Sam35 of the Mitochondrial Protein Sorting and Assembly Machinery Is a Peripheral Outer Membrane Protein Essential for Cell Viability*

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The mitochondrial outer membrane contains two integral proteins essential for cell viability, Tom40 of the translocase of the outer membrane (TOM complex) and Sam50 of the sorting and assembly machinery (SAM complex). Here we report the identification of Sam35, the first peripheral mitochondrial outer membrane protein that is essential for cell viability. Sam35 (encoded by the Saccharomyces cerevisiae ORF YHR083w) is a novel subunit of the SAM complex and is crucial for the assembly pathway of outer membrane β-barrel proteins, such as the precursors of Tom40 and porin. Sam35 is not required for the import of inner membrane or matrix targeted proteins. The presence of two essential proteins in the SAM complex, Sam35 and Sam50, indicates that it plays a central role in mitochondrial biogenesis.

Two major pathways of import of nuclear-encoded proteins into mitochondria have been characterized in detail, the presequence pathway and the carrier pathway (1–6). Both mitochondrial import pathways start with the translocase of the outer mitochondrial membrane (TOM complex) that contains receptors and a general import pore. Of the seven different subunits of the TOM complex, the pore-forming protein Tom40 is the only one that is strictly essential for cell viability (7–9). After passing through the Tom40 pore, presequence-containing proteins are directed to the presequence translocase of the inner membrane (TIM23 complex) with the associated motor (PAM). The hydrophobic carrier proteins are synthesized in the cytosol without a presequence. After passing the TOM complex, the carrier precursors are guided by small Tim proteins through the intermembrane space and are inserted into the inner membrane by the twin-pore translocase (TIM22 complex). A number of subunits of the TIM and PAM machineries are essential for cell viability.

A third mitochondrial protein import pathway has been found by the discovery that the outer membrane protein Mas37 functions in the sorting and assembly of β-barrel proteins (10). The mitochondrial β-barrel proteins are located in the outer membrane and include Tom40 and porin, the most abundant outer membrane protein (4, 11–15). Mas37 is not associated with the TOM complex but is present in a different complex, termed the protein sorting and assembly machinery (SAM complex) (6, 10, 14, 15). Cells lacking Mas37 show growth defects but are still viable except for elevated temperatures (10, 16). Recently, a second subunit of the SAM complex has been found, the integral outer membrane protein Sam50 (Omp85, Tob55) (17–20). Sam50 is essential for yeast cell viability under all growth conditions and related to the bacterial export component Omp85, suggesting that principles of the SAM pathway have been conserved from bacteria to mitochondria.

The precursors of β-barrel proteins are first imported via the TOM complex, like all other nuclear-encoded mitochondrial proteins (10, 13, 21–24). The proteins are translocated to the intermembrane space side of the outer membrane and with the help of small Tim proteins are transferred to the SAM complex (10, 24–27). The SAM complex then promotes the insertion of the proteins into the outer membrane (10, 18, 20, 24).

We have identified a new subunit of the SAM complex. Sam35 is essential for cell viability and shows the characteristics of a peripheral outer membrane protein. Sam35 is needed for formation of an assembly intermediate of Tom40 at the SAM complex and thus plays a specific role in the biogenesis of mitochondrial β-barrel proteins.

EXPERIMENTAL PROCEDURES

Yeast Strains—A triple HA tag (pYM2) (28) or a Hisα tag (pFA-His10-HIS3MX6) (29) were integrated 3‘ to the Sam35 ORF (YHR083w/FMP20) into the Saccharomyces cerevisiae strain YPH409, yielding the strains Sam35α (Mata, ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, sam35::SAM35HA-HIS3) and Sam35αtrp1 (Mata, ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, sam35::SAM35His10-HIS3). For disruption of genomic Sam35 by homologous recombination, a DNA fragment containing sam35::ADE2 was transformed into the YPH409 strain carrying the 2μ URA3-containing plasmid Yepl32 with the Sam35 ORF between the MET75 promoter and the CYC1 terminator, yielding sam35-delta (Mata, ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, sam35::ADE2) (Yepl35Sam35). The temperature-sensitive yeast strain sam35-2 (Mata, ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, sam35::ADE2) (pPLfomp4-Ts5-2-CEN) was generated by low fidelity PCR of the Sam35 ORF, followed by cloning through gap repair and
subsequent plasmid shuffling of the sam35-2 mutant allele plasmid pH8omp4-Ts5-2-CEN against the wild-type Yep-SAM35 plasmid in the sam35-delta strain according to published procedures (30). Yeast strains were grown at 24 or 30 °C in YPG medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, and 2% (w/v) glucose). The materials used for the chromatography. A, WT, Sam35HA, and Sam50HA, mitochondria were lysed in digitonin buffer, subjected to BN-PAGE, and analyzed by Western blot analysis with antibodies against Mas37, HA, and Tom40.

**RESULTS AND DISCUSSION**

**Sam35 Is a Peripheral Outer Membrane Protein**—We scaled up the purification of the SAM complex from isolated *S. cerevisiae* mitochondria via a tagged Mas37, carrying protein A (17) at the N terminus (17). A mass spectrometric analysis revealed the presence of a further protein, termed Sam35, besides the known subunits Mas37 and Sam50 (Fig. 1A). The new protein is encoded by the *S. cerevisiae* ORF YHR083w and is predicted to contain 329 amino acid residues without longer hydrophobic segments (Fig. 1B). Sam35 possesses homologues in *Saccharomyces bayanus*, *Saccharomyces mikatae*, *Schachomyces paradoxus*, and *Saccharomyces castellii* and, via the proteins ADR303Wp of *Ashbya gossypii*, FG10106.1 of *Gibberella zeae*, AN0657.2 of *Aspergillus nidulans*, and SPAC589.04 of *Gibberella zeae*, AN0657.2 of *Aspergillus nidulans*, and SPAC589.04 of *Gibberella zeae*.

**Miscellaneous**—Isolation of the SAM complex via a protein A tag was performed by adaptation and scaling up of a described method (17, 31). Isolation of the SAM complex via a HA tag followed a described procedure (29) with the following modifications: mitochondria were lysed in 1% digitonin; 0.5% digitonin was used in subsequent buffers; and 30–40 mM imidazole was used for washing. Sonication was performed as described previously (17). For treatment with carbonate, samples were incubated for 30 min on ice. For some figures, non-relevant gel lanes were excised digitally. Western transfers onto polyvinylidene difluoride membranes and immunodetection were done according to standard techniques. Enhanced chemiluminescence was used for detection (Amersham Biosciences).

**Fig. 1.** Sam35 is a subunit of the SAM complex of the outer mitochondrial membrane. A, purification of the SAM complex. Mitochondria were isolated from wild-type (WT) yeast cells and from a yeast strain expressing Mas37 with a protein A tag (17). The mitochondria were lysed in 1% digitonin buffer and subjected to IgG chromatography, followed by elution with 500 mM sodium acetate, pH 3.4. After separation by SDS-PAGE, proteins were stained with colloidal Coomassie Brilliant Blue G-250. Sam35 was identified by mass spectrometry (31). Mas37*, fragment of Mas37 proximal to the N terminus (17), and Sam35 are a peripheral membrane protein. Mitochondria from yeast expressing an HA-tagged Sam35 were sonicated in the presence of 500 mM NaCl or subjected to 100 mM Na2CO3 treatment at pH 11.5 or pH 10.8. Samples were separated by 100,000 × g centrifugation into pellet (P) and supernatant (S) fractions or left untreated (T), and analyzed by SDS-PAGE and Western blot analysis. D, Sam35 is exposed on the mitochondrial surface. Sam35HA mitochondria were treated with protease K and analyzed by SDS-PAGE and Western blotting. E, Sam35 of the SAM complex co-purifies with Sam35. Mitochondria were isolated from a yeast strain containing a His6 tag at Sam35, lysed with digitonin buffer, and subjected to nickel-nitritotriacetic acid chromatography, followed by elution with imidazole. The fractions were separated by SDS-PAGE, and analyzed by Western blotting with the indicated antibodies. Lead represents 0.5%, 1% and 2% of the material used for the chromatography. F, WT, Sam35HA, and Sam50HA mitochondria were lysed in digitonin buffer, subjected to BN-PAGE, and analyzed by Western blot analysis with antibodies against Mas37, HA, and Tom40.
of salt, Sam35 was retained in the membrane fraction like the ADP/ATP carrier (AAC) and Tim44, while the matrix protein Mge1 was released to the supernatant (Fig. 1C, lanes 1 and 2). When mitochondria were treated at alkaline pH, however, Sam35 was extracted from the membranes both under strong (pH 11.5) and mild conditions (pH 10.8) like the peripheral membrane protein Tim44 (Fig. 1C, lanes 5 and 8), while the integral membrane protein AAC remained in the membrane sheets (Fig. 1C, lanes 4 and 7). Thus Sam35 shows the typical characteristics of a peripheral membrane protein that is associated with the membrane via interaction with other membrane proteins. A treatment of isolated mitochondria with protease rapidly removed the Sam35 signal, indicating that at least the HA tag was degraded (Fig. 1D). Under the same conditions, Tim23 and Tim22 were not affected by the protease treatment (Fig. 1D). Since Tim23 is exposed to the intermembrane space, the outer membrane barrier remained intact during the protease treatment, indicating that Sam35 is exposed on the mitochondrial surface.

To determine whether Sam35 is specifically associated with the SAM complex, we generated a yeast strain carrying a 10-histidine tag at the C terminus of Sam35. Mitochondria were isolated, lysed with digitonin, and subjected to nickel-nitrilotriacetic acid chromato­graphy. The eluate contained Sam35 and Mas37 but neither Tom proteins, Tim proteins, nor the abundant membrane proteins porin and AAC (Fig. 1E, lane 6), demonstrating a specific association of Sam35 and Mas37.

In an independent approach, digitonin-lysed mitochondria were separated by blue native electrophoresis (10, 17). Sam35 migrated in a distinct band, termed SAM core complex (Fig. 1F, lane 2). Its mobility was indistinguishable from Mas37 and Sam50 (in the latter case, a yeast strain carrying HA-tagged Sam50 was used (17)), while the TOM complex showed a slower mobility (Fig. 1F). Taken together, these results show that Sam35 is a peripheral outer membrane protein that represents a new subunit of the SAM complex.

**Sam35 Is Essential for Viability of Yeast Cells—Deletion of the SAM35 gene was lethal to yeast cells (34) (not shown). We generated yeast mutant cells by low fidelity PCR of the SAM35 ORF and plasmid shuffling. A mutant strain carrying the temperature-sensitive allele *sam35-2* was selected. The *sam35-2* cells grew like wild-type cells at 24–30 °C on both fermentable and non-fermentable medium but stopped growth at the non-permissive temperature of 37 °C (Fig. 2A). To minimize possible indirect effects of the *sam35-2* mutation on cellular growth and mitochondrial function, the mutant cells were grown at the permissive temperature of 24 °C and mitochondria were isolated. The mutant mitochondria contained marker proteins for the outer membrane (Tom40, Tom22, porin) and inner membrane (AAC) in roughly similar amounts as wild-type mitochondria (Fig. 2B). The SAM core complex, analyzed by blue native electrophoresis and Western blotting with antibodies against Mas37, remained intact also after incubation of the mutant mitochondria at 37 °C (Fig. 2C).

**Sam35 Is Needed for Formation of a Tom40 Assembly Intermediate at the SAM Complex**—To determine whether Sam35 was involved in the biogenesis of the precursor of Tom40, we induced the mutant phenotype by a preincubation of the mitochondria at 37 °C and imported the [35S]-labeled precursor protein of Tom40. In wild-type mitochondria, the assembly pathway of Tom40 into the mature TOM complex of about 450 kDa involves two intermediates of 250 and 100 kDa, respectively (Fig. 2D, lanes 1–3) (10, 17–19, 24, 26). In *sam35-2* mitochondria, the assembly of Tom40 was blocked already at the level of intermediate I (Fig. 2D, lanes 4–6). The assembly intermediate I has been shown to be associated with Mas37 and Sam50 (10, 18), indicating that it represented the SAM-intermediate of Tom40 biogenesis.

To directly determine whether Sam35 was present in a functional SAM complex engaged in precursor binding, we used the method of antibody shift blue native electrophoresis (10, 30). Mitochondria carrying Sam35 with an HA tag were incubated with the radiolabeled precursor of Tom40 to generate the assembly intermediate I, followed by incubation with anti-HA antibodies and blue native electrophoresis. The antibodies efficiently shifted the assembly intermediate I to a high molecu-
lar weight range (Fig. 2E, lane 2). In a parallel reaction, the radiolabeled Tom40 was fully assembled into the mature TOM complex. Anti-HA antibodies did not shift the TOM complex (Fig. 2E, lane 4), demonstrating the specificity of the antibody shift and confirming the absence of Sam35 from the TOM complex. We conclude that Sam35 is a subunit of the active SAM complex.

**Specificity of Sam35 Function in Mitochondrial Protein Biogenesis**—We studied the specificity of the role of Sam35 in mitochondrial protein import by analyzing the biogenesis of five different proteins. The assembly of the β-barrel protein porin (13) was strongly inhibited in sam35-2 mitochondria compared with wild-type mitochondria when both mitochondria were preincubated at 37 °C (Fig. 3A, lanes 4–6 versus lanes 1–3). Without preincubation at 37 °C, porin assembly was similar in wild-type and mutant mitochondria (Fig. 3A, lanes 7–12), indicating that the mutant phenotype of sam35-2 mitochondria was selectively induced by the heat treatment. The outer membrane precursor Tom20, which contains a single transmembrane segment (4), efficiently assembled into the TOM complex in both wild-type and sam35-2 mitochondria (Fig. 3B). Moreover, the import of precursor proteins via the presequence pathway or the carrier pathway was not affected (Fig. 3C and D, respectively). Since it was reported that metaxin is required for the biogenesis of Tom40, since it requires the functional cooperation of two topologically different, essential components. Sam35 and Mas37 are related to mammalian metaxins 2 and 1, respectively. Since it was reported that metaxin is required for tumor necrosis factor-induced cell death (35), it is tempting to speculate that the role of mitochondria in apoptotic events (36, 37) may also involve the SAM complex.

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**Fig. 3. Sam35 is required for the biogenesis of β-barrel outer membrane proteins but not for the import of presequence or carrier proteins.** A, the assembly of porin is inhibited in sam35-2 mitochondria. WT and sam35-2 mitochondria were preincubated at 37 °C for 15 min (heat shock) or used without heat shock for the in vitro import of 35S-labeled porin at 25 °C for the indicated times. The mitochondria were lysed in 0.5% digitonin and analyzed by BN-PAGE and digital autoradiography. B, the assembly of Tom20 was analyzed as described in the legend to A. C and D, import of presequence-containing proteins. After heat shock of WT and sam35-2 mitochondria, the radiolabeled precursors of F1-ATPase subunit β (F1β), and the Rieske Fe/S protein were imported in the presence or the absence of a membrane potential (Δψ). After treatment with proteinase K, the mitochondria were analyzed by SDS-PAGE and digital autoradiography. P, precursor; i, intermediate; m, mature. E, import of dicarboxylate carrier (Dic2). Wild-type (WT) and sam35-2 mitochondria were heat-shocked, incubated with dicarboxylate carrier, and analyzed by BN-PAGE. Where indicated, the membrane potential was dissipated.
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