et al*, 2005). We show that mitochondria deficient in Mdm12 or Mmm1 are competent in forming the SAM_{core} intermediate of β -barrel proteins, demonstrating that Mdm12/Mmm1 operate after the SAM_{core} complex. The separation of the Tom40-specific pathway occurs after the action of Mdm12/Mmm1.

Interestingly, Mdm10 of the Tom40-specific pathway can associate with either complex of the major β -barrel pathway, the SAM_{core} complex and the Mdm12/Mmm1 complex, forming the larger SAM_{holo} complex and the Mdm12/Mmm1/Mdm10 complex, respectively. This dual localization of Mdm10 solves the previous controversy on its localization (Boldogh *et al*, 2003; Meisinger *et al*, 2004) and indicates a dynamic interplay between the components of the major β -barrel pathway and those of the Tom40-specific pathway. The association of Mdm10 with a fraction of SAM_{core} and Mdm12/Mmm1 complexes may help to regulate the substrate flow in the β -barrel pathways. It is conceivable that Mdm10 acts as a switch that shuttles between both complexes and channels

Figure 6 Partial suppression of *sam37Δ* mutant cells by overexpression of Mmm1, Mdm10 or Mdm12. The *sam37Δ* yeast strain was transformed with the control plasmid YE352 or with plasmids containing the endogenous promoter, open reading frame and terminator region of *MMM1*, *MDM10*, *MDM12*, *SAM37*, *SAM35* and *SAM50*. Growth was analyzed on agar plates at fermentable (YPD) or non-fermentable (YPEG) conditions at 24 and 37°C.

precursor proteins into the TOM-specific pathway, that is, for association with other TOM proteins. Indeed, Meisinger *et al* (2006b) showed that Tom7 controls the association of Mdm10 with the SAM complex and thus differentially regulates the assembly efficiency of porin and Tom40. We did not find Mim1 in association with SAM components or Mdm/Mmm components (data not shown), supporting the view that Mim1 is located in a distinct outer membrane complex dedicated to Tom40 assembly (Ishikawa *et al*, 2004; Waizenegger *et al*, 2005).

Mutants of *MDM10*, *MDM12* or *MMM1* show a similar alteration of mitochondrial morphology, that is, the formation of condensed giant mitochondria (Burgess *et al*, 1994; Sogo and Yaffe, 1994; Berger *et al*, 1997; Dimmer *et al*, 2002; Meisinger *et al*, 2004, 2006b). Our findings that mutants of these proteins are impaired in mitochondrial β -barrel assembly raise the question whether the primary function of this group of Mdm/Mmm proteins is assembly of proteins or maintenance of mitochondrial morphology. First, the requirement for these Mdm/Mmm proteins at defined stages of β -barrel assembly argues against an unspecific/indirect impairment of protein assembly in the mutant mitochondria. Second, we asked if alteration of mitochondrial morphology causes defects in β -barrel assembly and analyzed *Saccharomyces cerevisiae* mutant strains with defined defects in mitochondrial morphology, that is, the mitochondrial outer membrane components that are directly required for mitochondrial fission and fusion. Mutants of these fission and fusion components (*mdv1Δ*, *fis1Δ*, *dnm1Δ*, *fzo1Δ*, *ugo1Δ*) did not display any significant defect in β -barrel assembly, demonstrating that the defects in mitochondrial morphology do not cause an impairment of outer membrane protein assembly. Third, we used a temperature-conditional mutant of *MMM1* and found a sequential induction of phenotypes with the β -barrel assembly defect occurring significantly before the morphological alteration. These findings indicate that defects in the β -barrel pathway lead to impaired assembly of protein complexes of the mitochondrial outer membrane, including protein complexes that are required to maintain a proper mitochondrial morphology. Indeed, mutants of SAM_{core} components as well as TOM subunits show

similar morphological alterations (giant mitochondria) like mutants of Mdm10, Mdm12 and Mmm1 (Dimmer *et al*, 2002; Altmann and Westermann, 2005; Meisinger *et al*, 2004, 2006b) and overexpression of Mdm10, Mdm12 and Mmm1 can partially suppress the growth defect of mutants impaired in the SAM_{core} complex.

In summary, we have identified a new stage of the major β -barrel assembly pathway of mitochondria that requires the morphology proteins Mdm12/Mmm1. The SAM_{core} complex as well as Mdm12/Mmm1 can associate with Mdm10 that functions in the Tom40-specific route, indicating a dynamic cooperation of the machineries required for mitochondrial β -barrel assembly. Our findings suggest that these Mdm/Mmm proteins play a primary role in assembly of protein complexes of the mitochondrial outer membrane whereas the maintenance of mitochondrial morphology is a secondary consequence of the assembly function.

Materials and methods

Yeast growth and isolation of mitochondria

The *S. cerevisiae* strains *mmm1-1* (Burgess *et al*, 1994), *sam37 Δ* (*tom37 Δ*) (Ryan *et al*, 1999), *Sam35-His₁₀* (Milenkovic *et al*, 2004) and the strain expressing Mdm10-His₁₀ (Meisinger *et al*, 2004) have been described before. The strains Mdm12-His₁₀ (background YPH499, C-terminal deca-histidine tag) and ProtA-Mmm1 (BY4741, N-terminal protein A-tag) were generated by homologous recombination with PCR products from affinity tag replacement cassettes (Meisinger *et al*, 2001; Kozjak *et al*, 2003). Other yeast deletion strains were obtained from Euroscarf (BY4741). To avoid adaptation/suppression, the strains were used directly from the stock. For overexpression, the open reading frames of *MMM1*, *MDM10*, *MDM12*, *SAM37*, *SAM35* and *SAM50* under control of the endogenous promoter and terminator were cloned into the vector YEpl352 (Hill *et al*, 1986). The resulting plasmids were able to complement the growth phenotype of the respective deletion strains. After transformation of the *sam37 Δ* strain and selection on medium without uracil, yeast clones were tested as described in the legend of Figure 6.

Yeast cells were grown in medium (1% (w/v) yeast extract, 2% (w/v) bacto-peptone) with 2% (w/v) glucose (YPD), 2% (w/v) sucrose (YPS), 3% (w/v) glycerol (YPG) or 2% (v/v) ethanol and 2% (w/v) glycerol (YPEG) at 20–33°C. For isolation of mitochondria used in import experiments, *mdm12 Δ* yeast and the corresponding wild-type strain were grown at 20°C, and *mmm1 Δ* yeast and the corresponding wild-type strain were grown at 23°C. Cells of *mmm1-1* yeast and the corresponding wild-type strain were cultured at 24°C in YPG medium to an optical density (OD) of 1 (mid-log-phase). The cultures were split and diluted with fresh medium to an OD of 0.33. The fresh medium had a temperature of either 24 or 38°C in order to instantly set permissive (24°C) and non-permissive (33°C) growth conditions for the *mmm1-1* mutant. Yeast cells were further incubated for 30 and 90 min. Mitochondria were stained by direct application of 150 nM of the lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC₆) (Pringle *et al*, 1989) to the growth medium. Mitochondrial morphology was analyzed immediately with an epifluorescence or confocal microscope. Images of mitochondria from cells with a representative phenotype were taken and processed as described (Meisinger *et al*, 2004). Mitochondria were isolated by differential centrifugation, resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/

KOH, pH 7.2) and aliquots were frozen at –80°C (Meisinger *et al*, 2006a).

In vitro import and rupturing of the outer membrane

pGEM vectors carrying open reading frames of mitochondrial precursor proteins were used for *in vitro* transcription with SP6 RNA polymerase (Stratagene). Radiolabeled precursor proteins were synthesized *in vitro* in rabbit reticulocyte lysate in the presence of [³⁵S]methionine/cysteine (GE Healthcare). For *in vitro* import, isolated mitochondria (25–50 μ g protein) were incubated with radiolabeled precursor proteins in 100 μ l import buffer (3% (w/v) BSA, 80 mM KCl, 5 mM MgCl₂, 2 mM NADH, 5 mM creatine phosphate, 100 μ g/ml creatine kinase, 10 mM MOPS/KOH, pH 7.2, 2–4 mM ATP) at 25°C. Import reactions were stopped on ice by addition of 8 μ M antimycin A, 20 μ M oligomycin and 1 μ M valinomycin (Sigma). Mitochondria were reisolated and washed with SEM buffer.

To selectively rupture the outer membrane and generate mitoplasts by swelling, mitochondria were resuspended in SEM buffer and the suspension was diluted with 9 volumes of EM buffer (1 mM EDTA, 10 mM MOPS/KOH, pH 7.2) and incubated on ice for 15 min. Mitochondria or mitoplasts were treated with proteinase K (5–50 μ g/ml) for 15 min on ice. The protease was inactivated by addition of 2 mM PMSF and incubation for 5 min on ice. Further details of the methods are described in Wiedemann *et al* (2006b).

Isolation of tagged protein complexes, SDS-PAGE and blue native electrophoresis

Protein A- or decahistidine-tagged protein complexes were isolated essentially as described (Meisinger *et al*, 2001, 2004). In brief, mitochondria were solubilized at 4°C in 1% (w/v) digitonin buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (w/v) glycerol), centrifuged at 12000 g at 4°C for 15 min and the supernatant was subjected to Ni-NTA or IgG chromatography. The IgG column was washed with digitonin buffer and the protein A tag was cleaved with TEV protease for elution. The Ni-NTA column was washed with digitonin buffer with 200 mM NaCl and 50–70 mM imidazole whereas decahistidine-tagged proteins were eluted with 200 mM imidazole. Eluates or isolated mitochondria (25–50 μ g protein) were resuspended in SDS sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 60 mM Tris-HCl, pH 6.8, 0.2–1% (v/v) β -mercaptoethanol, 0.01% bromophenol blue), heated to 95°C for 5 min and subjected to SDS-PAGE. For separation of protein complexes, mitochondria were resuspended in digitonin buffer (0.5–1% (w/v) digitonin for porin, 1% (w/v) digitonin for TOM) and incubated for 15 min on ice. A 5 μ l portion of blue native-sample buffer (5% (w/v) Coomassie brilliant blue G-250, 100 mM Bis-Tris/HCl, pH 7.0, 500 mM ϵ -amino-n-caproic acid) was added, samples were centrifuged at 12000 g and 4°C for 15 min and the supernatants were applied to a 6–16.5% blue native electrophoresis gradient gel at 4°C. Radiolabeled proteins were detected by digital autoradiography. Non-relevant gel lanes were excised digitally. Endogenous proteins or protein complexes were analyzed by Western blotting on PVDF membranes, decoration with specific antibodies and detection by ECL (GE Healthcare).

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