An Essential Role of Sam50 in the Protein Sorting and Assembly Machinery of the Mitochondrial Outer Membrane*

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The preprotein translocase of the outer mitochondrial membrane (TOM complex) contains one essential subunit, the channel Tom40. The assembly pathway of the precursor of Tom40 involves the TOM complex and the sorting and assembly machinery (SAM complex) with the non-essential subunit Mas37. We have identified Sam50, the second essential protein of the mitochondrial outer membrane. Sam50 contains a β-barrel domain conserved from bacteria to man and is a subunit of the TOM complex. Yeast mutants of Sam50 are defective in the assembly pathways of Tom40 and the abundant outer membrane protein porin, while the import of mitochondrial precursor proteins is not affected. Thus the protein sorting outer membrane protein porin, while the import of mitochondrial precursor proteins in the cytosol and must be imported into the mitochondrial membrane protein Mas37 (20) is involved in insertion by the TOM complex (13, 15, 17–19). However, the way of Tom40 involves recognition of the precursor and initial insertion by the TOM complex (26) and is the only known subunit of an outer membrane complex of ~200 kDa, termed the sorting and assembly machinery (SAM complex) (21). In contrast to Tom40, Mas37 is not essential for cell viability (20, 21). This suggests either that assembly via the SAM complex is not essential for the function of Tom40 or that the SAM complex contains an unknown essential subunit.

For this report, we have purified the SAM complex and identified a new subunit, Sam50. Sam50 is essential for viability of the yeast Saccharomyces cerevisiae and crucial for the assembly pathway of Tom40 and the outer membrane protein porin. Our results suggest that protein assembly is an essential function of the mitochondrial outer membrane.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—A three-way ligation of the EcoRI, BamHI, HISSMX6-fragment of pFA-His10-HISSMX6 (23) with the BamHI and HindIII fragment of PR14_11 containing the NOP1 promoter-ProATEV (24) and pGEM-4Z (Promega) previously cut with EcoRI and HindIII yielded pGEM-HISSMX6-ProATEV (NW4011). The HISSMX6-ProATEV fragment was integrated 5’ to the SAM50 open reading frame into the chromosome leading to SAM50HA (NW40111) (Mat, ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2–801, mas37::HIS3-PRNP1-ProATEV-MAS37). A triple HA-tag (YPM2) (25) was integrated 3’ to the SAM50 open reading frame (open reading frame YNL026W) into the chromosome leading to SAM50HA (Mat, ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2–801, sam50::SAM50/HA-HIS3). Conditional alleles of SAM50 were obtained by error prone PCR (26) and introduced into a SAM50 deletion strain (covered by a plasmid containing SAM50; YEpSAM50) by gap repair and plasmid shuffle, yielding the mutants YPH-BG-FOMP1 (Mat, ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2–801, sam50::ADE2, pFLfomp1-Ts CEN7). Mutants 1 and 3 were selected and termed sam50-1 and sam50-3. Yeast cells were grown on YPG (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 3% (w/v) glucose) at 23 or 37 °C.

Biochemical Methods—The following methods were performed according to published procedures (22, 24, 27): isolation of mitochondria, synthesis of 35S-labeled precursor proteins in rabbit reticulocyte lysate, protein import into mitochondria at 25 °C (upon preincubation at 37 °C), dissipation of the membrane potential Δψ, protease treatment of mitochondria, SDS-PAGE, blue native electrophoresis (BN-PAGE) of mitochondria lysed with digitonin, digital autoradiography, Western blotting, sonication of mitochondria, treatment with 0.1 M Na2CO3, pH 11.5, separation of membranes and supernatant, and analysis by mass spectrometry. For Figs. 2 and 3, non-relevant gel lanes were excised by digital processing.

For sub mitochondrial fractionation, 9 ml of swelling buffer (20 mM HEPES-KOH, pH 7.4, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) were added to mitochondria in SE buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2). After 30-min incubation on ice, the mixture was centrifuged at 150,000 × g for 90 min at 4 °C. The pellet was resuspended in a 10 mM citrate buffer containing 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride. After a clarification step (20,000 × g, 15 min), the supernatant was loaded onto a discontinuous sucrose gra-

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† The abbreviations used are: TOM complex, translocase of outer mitochondrial membrane; BN-PAGE, blue native electrophoresis; SAM complex, sorting and assembly machinery; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid.
amino acid residues with an 1). The primary structure of Sam50 predicts a protein of 484 B

a novel protein, termed Sam50 (ORF YNL026W) (Fig. 1 A). When mitochondria were

segment; red, predicted /H9252 /H11003 tation at pH 11.5. Samples were left untreated or subjected to 100,000

gradient (11 ml, 29.1%, 37.65%, 46.2%, 54.77% (w/v) sucrose in 5 m M

pectrometry. Samples 3

dient (11 ml, 29.1%, 37.65%, 46.2%, 54.77% (w/v) sucrose in 5 m M

HEPES/KOH, pH 7.4, 10 m M KCl). After centrifugation (100,000
g. 4 °C, 16 h, 4 °C), 750-μl fractions were collected from top to bottom.

RESULTS AND DISCUSSION

Sam50, an Essential Protein of the Mitochondrial Outer Membrane—To identify further subunits of the SAM complex, we constructed a S. cerevisiae strain with a protein A tag at Sam50. The growth behavior of the strain was indistinguishable from wild-type yeast. Mitochondria were isolated and lysed with digitonin. The SAM complex was purified by IgG-affinity chromatography. Two protein bands were specifically eluted and identified by mass spectrometry as Mas37ProtA and an novel protein, termed Sam50 (ORF YNL026W) (Fig. 1A, lane 1). The primary structure of Sam50 predicts a protein of 484 amino acid residues with an α-helical segment in the N-terminal region, followed by multiple β-strands (β-barrel) (Fig. 1B).
The C-terminal Sam50 domain of ~30 kDa is conserved from bacteria to all eukaryotic kingdoms. Bacterial proteins containing this conserved domain include Omp1, Omp85, Oma87, and D-15, while the eukaryotic homologues of Sam50 have not been further characterized. Omp85 is also related to the chloroplast channel Toc75 (28–30); however, the relation of Sam50 to Toc75 is much weaker than that to its not yet characterized eukaryotic homologues. Deletion of SAM50 is lethal to yeast cells (not shown).

We generated a yeast strain with an HA-tag at Sam50. The strain grew like wild-type yeast. When mitochondria were lysed with digitonin and separated by BN-PAGE, Sam50 migrated like Mas37, confirming its presence in the 200-kDa SAM complex (Fig. 1A, lanes 3 and 4). Upon sonication of mitochondria in the presence of salt, Sam50 remained in the membrane pellet like the membrane proteins ADP/ATP carrier and Tim44 (translocase of inner membrane), while the soluble protein Mge1 was extracted (Fig. 1C, lanes 3–6). Treatment of mitochondria at pH 11.5 extracted Mge1 and the peripheral membrane protein Tim44, while Sam50 and the ADP/ATP carrier remained in the membrane sheets (Fig. 1C, lanes 4–6), indicating that Sam50 is an integral membrane protein in agreement with the secondary structure prediction. Upon separation of mitochondrial outer and inner membrane vesicles on a sucrose gradient (24), Sam50 was found in the outer membrane fraction like porin (VDAC) and the receptor Tom70, while Tim23 was present in the inner membrane fractions (Fig. 1D).

We conclude that Sam50 is an integral protein of the outer membrane associated with Mas37. Sam50 is thus the second mitochondrial outer membrane protein essential for cell viability of yeast.

Sam50 Is Required for the Assembly Pathway of Mitochondrial Outer Membrane Proteins—We generated temperature-sensitive alleles of SAM50 by error-prone PCR and selected the mutant strains sam50-1 and sam50-3. At 23 °C the growth of the mutant cells was similar to wild-type cells, while they were unable to grow at 37 °C (Fig. 2A). To minimize indirect effects of the sam50 mutations on cellular and mitochondrial functions, cells were grown at the permissive temperature of 23 °C, and the isolated mitochondria were incubated for 15 min at 37 °C to induce the mutant phenotype. The isolated mitochondria contained wild-type levels of the marker proteins analyzed, including matrix Hsp70, Tom70, Tom40, and porin (Fig. 2B). Similarly, the pre-existing TOM complex analyzed by BN-

FIG. 1. Identification of the mitochondrial outer membrane protein Sam50. A, samples 1 and 2, mitochondria from wild-type (WT) and yeast cells expressing protein A-tagged Mas37 were lysed in digitonin-buffer. After IgG-chromatography, the SAM-complex was eluted by 0.5 m sodium acetate, pH 3.4, separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The indicated bands were identified by mass spectrometry. Samples 3 and 4, mitochondria from cells expressing Sam50 with HA-tag were separated by BN-PAGE and the SAM complex was detected by Western blotting with antibodies directed against HA or Mas37. B, deduced primary structure of Sam50. Blue, predicted α-helical segment; red, predicted β-strands (also in eukaryotic Sam50 homologues) according to BetaTPred2 (34).

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PAGE and Western blotting for Tom40 was comparable between wild-type and mutants (Fig. 2C, upper panel). Mas37 was present in wild-type amounts in sam50-1 mitochondria (Fig. 2B). The amount of SAM complex of sam50-1 mitochondria, analyzed by BN-PAGE and antibodies against Mas37, was reduced by about 50% (Fig. 2C, lower panel), indicating that the assembly of the SAM complex was partially impaired in the mutant mitochondria. In sam50-3 mitochondria, the amount of both Mas37 and SAM complex was moderately reduced compared with wild-type mitochondria (Fig. 2B and C). Thus, both mutant mitochondria still contain a SAM complex, but in reduced amounts.

To analyze the effect of the sam50 mutations on the assembly pathway of Tom40, we used an in organello assay with 35S-labeled precursor of Tom40. In wild-type mitochondria, the assembly into the 450-kDa TOM complex occurs in two intermediate steps of 250 kDa (assembly intermediate I) and 100 kDa (assembly intermediate II) that can be separated by BN-PAGE of digitonin-lysed mitochondria (Fig. 2D, lanes 1–3) (15, 21). It has been shown that the mature TOM complex is in continuous exchange with the assembly intermediate II (15). Therefore, newly synthesized individual subunits like Tom40 can be incorporated into pre-existing TOM complexes (15, 18, 21). In both sam50 mutant mitochondria, the assembly of Tom40 was nearly blocked, already at the level of the intermediate I (Fig. 2D, lanes 4–9). Since the assembly intermediate I represents the SAM complex-bound precursor of Tom40 (21), this result indicates that a functional Sam50 is important for the action of the SAM complex. A working hypothesis for the composition of the SAM complex includes the observation that mas37/H9004 mitochondria accumulate a crippled (smaller) form of the assembly intermediate I consistent with the lack of one molecule of Mas37 (21). Thus, the SAM complex may contain one molecule of Mas37 and up to three molecules of Sam50 (Mas37ProtA was overexpressed in the strain used for Fig. 1A).

To determine the specificity of Sam50 function, we analyzed the biogenesis of three further mitochondrial proteins, the most...
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abundant outer membrane protein porin (VDAC, Por1) and two proteins that are translated across the outer and inner membranes to the matrix. Mature, assembled porin has been shown to form complexes in the size range of ~200–400 kDa (27). In wild-type mitochondria, the assembly of porin was strongly reduced (Fig. 3A, lanes 4–9). In contrast, the import of two matrix proteins, α-subunit of mitochondrial processing peptidase and the model protein Su9-DHFR with the presence of Fd-ATPase subunit 9 fused to dihydrofolate reductase, was not affected by the sam50 mutations (Fig. 3, B and C). We conclude that Sam50 is required for the biogenesis of β-barrel proteins of the mitochondrial outer membrane (Tom40 and porin) but not for the translocation of preproteins across the outer and inner membranes into the matrix.

Conclusions—We have identified the second essential protein of the mitochondrial outer membrane. Unlike the essential protein Tom40 that is required for the import of virtually all mitochondrial proteins (7, 12, 27), Sam50 plays a specific role in the assembly pathways of outer membrane proteins. Together with the non-essential Mas37, Sam50 forms the SAM complex of the outer membrane. Sam50 contains a β-barrel domain that is conserved in evolution from bacteria to man. Recently, two different views have been reported for the function of the bacterial homologue Omp85, involvement in transport of either proteins or lipids to the outer membrane of Gram-negative bacteria (30, 33). A reason for the controversial views is that the bacteria had to be incubated under non-induced conditions for several hours to deplete the cells of Omp85. With the in organello assembly assay of mitochondria, we can grow the cells at permissive temperature and induce the phenotype by shifting the isolated mitochondria to non-permissive temperature for only 15 min, thus minimizing indirect effects. We thereby showed that Sam50 is a crucial subunit of the assembly machinery for outer membrane proteins. Our results raise the exciting possibility that the mitochondrial outer membrane has retained a conserved domain for the assembly of β-barrel proteins.

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