THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 21, Issue of May 21, pp. 22781–22785, 2004
Printed in U.S.A.

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

Received for publication, March 22, 2004, and in revised form, March 30, 2004
Published, JBC Papers in Press, April 2, 2004, DOI 10.1074/jbc.C400120200

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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)[1] 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, 11–13). 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 His_{10} tag (pFA-His10-HIS3MX6) (29) were integrated 3′ to the *SAM35* ORF (YHR083w/*FMP20*) into the *Saccharomyces cerevisiae* strain YPH499, yielding the strains $SAM35_{HA}$ (*Mat***a**, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*, *sam35::SAM35HA-HIS3*) and $SAM35_{His}$ (*Mat***a**, *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 YPH499 strain carrying the 2μ *URA3*-containing plasmid YEp352 with the *SAM35* ORF between the *MET25* promoter and the *CYC1* terminator, yielding *sam35-delta* (*Mat***a**, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, sam35::ADE2,* [YEp-*SAM35*]). The temperature-conditional yeast strain *sam35-2* (*Mat***a**, *ade2-101, his3-Δ200, leu2-Δ1, ura3-52, trp1-Δ63, lys2-801, sam35::*ADE2, [pFLfomp4-Ts5-2-CEN]) was generated by low fidelity PCR of the *SAM35* ORF, followed by cloning through gap repair and

* This work was supported by the Deutsche Forschungsgemeinschaft, the Sonderforschungsbereich 388, Max Planck Research Award/Alexander von Humboldt Foundation, Bundesministerium für Bildung und Forschung, Nationales Genomforschungsnetz, and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TOM, translocase of outer mitochondrial membrane; AAC, ADP/ATP carrier; BN, blue native; HA, hemagglutinin; ORF, open reading frame; SAM, sorting and assembly machinery; TIM, translocase of inner mitochondrial membrane; PAM, presequence translocase-associated motor; MOPS, 4-morpholinepropanesulfonic acid.

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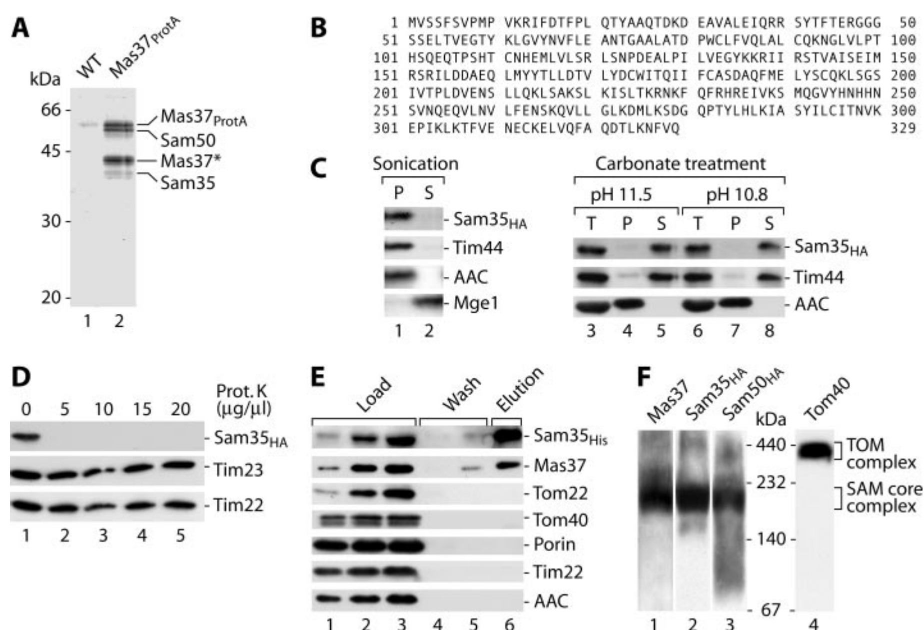

FIG. 1. **Sam35 is a subunit of the SAM complex of the outer mitochondrial membrane.** *A*, purification of the SAM complex. Mitochondria were isolated from wild-type (*WT*) yeast cells and from a yeast strain expressing Mas37 with a protein A tag (17). The mitochondria were lysed in 1% digitonin buffer 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 $Mas37_{ProtA}$. *B*, predicted primary structure of Sam35. *C*, Sam35 is 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 Na_2CO_3 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. $Sam35_{HA}$ mitochondria were treated with proteinase K and analyzed by SDS-PAGE and Western blotting. *E*, Mas37 of the SAM complex co-purifies with Sam35. Mitochondria were isolated from a yeast strain containing a His_{10} tag at Sam35, lysed with digitonin buffer, and subjected to nickel-nitrilotriacetic acid chromatography, followed by elution with imidazole. The fractions were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies. *Load* represents 0.5%, 1 and 2% of the material used for the chromatography. *F*, WT, $Sam35_{HA}$, and $Sam50_{HA}$ mitochondria were lysed in digitonin buffer, subjected to BN-PAGE, and analyzed by Western blot analysis with antibodies against Mas37, HA, and Tom40.

subsequent plasmid shuffling of the *sam35-2* mutant allele plasmid pFLfomp4-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 3% (v/v) glycerol) or YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, and 2% (w/v) glucose). Mitochondria were isolated by differential centrifugation and adjusted to a protein concentration of 10 mg/ml in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) and stored at −80 °C.

In Vitro Protein Import—Radiolabeled precursor proteins were generated by *in vitro* transcription, using SP6 RNA polymerase, and translation in rabbit reticulocyte lysate in the presence of [^{35}S]methionine/cysteine (Amersham Biosciences). Mitochondria in BSA buffer (3% (w/v) fatty acid-free BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS-KOH, pH 7.2, 5 mM methionine, 2 mM KH_2PO_4) were preincubated at 37 °C for 15 min where indicated. The samples were transferred to 25 °C for 2 min and 2–5 mM ATP, 2 mM NADH, 100 µg/ml creatine kinase, and 5 mM creatine phosphate were added when required. To dissipate the membrane potential before import, 1 µM valinomycin was added. The import was started by addition of reticulocyte lysate (5–20% (v/v) of import reaction). After the indicated times, import was stopped by addition of 1 µM valinomycin, and samples were chilled on ice. Where indicated, mitochondria were treated with 5–50 µg/ml proteinase K for 15 min on ice. After addition of 2 mM phenylmethylsulfonyl fluoride and 10-min incubation on ice, mitochondria were reisolated and washed with SEM buffer containing phenylmethylsulfonyl fluoride. Mitochondria were resuspended in Laemmli buffer for SDS-PAGE or in digitonin buffer (0.4–1% (w/v) digitonin, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (v/v) glycerol) for BN-PAGE (10, 24). Radiolabeled proteins were detected by digital autoradiography (Amersham Biosciences).

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 His_{10} 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, nonrelevant gel lanes were excised digitally. Western transfers onto polyvinylidene difluoride membranes and immunodecoration were done according to standard techniques. Enhanced chemiluminescence was used for detection (Amersham Biosciences).

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 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. 1*A*). 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. 1*B*). Sam35 possesses homologues in *Saccharomyces bayanus*, *Saccharomyces mikatae*, *Saccharomyces paradoxus*, and *Saccharomyces castellii* and, via the proteins ADR303Wp of *Ashbya* (*Eremothecium*) *gossypii*, FG10106.1 of *Gibberella zeae*, AN0657.2 of *Aspergillus nidulans*, and SPAC589.04 of *Schizosaccharomyces pombe*, is related to mammalian metaxin 2. The function of the mitochondrial outer membrane protein metaxin 2 is unknown, yet it has been found by its similarity to and interaction with metaxin 1 (32). Metaxin 1 shows a weak similarity to Mas37 and has been implicated to be involved in mitochondrial protein import (33). Our finding that Sam35 is present in the SAM complex together with Mas37 raises the possibility that mammalian metaxins 1 and 2 may be involved in the biogenesis of mitochondrial outer membrane proteins.

We constructed a *S. cerevisiae* strain with an HA tag at the C terminus of Sam35. The resulting cells grew like wild-type yeast. Upon sonication of isolated mitochondria in the presence

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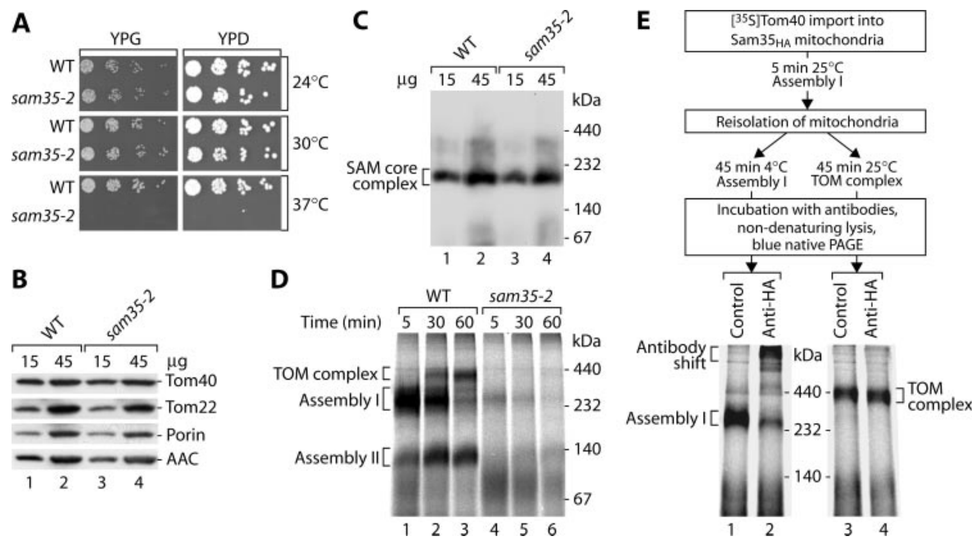

FIG. 2. **The essential Sam35 is required for Tom40 assembly and forms a constituent of the assembly I complex.** *A*, growth of wild-type (*WT*) and *sam35-2* yeast cells on YPG and YPD media at the indicated temperatures. *B*, mitochondrial proteins (μg) of WT and *sam35-2* mitochondria were subjected to SDS-PAGE and Western blot analysis. *C*, the SAM complex of *sam35-2* mitochondria is comparable with that of WT mitochondria. Isolated mitochondria were preincubated for 15 min at 37 °C (heat shock), separated by BN-PAGE, and analyzed with anti-Mas37 antibodies. *D*, Tom40 assembly is blocked in *sam35-2* mitochondria. WT and *sam35-2* mitochondria were heat-shocked and incubated with radiolabeled Tom40 for the indicated times at 25 °C. Protein complexes were analyzed by BN-PAGE and digital autoradiography. *E*, Sam35 is present in the assembly I complex of Tom40. ^{35}S-Labeled Tom40 was imported into $Sam35_{HA}$ mitochondria for 5 min to generate the assembly intermediate I (10). After reisolation, the mitochondria were incubated for 45 min at 4 °C (*lanes 1* and *2*) or 25 °C to chase the Tom40 precursor to the TOM complex (*lanes 3* and *4*). For the antibody shift experiment, mitochondria were incubated with antibodies against HA (*lanes 2* and *4*) or were left untreated (*lanes 1* and *3*, control) and were analyzed by BN-PAGE and digital autoradiography.

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. 1*C*, *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. 1*C*, *lanes 5* and *8*), while the integral membrane protein AAC remained in the membrane sheets (Fig. 1*C*, *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. 1*D*). Under the same conditions, Tim23 and Tim22 were not affected by the protease treatment (Fig. 1*D*). 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. 1*E*, *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. 1*F*, *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. 1*F*). 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. 2*A*). 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. 2*B*). 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. 2*C*).

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 ^{35}S-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. 2*D*, *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. 2*D*, *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-

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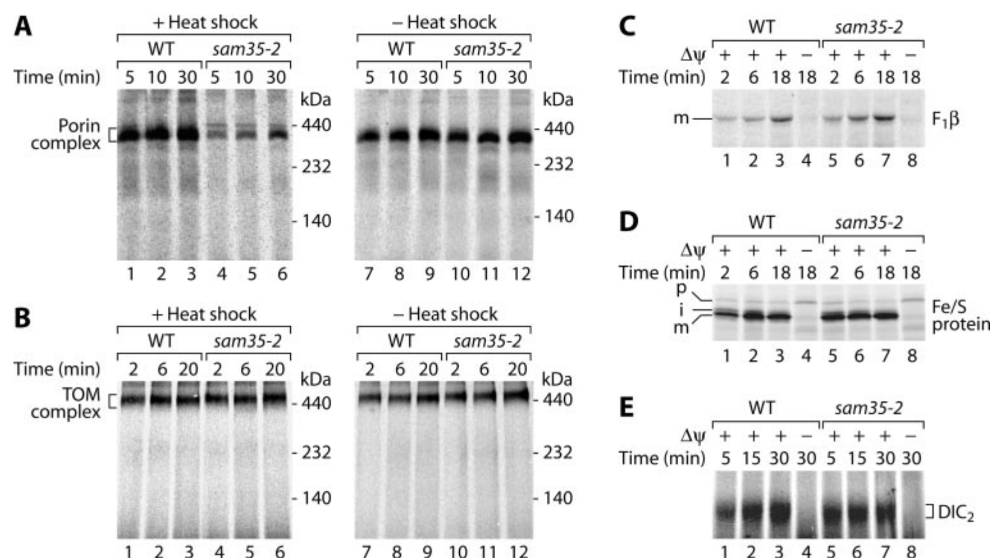

FIG. 3. **Sam35 is required for the biogenesis of β-barrel outer membrane proteins but not for the import of presequence or carrier proteins.** *A*, the assembly of porin is inhibited in *sam35-2* mitochondria. WT and *sam35-2* mitochondria were preincubated at 37 °C for 15 min (heat shock) or used without heat shock for the *in vitro* import of ^{35}S-labeled porin at 25 °C for the indicated times. The mitochondria were lysed in 0.4% 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 F_1-ATPase subunit β ($F_1β$) 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 (DIC). 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.

lar weight range (Fig. 2*E*, *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. 2*E*, *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. 3*A*, *lanes 4–6 versus lanes 1–3*). Without preincubation at 37 °C, porin assembly was similar in wild-type and mutant mitochondria (Fig. 3*A*, *lanes 7–12*), indicating that the mutant phenotype of *sam35-2* mitochondria was selectively induced by the heat treatment. The outer membrane receptor Tom20, which contains a single transmembrane segment (4), efficiently assembled into the TOM complex in both wild-type and *sam35-2* mitochondria (Fig. 3*B*). Moreover, the import of precursor proteins via the presequence pathway or the carrier pathway was not affected by the *sam35-2* mutation (heat-shocked mitochondria), as shown with two cleavable preproteins, matrix F_1-ATPase subunit β (Fig. 3*C*) and the Rieske Fe/S protein of the inner membrane bc_1 complex (Fig. 3*D*), and the non-cleavable dicarboxylate carrier of the inner membrane (Fig. 3*E*). These results suggest a specific role of Sam35 in the biogenesis of β-barrel proteins and thus a genuine function in the SAM complex.

Conclusions—Sam35 is a novel subunit of the SAM complex and is required for the assembly pathway of β-barrel proteins of the mitochondrial outer membrane. The SAM complex contains two subunits that are essential for cell viability, Sam35 and Sam50, whereas only one subunit of the TOM complex, Tom40, is essential, emphasizing a crucial role of the SAM complex in mitochondrial biogenesis. While Sam50 and Tom40 are both integral outer membrane proteins with β-barrel structure, Sam35 shows the typical characteristics of a peripheral membrane protein exposed on the mitochondrial surface. Sam35 is present in the active SAM complex engaged in binding of the Tom40 precursor at an early assembly stage, and Sam50 is required at the same assembly stage I. We conclude that the generation of assembly intermediate I is of major importance in 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.

Acknowledgments—We thank Drs. P. Rehling, A. Chacinska, and A. E. Frazier for discussion and experimental advice. We are grateful to B. Schönfisch and N. Zufall for excellent technical assistance.

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