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Dissecting Membrane Insertion of Mitochondrial β-Barrel Proteins

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SUMMARY

Communication of mitochondria with the rest of the cell requires β-barrel proteins of the outer membrane. All β-barrel proteins are synthesized as precursors in the cytosol and imported into mitochondria by the general translocase TOM and the sorting machinery SAM. The SAM complex contains two proteins essential for cell viability, the channel-forming Sam50 and Sam35. We have identified the sorting signal of mitochondrial β-barrel proteins that is universal in all eukaryotic kingdoms. The β-signal initiates precursor insertion into a hydrophilic, proteinaceous membrane environment by forming a ternary complex with Sam35 and Sam50. Sam35 recognizes the β-signal, inducing a major conductance increase of the Sam50 channel. Subsequent precursor release from SAM is coupled to integration into the lipid phase. We propose that a two-stage mechanism of signal-driven insertion into a membrane protein complex and subsequent integration into the lipid phase may represent a general mechanism for biogenesis of β-barrel proteins.

INTRODUCTION

Many organelles, like the endoplasmic reticulum, peroxisomes, and lysosomes, are confined by one membrane, harboring α-helical channel proteins. Mitochondria and chloroplasts are additionally bordered by an outer membrane, which contains channel proteins of β-barrel structure (Schleiff and Soll, 2005; Dolezal et al., 2006). The precursors of most mitochondrial proteins are synthesized on cytosolic ribosomes and transported to the translocase of the outer mitochondrial membrane (TOM complex). From here, transport pathways diverge and the precursor proteins are transported to their submitochondrial destination with the help of target-specific recognition sequences (Rehling et al., 2004; Neupert and Herrmann, 2007). N-terminal or internal signals direct α-helical membrane proteins to the inner mitochondrial membrane, into which they are inserted by translocases of the inner membrane (TIM).

The precursors of outer membrane β-barrel proteins are transferred from the TOM complex to the sorting and assembly machinery (SAM complex) (Wiedemann et al., 2003; Johnson and Jensen, 2004; Dolezal et al., 2006; Neupert and Herrmann, 2007). The SAM complex contains two proteins that are essential for cell viability, the integral membrane protein Sam50 (Tob55/Omp85) (Kozjak et al., 2003; Paschen et al., 2003; Gentle et al., 2004; Humphries et al., 2005) and Sam35 (Tob38/Tom38), which behaves as a peripheral membrane protein (Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004). The third subunit of the SAM complex, Sam37, is not essential for cell viability.

The mechanism of sorting and membrane integration of mitochondrial β-barrel proteins is poorly understood. Sam50 is homologous to Omp85/YaeT from Gram-negative bacteria, implying that these proteins function in a related manner in β-barrel insertion (Schleiff and Soll, 2005; Dolezal et al., 2006; Robert et al., 2006; Sklar et al., 2007). Bacterial β-barrel proteins contain a C-terminal signature motif that interacts with Omp85. The motif typically consists of the terminal amino acid phenylalanine and further hydrophobic residues close to the C terminus (Robert et al., 2006). Despite extensive searches, the signal has not been identified in mitochondrial β-barrel proteins. Mutational analysis yielded various regions spread over the mitochondrial precursors that influenced the formation of mature β-barrel proteins; however, no consensus was found (Court et al., 1996; Rapaport and Neupert, 1999; Rapaport et al., 2001; Taylor et al., 2003; Humphries et al., 2005; Sherman et al., 2006). Habib et al. (2007) concluded that a linear consensus sequence as a sorting signal is unlikely in the case of mitochondria. The membrane environment for β-barrel proteins changed during evolution, as the outer leaflet of the bacterial but not the mitochondrial outer membrane is composed of lipopolysaccharides. Omp85/YaeT forms a complex with four lipoproteins (Sklar et al., 2007), yet the SAM complex does not contain homologs of those proteins (Dolezal et al., 2006). Thus the similarity between bacterial and mitochondrial β-barrel biogenesis seems to be limited to the homology of Omp85 and Sam50 while the sorting signal, membrane environment, and partner proteins are different.

Mutants of Sam50, Sam35, and Sam37 are impaired in substrate interaction with the SAM complex in organello

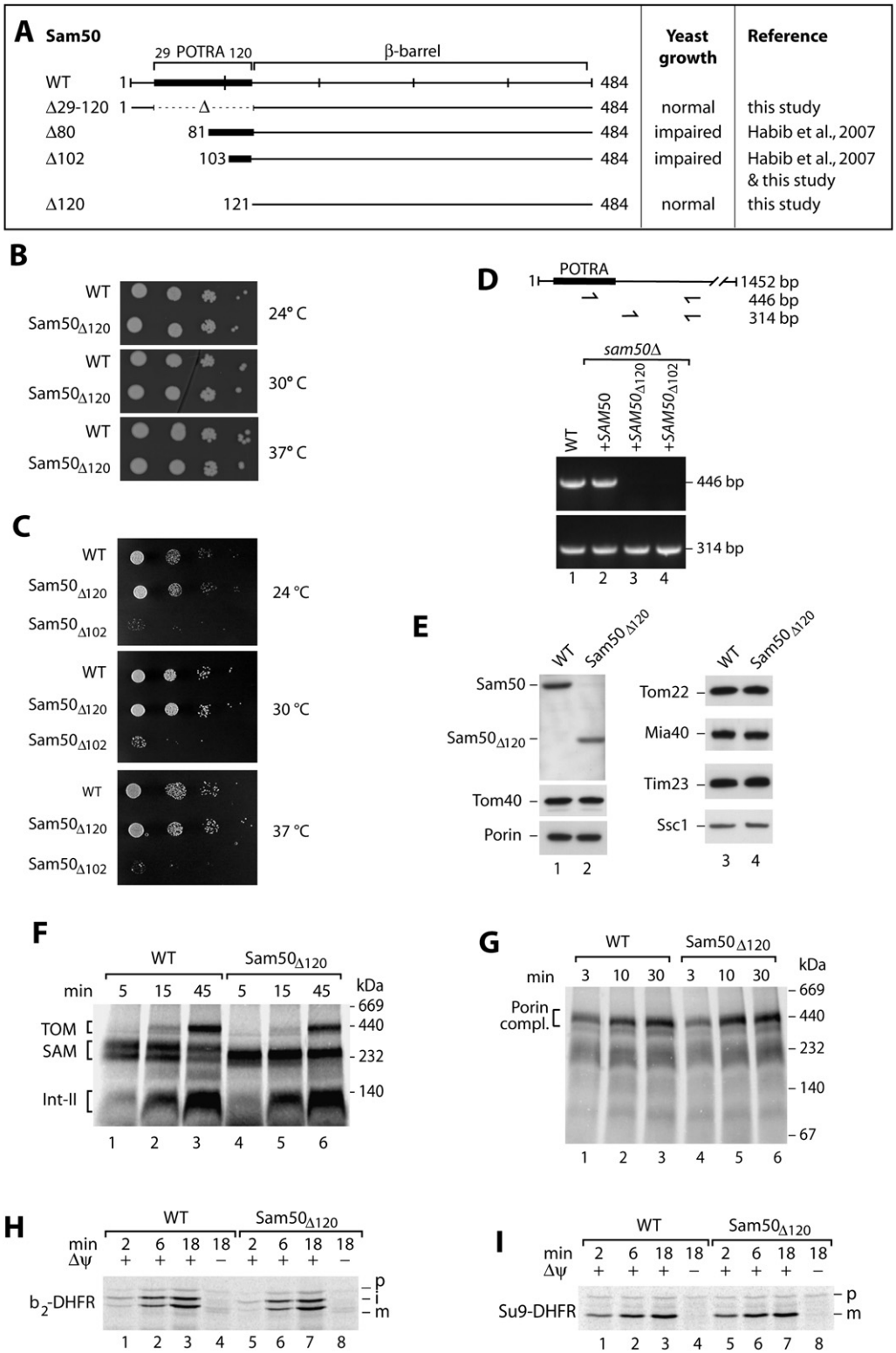

Figure 1. Sam50 POTRA Domain Is Dispensable for β-Barrel Protein Biogenesis

(A) Scheme of wild-type (WT) and Sam50 POTRA yeast deletion mutants.

(B) Growth of WT and Sam50$_{\Delta 120}$(pFL39) yeast on YPG.

(C) Growth of WT, Sam50$_{\Delta 120}$(pRS413), and Sam50$_{\Delta 102}$ yeast on YPG.

(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; Chan and Lithgow, 2008); however, a molecular mechanism of function has only been reported for Sam50. The large β-barrel domain forms a channel like Omp85 (Paschen et al., 2003; Robert et al., 2006) while the N-terminal α-helical domain contains one polypeptide transport associated (POTRA) domain, which is present in five copies in Omp85/YaeT (Sánchez-Pulido et al., 2003; Dolezal et al., 2006; Bos et al., 2007; Kim et al., 2007). Habib et al. (2007) reported that deletion of a major portion of the N-terminal domain of Sam50 inhibited the growth of yeast cells and the interaction of β-barrel precursors with SAM. They concluded that the POTRA domain plays a receptor-like function in β-barrel biogenesis. Chan and Lithgow (2008) proposed that the two peripheral membrane proteins Sam35 and Sam37 provide a protective environment for assembly of the extramembrane (cytosolic) domains of mitochondrial β-barrel proteins. As the mitochondrial outer membrane contains only three proteins essential for cell viability, the two channel-forming proteins Tom40 and Sam50 and Sam35 (Milenkovic et al., 2004; Dolezal et al., 2006), it will be of particular importance to understand the molecular role of Sam35 in β-barrel biogenesis.

Unexpectedly, we found that deletion of the entire N-terminal domain of Sam50 did not inhibit growth of yeast cells and precursor binding to SAM, excluding that the POTRA domain was crucial for recognition of β-barrel precursors. We identified the β-sorting signal that is present in mitochondrial β-barrel proteins of all eukaryotic kingdoms. We show that Sam35 recognizes the β-signal, inducing opening of the channel formed by Sam50. Our findings imply a two-step mechanism for recognition and insertion of β-barrel precursors.

RESULTS

The POTRA Domain of Sam50 Is Dispensable for Cell Growth and β-Barrel Biogenesis

The predicted POTRA domain of *Saccharomyces cerevisiae* Sam50 consists of amino acid residues 29–120 (Sánchez-Pulido et al., 2003). We deleted the entire POTRA domain, but the resulting mutant yeast grew like wild-type (WT) cells (Figure 1A) and the mitochondria efficiently imported precursor proteins (data not shown), in difference to the findings by Habib et al. (2007). In that study the first N-terminal 80 or 102 residues, respectively, were deleted, raising the possibility that the N-terminal 28 residues of Sam50 were crucial for β-barrel biogenesis. We deleted the entire N-terminal domain of Sam50 (120 residues in front of the predicted β-barrel domain), yet the yeast mutant still grew like WT (Figure 1B). To exclude a cloning artifact, we generated a second $Sam50_{\Delta 120}$ strain using different vectors and markers yet obtained the same WT growth (Figure 1C). We thus generated the Δ102 partial deletion of the POTRA domain used by Habib et al. (2007) and indeed observed a severe growth defect (Figure 1C), indicating that the remaining 18 residue POTRA segment was responsible for the reported defect. The truncation of $Sam50_{\Delta 120}$ was confirmed by PCR (Figure 1D) and western blot analysis (Figure 1E). The steady-state levels of the β-barrel proteins Tom40 and porin, as well as of marker proteins for different mitochondrial subcompartments, were unchanged between WT and $Sam50_{\Delta 120}$ mitochondria (Figure 1E). We used the radiolabeled precursors of Tom40 and porin to study the biogenesis of β-barrel proteins in isolated mitochondria and analyzed them by blue native electrophoresis (BN-PAGE) of digitonin-lysed mitochondria. The precursor of Tom40 assembles via two intermediate stages, first an intermediate-I of ~250 kDa, representing interaction of the precursor with the SAM complex, and second an intermediate-II of ~100 kDa, representing insertion of Tom40 into the lipid phase of the outer membrane (Model et al., 2001; Paschen et al., 2003; Wiedemann et al., 2003; Gentle et al., 2004; Ishikawa et al., 2004; Waizenegger et al., 2004). Subsequently, the mature TOM complex of ~450 kDa is formed (Figure 1F). $Sam50_{\Delta 120}$ mitochondria efficiently formed the SAM intermediate of Tom40, which migrated faster on the native gels in agreement with the truncation of Sam50 (Figure 1F). The precursor of porin does not form stable intermediates with SAM (Wiedemann et al., 2003) and thus assembly into the mature porin complexes was monitored (Figure 1G). The formation of mature porin and TOM complexes occurred in the mutant mitochondria with an efficiency that was close to that of WT mitochondria (Figures 1F and 1G). As expected, control proteins transported to the intermembrane space (Figure 1H) and matrix (Figure 1I) were not affected in $Sam50_{\Delta 120}$ mitochondria. We conclude that the POTRA domain does not play an essential role for precursor binding to SAM and mitochondrial protein import.

Identification of the Mitochondrial β-Signal

To screen for a potential sorting signal of β-barrel precursors, we systematically deleted N- and C-terminal segments of Tom40 and monitored import of the radiolabeled constructs into isolated mitochondria by BN-PAGE (Figures 2A–2C). Thereby, binding to SAM and formation of the mature TOM complex could be directly differentiated. N-terminally truncated versions of Tom40 led to formation of a SAM intermediate while the subsequent assembly steps were blocked (Figure 2B). To directly determine if the truncated versions were bound to SAM, we used mitochondria carrying a hemagglutinin (HA)-tagged Sam35 (Milenkovic et al., 2004). Anti-HA antibodies depleted the N-terminally truncated versions of Tom40 as well as WT Tom40 on BN-PAGE, demonstrating the accumulation at SAM (Figure 2B). In contrast, Tom40 constructs with C-terminal truncations were blocked in association with SAM (Figure 2C, upper panel); the constructs were still targeted to mitochondria, determined as total mitochondria-bound precursor (Figure 2C, bottom panel), excluding

(D) DNA was isolated and used for verification of POTRA deletions by PCR.

(E) Mitochondria were isolated from WT and $Sam50_{\Delta 120}$ yeast and subjected to SDS-PAGE and immunodecoration.

(F and G) Isolated mitochondria were incubated with [^{35}S]Tom40 or porin, solubilized in digitonin, and analyzed by BN-PAGE and autoradiography. SAM, precursor-SAM complex intermediate-I; Int-II, 100 kDa assembly intermediate-II.

(H and I) Mitochondria were incubated with [^{35}S]precursors, treated with proteinase K, and analyzed by SDS-PAGE. p, precursor; i, intermediate; m, mature.

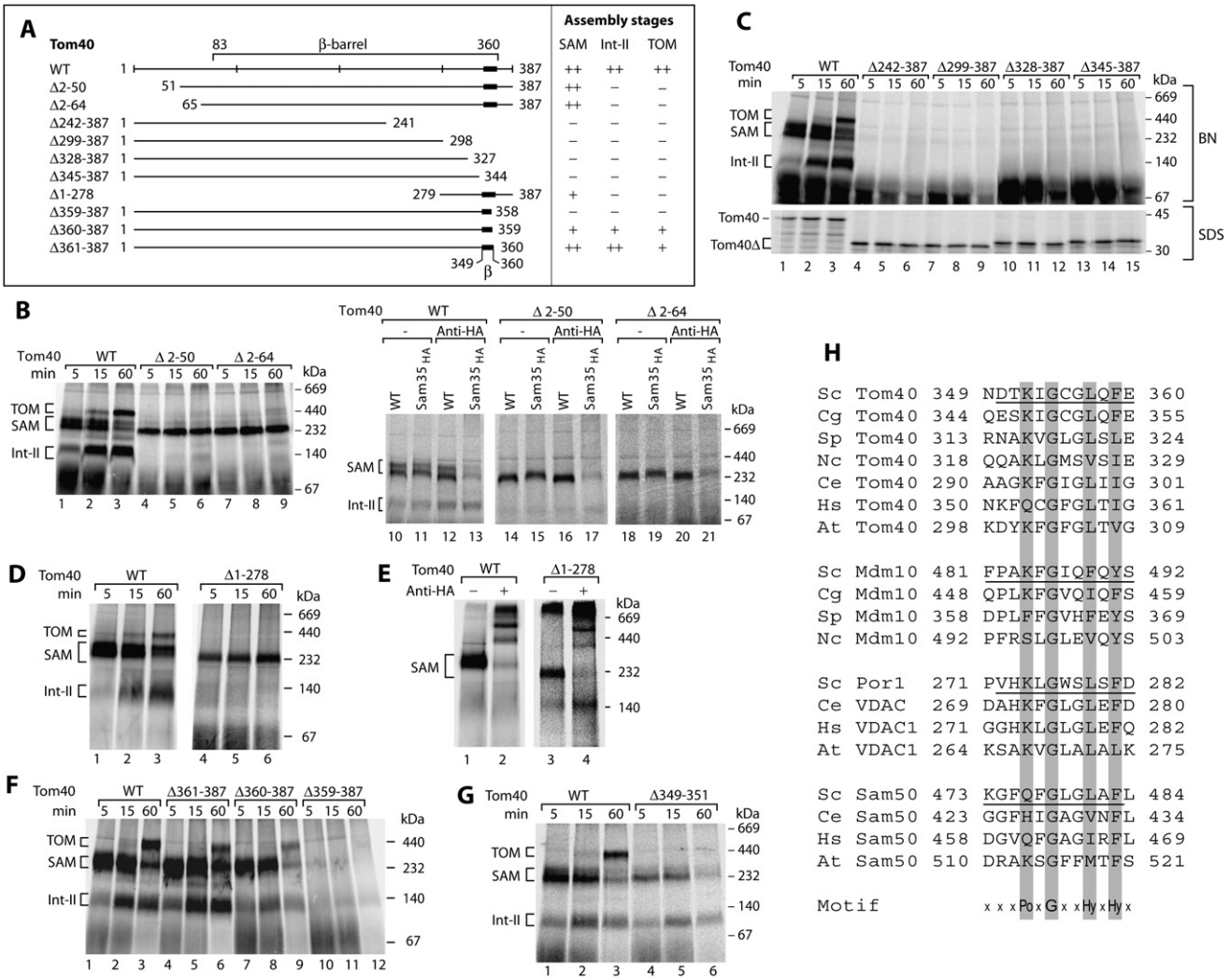

Figure 2. C-Terminal Fragment of Tom40 Is Sufficient for SAM Binding

(A) Scheme of truncated Tom40 constructs.

(B) Isolated WT mitochondria were incubated with [^{35}S]precursors and analyzed by BN-PAGE (left panel). Samples 10–21, mitochondria from WT and Sam35$_{HA}$ yeast were used; where indicated anti-HA antibody was added.

(C) Precursors were imported into WT mitochondria and analyzed by BN-PAGE (upper panel) and SDS-PAGE (lower panel).

(D) Tom40 and Tom40$_{\Delta 1\text{-}278}$ were incubated with WT mitochondria and analyzed by BN-PAGE.

(E) Tom40 and Tom40$_{\Delta 1\text{-}278}$ were incubated with Sam35$_{HA}$ mitochondria. Where indicated anti-HA antibody was added.

(F and G) Tom40 constructs were incubated with WT mitochondria.

(H) Alignment of last predicted β-strand (underlined). *S. cerevisiae* (*Sc*), *Candida glabrata* (*Cg*), *Schizosaccharomyces pombe* (*Sp*), *Neurospora crassa* (*Nc*), *Caenorhabditis elegans* (*Ce*), *Homo sapiens* (*Hs*), *Arabidopsis thaliana* (*At*). Po, polar; Hy, hydrophobic.

a degradation of the constructs. A fragment consisting of the C-terminal 109 residues of the 387 residue Tom40 formed a complex with the expected size of a SAM intermediate (Figure 2D). When imported into Sam35$_{HA}$ mitochondria, anti-HA antibodies shifted the complex formed by Tom40$_{\Delta 1-278}$, demonstrating that it was accumulated at SAM (Figure 2E, lane 4). Thus, the C-terminal 109 amino acid residues contain sufficient and necessary information for directing Tom40 to SAM.

We performed a detailed residue per residue truncation of Tom40 from the C terminus. Deletion of the C-terminal 27 residues (361–387) did not impair SAM targeting and membrane insertion (intermediate-II), only the final maturation to the mature TOM complex was delayed (Figure 2F). However, the additional deletion of residues 360 and 359 severely affected the sorting of Tom40. Upon lack of Glu360, the precursor was still targeted to SAM, yet formation of assembly intermediate-II was inhibited (Figure 2F). Deletion of Phe359 blocked sorting to SAM and the further assembly steps of Tom40 (Figure 2F). Residues 359 and 360 are located in the C-terminal region of the predicted last transmembrane β-strand of Tom40. A deletion of N-terminal residues of this predicted β-strand also inhibited sorting of Tom40 to SAM (Figure 2G). A detailed search using different mitochondrial

β-barrel proteins led to identification of a conserved sequence located in the last predicted transmembrane β-strand (Figure 2H). Characteristic features of the consensus sequence, termed β-signal, are a large polar residue (predominantly lysine or glutamine), an invariant glycine, and two large hydrophobic residues.

Two-Step Sorting of Tom40

We replaced each of the four conserved residues of the Tom40 β-signal by alanine. The radiolabeled constructs were incubated with isolated mitochondria and analyzed by BN-PAGE. Tom40 precursors lacking Lys352, Leu357, or Phe359 were significantly inhibited in formation of the SAM intermediate and the subsequent assembly steps (Figures 3A–3C, lanes 4–6). The initial targeting of these constructs to mitochondria occurred with an efficiency close to that of the WT precursor (Figures 3A–3C, bottom panels). We replaced conserved polar and large hydrophobic residues in other predicted β-strands of Tom40. Each of the precursors with amino acid replacements outside the last β-strand still formed the SAM intermediate (Figures 3D and 3E), supporting a critical function of the residues in the last β-strand for Tom40 sorting to SAM.

When Gly354 of the β-signal was replaced by alanine, the precursor accumulated at SAM while membrane insertion at intermediate-II and maturation to the mature TOM complex were inhibited (Figure 3F). We replaced all other invariant glycines of Tom40. Replacement of Gly83 and Gly212, which are located in predicted transmembrane β-strands, also led to a SAM arrest, while replacement of Gly129 (interstrand loop) did not inhibit Tom40 import and assembly (Figure 3F). WT Tom40 accumulated at SAM is extracted at alkaline pH since it is not inserted into the lipid phase of the membrane, while fully assembled Tom40 in the mature TOM complex as well as a major fraction of intermediate-II are resistant to extraction at pH 11.5, indicating integration into the lipid phase (Figure 3G, lane 2; Model et al., 2001). $Tom40_{G354A}$ and $Tom40_{G83A}$, accumulated at the SAM complex, were extracted at pH 11.5, while $Tom40_{G129A}$ was efficiently integrated into the TOM complex and not extracted (Figure 3G), demonstrating that the β-strand glycines were critical for membrane insertion.

We conclude that the four conserved residues of the β-signal are involved in distinct steps. The three large residues are required for sorting to SAM, while the subsequent membrane insertion depends not only on the β-signal but also on other regions of Tom40. The conserved glycine residues in β-strands of Tom40 are required for membrane insertion.

General Role of the β-Signal for Biogenesis of β-Barrel Proteins

We analyzed the critical residues of the β-signal in two further β-barrel proteins, Mdm10 and porin, which do not show sequence homology to Tom40 besides the similarity in the β-signal. Stable SAM intermediates have not been reported for yeast β-barrel proteins other than Tom40. Therefore assembly of the radiolabeled proteins was monitored by formation of the mature complexes on BN-PAGE (Paschen et al., 2003; Wiedemann et al., 2003; Gentle et al., 2004). We generated C-terminal truncations of both precursors. When the truncations included the last hydrophobic residue of the β-signal (Tyr491 of Mdm10, Phe281 of porin), formation of the mature complexes was blocked (Figures 4A and 4B, upper panels) while the mutant precursors were still targeted to mitochondria (Figures 4A and 4B, lower panels). (In case of porin and Tom40, deletion of residues C-terminal to the β-signal delayed formation of the mature complexes [Figures 2F and 4B], suggesting that these residues were involved in membrane integration or assembly.) The predicted β-signals of Mdm10 and porin, like the one of Tom40, possess one conserved polar residue (Lys484 of Mdm10/Lys274 of porin) and two conserved hydrophobic residues (Phe489 and Tyr491/Leu279 and Phe281) (Figure 2H). Replacement of these residues impaired formation of the mature complexes (Figures 4C and 4D, upper panels), demonstrating that the conserved residues of the β-signal are crucial for β-barrel biogenesis.

We also studied the role of the invariant glycine 276 of the porin β-signal. Replacement by alanine delayed the formation of mature porin complexes and led to formation of an additional form at 250 kDa (Figure 4E, upper panel). Replacement of the glycine by isoleucine blocked formation of mature porin and led to accumulation of the 250 kDa form after a short-term import (and turnover after longer import) (Figure 4E, upper panel). We suspected that the 250 kDa form may represent accumulation of porin at SAM and used *sam37*Δ mitochondria, as well as $Sam35_{HA}$ mitochondria, for analysis. $Porin_{G276A}$ was indeed found in a smaller form in *sam37*Δ mitochondria in agreement with the size reduction of the SAM complex (Figure 4F; Wiedemann et al., 2003). In $Sam35_{HA}$ mitochondria, accumulated $porin_{G276I}$ was shifted by anti-HA-antibodies like SAM-accumulated Tom40 while the mature porin complex was not shifted (Figure 4G). In addition, mitochondria carrying accumulated $porin_{G276I}$ were incubated with the crosslinking agent 1,4-di-[3′-(2′-pyridyldithio)propionamido]butane (DPDPB). One of the crosslinking products was shifted in size when mitochondria containing a Protein A-tag at Sam50 were used (Figure 4H, lanes 7 and 8), demonstrating that accumulated porin was in close proximity to Sam50 (the yield of this crosslinking product was significantly lower with WT porin precursor; Figure 4H, lanes 3 and 4). Thus, like for Tom40, the invariant glycine of the porin β-signal is not required for recognition by SAM but for subsequent release of the precursor.

To obtain further evidence that the β-signal was crucial for directing β-barrel sorting we first performed signal-swapping experiments. The β-signal of porin, as well as that of Mdm10, rescued SAM sorting of a Tom40 construct lacking its endogenous signal (Figure 5A). Similarly, the β-signal of Tom40 conferred SAM sorting to a porin construct lacking the endogenous signal (Figure 5B). Second, we searched for mutations in the β-signal that enhanced the sorting yield over WT efficiency. We found that replacement of Phe359 of Tom40 by Tyr stimulated SAM binding and increased the formation of the subsequent assembly steps by 85%–90% (Figure 5C), providing positive evidence for the importance of the β-signal. In contrast, replacement of this residue by Gln completely blocked formation of the SAM intermediate (Figure 5C). Third, we synthesized a peptide containing the β-signal of Tom40 and a control peptide containing the same amino acid residues in scrambled order (Figure 5D). The β-signal peptide, but not the scrambled peptide, inhibited formation of the SAM intermediate of Tom40 (Figure 5E, upper panel) but

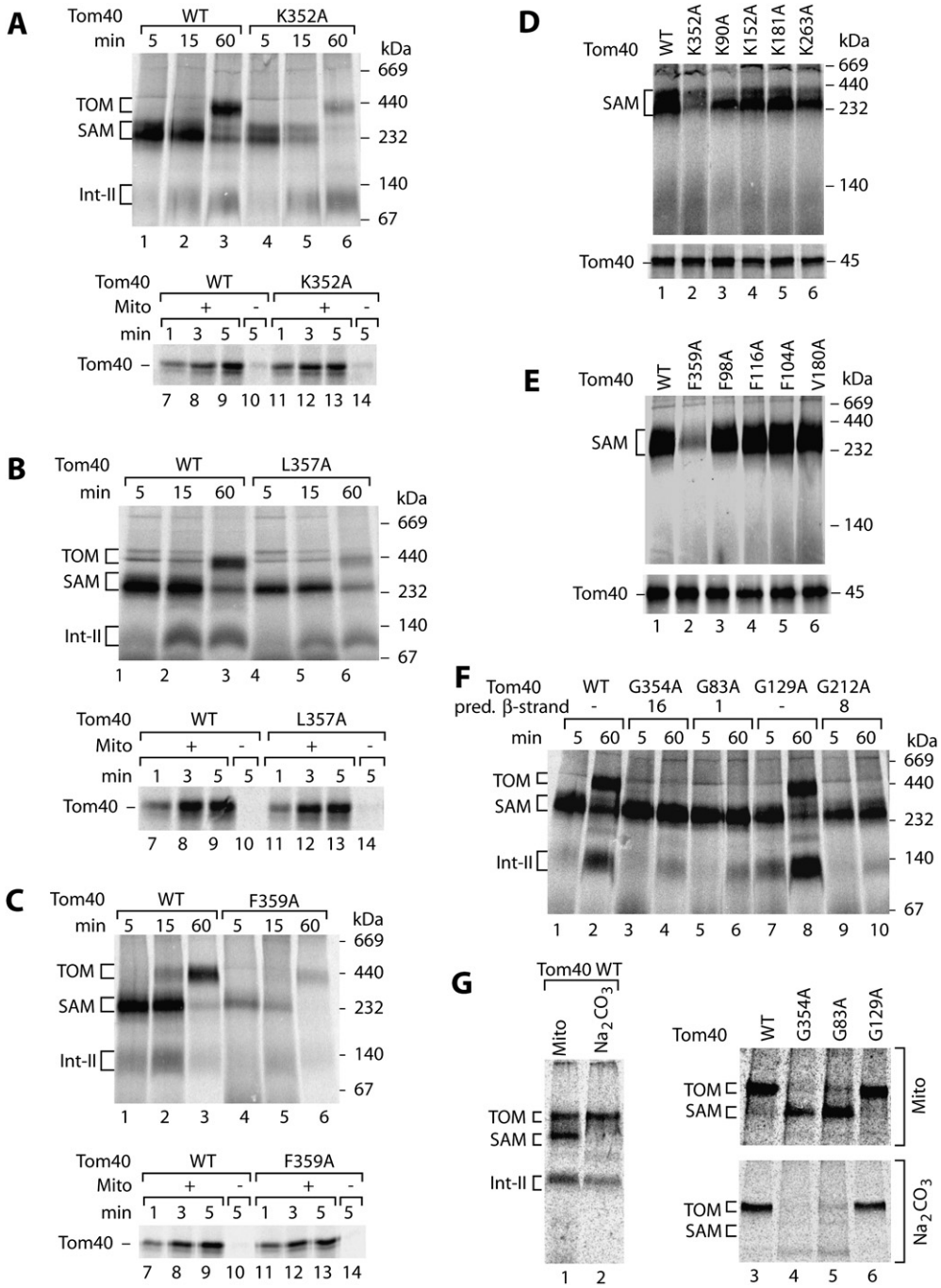

Figure 3. Conserved β-Signal Residues Are Required for SAM Binding and Assembly of Tom40

(A, B, and C) [^{35}S]Tom40 precursors were incubated with WT mitochondria and analyzed by BN-PAGE (top) and SDS-PAGE (bottom).

(D and E) Mitochondria were incubated with precursors for 5 min and separated by BN-PAGE (top). Loading control was analyzed by SDS-PAGE (bottom).

(F) Tom40 precursors were imported as in (A).

(G) Tom40 precursors were imported into WT mitochondria. Mitochondria were either directly separated by BN-PAGE or after treatment with sodium carbonate, pH 11.5.

not the targeting of Tom40 to mitochondria (Figure 5E, lower panel). A peptide, in which Phe359 was replaced by Gln (Figure 5D), did not inhibit formation of the SAM intermediate (Figure 5E, upper panel, lanes 8–10). Importantly, binding of porin$_{G276I}$ to SAM was also inhibited by the Tom40 β-signal peptide but not the control peptides (Figure 5F), underscoring the general function of the β-signal for β-barrel sorting. None of the other major mitochondrial protein import pathways were

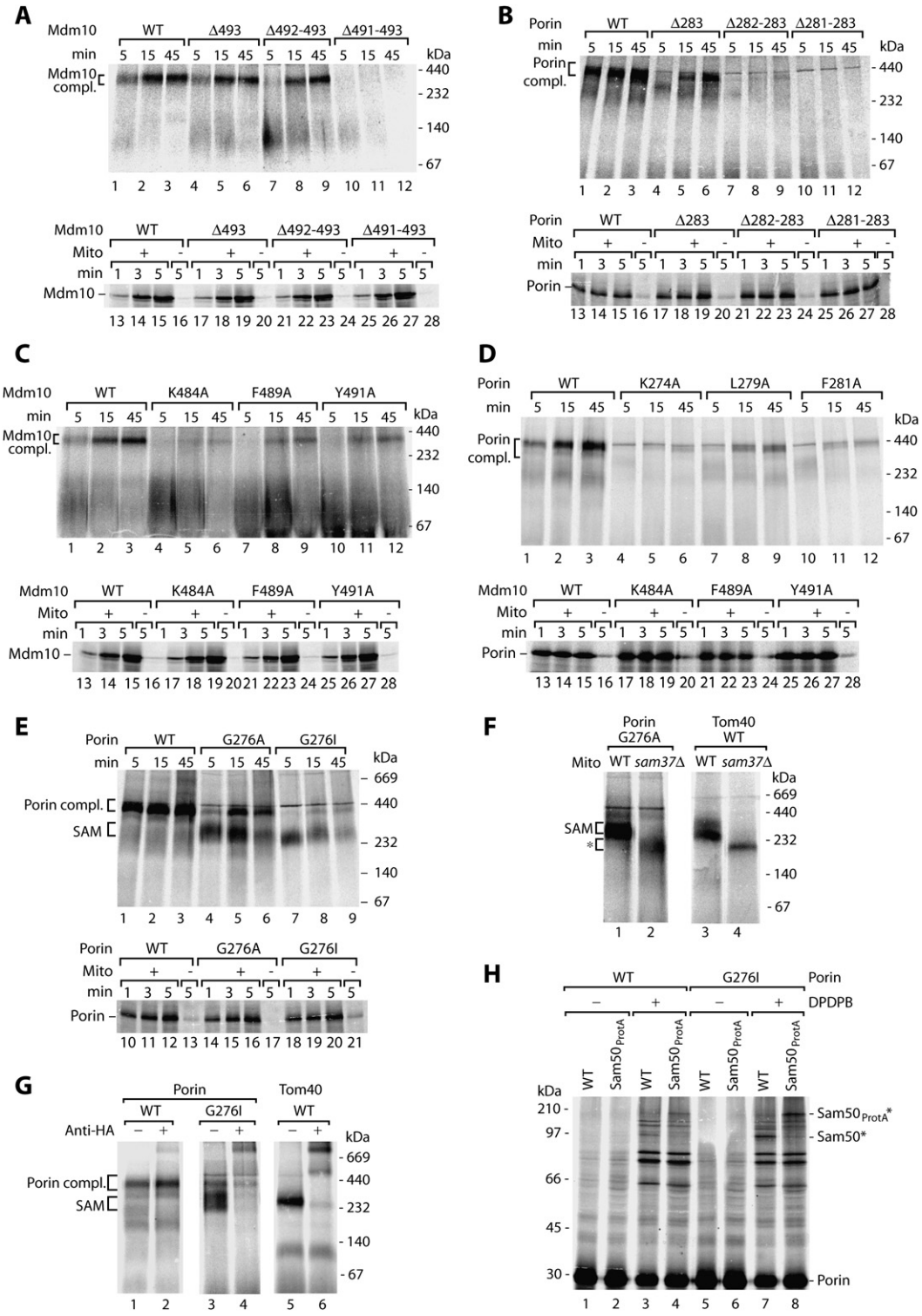

Figure 4. Mitochondrial β-Signal Is Functionally Conserved

(A–E) [^{35}S]precursors of Mdm10 and porin were incubated with isolated WT mitochondria and analyzed by BN-PAGE (upper panels) and SDS-PAGE (lower panels).

(F) Porin$_{G276A}$ and Tom40 were incubated with WT and *sam37*Δ mitochondria for 5 min, followed by BN-PAGE. Asterisk, SAM intermediate lacking Sam37.

(G) Porin, Porin$_{G276I}$, and Tom40 were incubated with Sam35$_{HA}$ mitochondria for 5 min. HA antibody was added where indicated, followed by BN-PAGE.

(H) Porin and Porin$_{G276I}$ were imported into WT and Sam50$_{ProtA}$ mitochondria for 5 min, followed by addition of DBDPB and SDS-PAGE. Asterisks, crosslinking products.

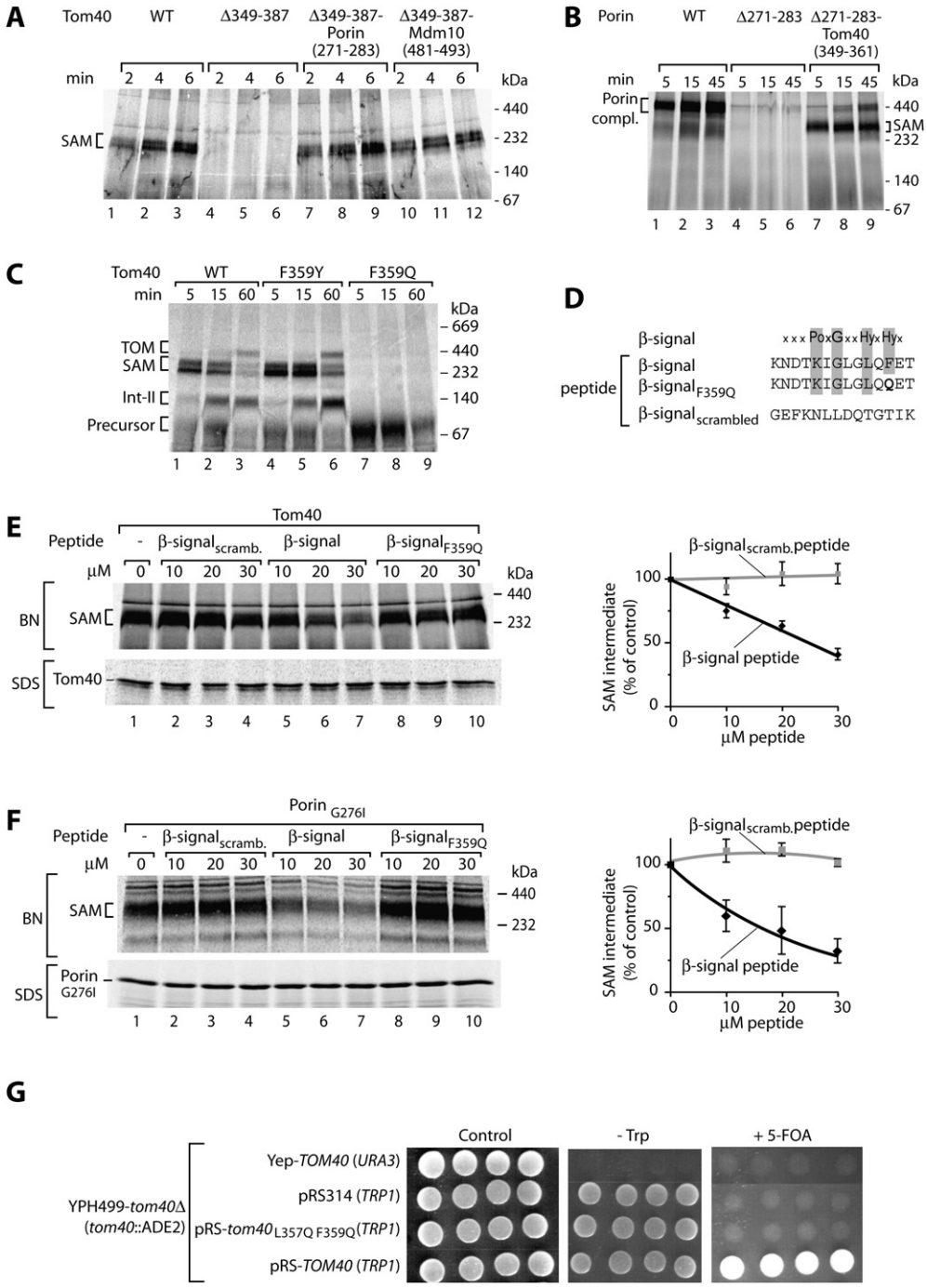

Figure 5. Specificity of the β-Signal

(A and B) Hybrids of Tom40, porin, and Mdm10 were imported into WT mitochondria and analyzed by BN-PAGE.

(C) Tom40 precursors were imported and analyzed by BN-PAGE.

(D) β-signal peptide derived from Tom40 (residues 348–361; Cys replaced by Leu), mutant peptide, and scrambled control peptide.

(E and F) Mitochondria were incubated with Tom40 and porin$_{G276I}$ in the presence of peptide for 5 min, followed by BN-PAGE. Quantification by digital autoradiography; data are represented as means ± SEM, n = 6 (E), n = 3 (F).

(G) Growth of WT and Tom40$_{L357Q\ F359Q}$ yeast cells. Trp, tryptophane.

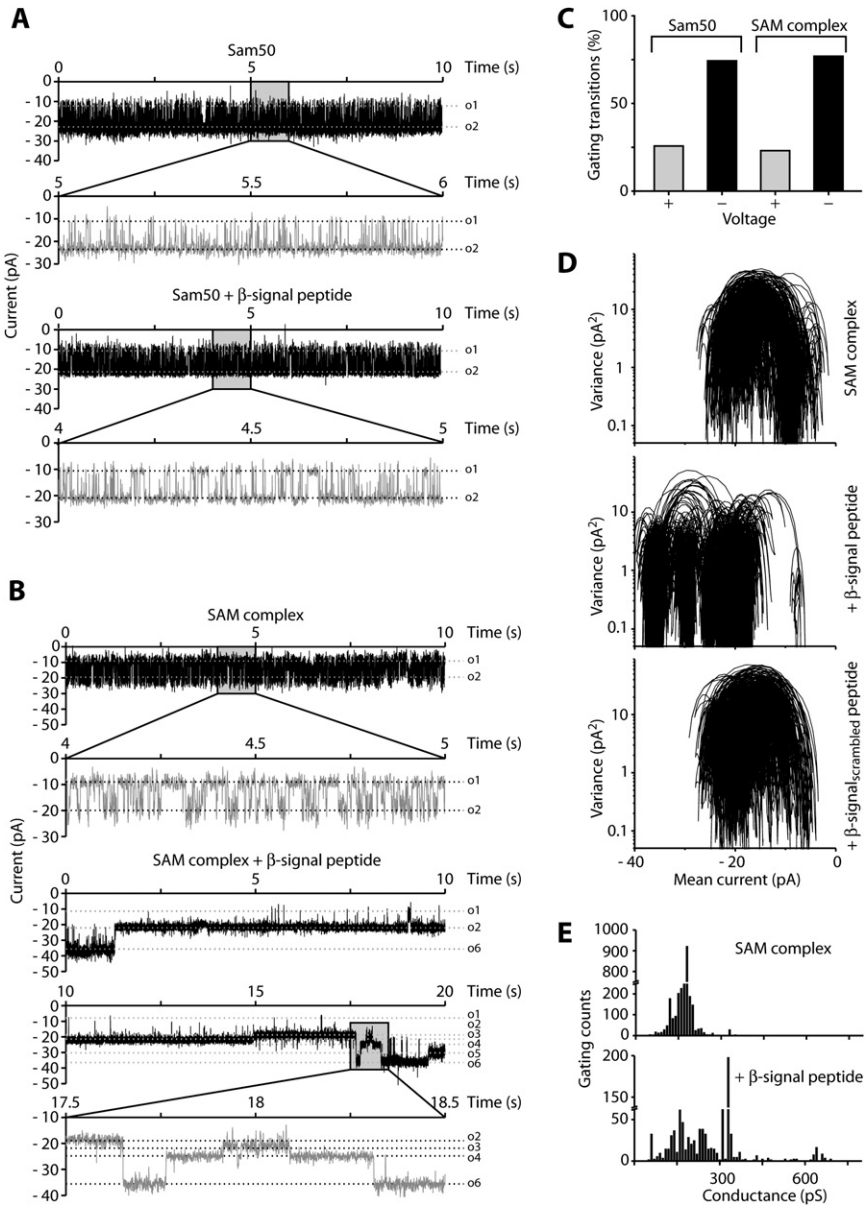

Figure 6. The SAM Complex but Not Purified Sam50 Forms a β-Signal-Sensitive Channel

(A) Single-channel current recordings of Sam50 ± β-signal peptide at a holding potential of −60 mV.

(B) Current recordings of a single SAM complex.

(C) Gating transitions in main conductance (50–250 pS) of Sam50 and SAM complex depend on the direction of the electric field (referring to transcompartment; $n_{gating} > 1500$).

(D) Purified SAM complex: mean current differences plotted against their variances.

(E) Conductance distribution of the SAM complex.

impaired by the β-signal (Figure S1 available online). We conclude that the β-signal is necessary and specific for sorting of outer membrane β-barrel proteins.

To determine if the Tom40 β-signal was essential in vivo, we performed a plasmid shuffling experiment. A haploid yeast strain with a chromosomal deletion of its *TOM40* gene was viable as long as Tom40 was expressed from a plasmid, but not when Tom40 containing a mutant β-signal was expressed (Figure 5G), demonstrating that the Tom40 β-signal is essential for cell viability.

Response of the Sam50 Channel to the β-Signal Requires a Further SAM Subunit

Recombinant Sam50 was purified and reconstituted into liposomes. Upon fusion with a planar lipid bilayer, a channel activity was observed (Figure 6A). The reversal potential of the channel was 30 mV, reflecting a slight preference for cations (K^+/Cl^- of 4:1). Addition of the β-signal peptide did not exert a major effect on the activity of the Sam50 channel (Figure 6A). We thus purified the SAM complex and reconstituted it into liposomes. Upon fusion with a planar lipid bilayer, we observed a channel with the same basic characteristics as recombinant Sam50 (E_{rev} = 30 mV; P_{K^+}: P_{Cl^-} = 4:1) (Figure 6B). Recombinant Sam50 and SAM complex showed the same asymmetric dependence on the direction of the electric field (Figure 6C), indicating that both were reconstituted in the same orientation. Addition of the β-signal peptide significantly altered the activity of the SAM complex channel by reducing the gating frequency (Figures 6B) and inducing the formation of channel activities with higher conductance (Figures 6D and 6E). As control, the scrambled peptide did not alter the channel properties of the SAM complex (Figure 6D). The major conductance of the SAM complex of 160 pS in the absence of the β-signal peptide was shifted to 320 pS in the presence of the β-signal (Figure 6E). Additionally, a less frequent large conductance state of ~640 pS was observed only in the presence of the β-signal (Figure 6E). As purified Sam50 did not show this reactivity to the β-signal peptide, we conclude that a further subunit of the SAM complex is required for regulation of the SAM channel by the β-signal.

Role of Sam35 in Recognition of the β-Signal

In order to identify the β-signal binding subunit(s) of the SAM complex we fused the C-terminal 51 residues of Tom40, including the β-signal, to glutathione-S-transferase (GST). The fusion protein was bound to sepharose beads and incubated with lysed mitochondria. Sam50 and Sam35 efficiently bound to GST-Tom40C but not to GST, while Sam37 and various control proteins did not bind (Figure 7A). To probe for the specificity of interaction, we

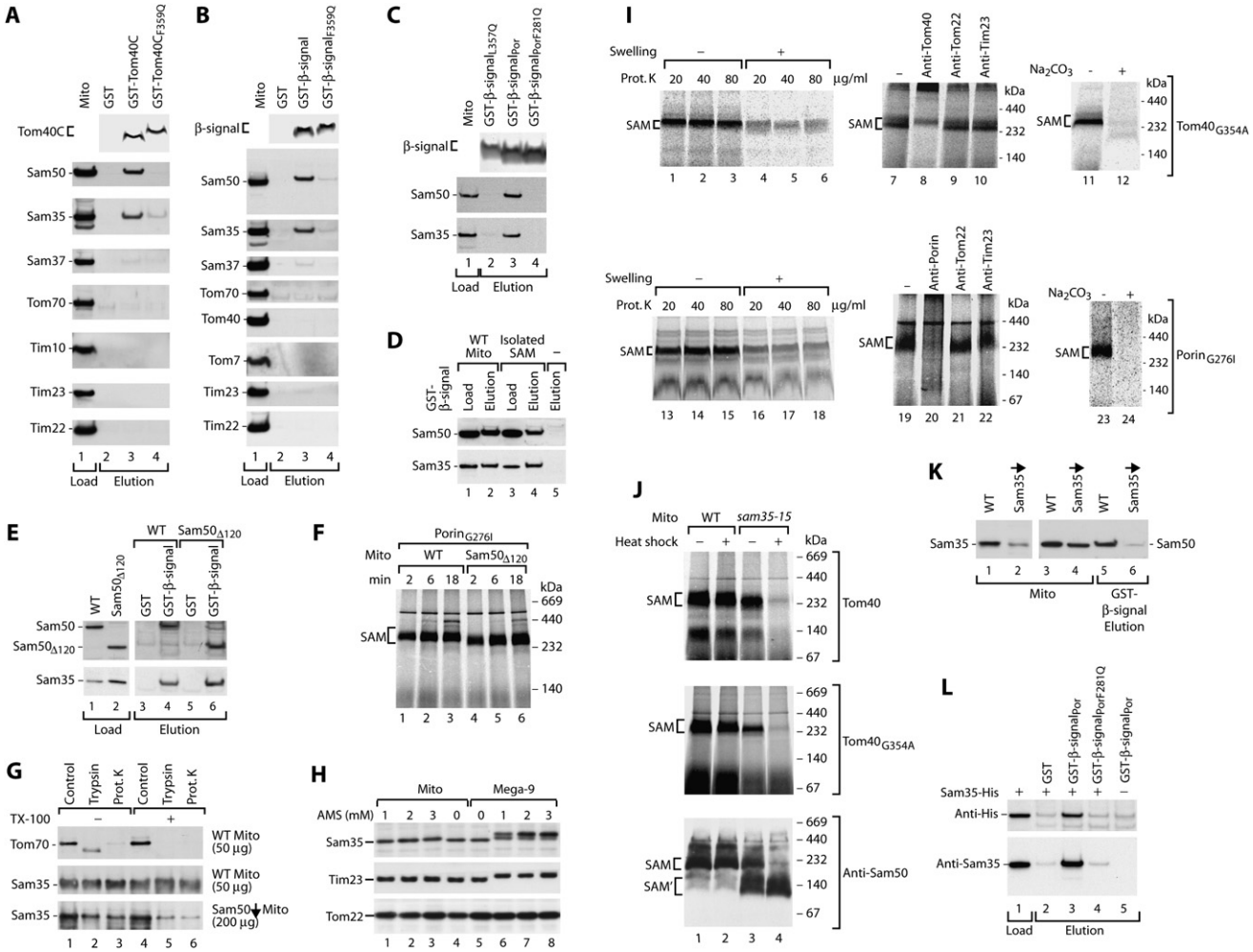

Figure 7. Sam35 Recognizes the β-Signal

(A) GST-Tom40C (Tom40 residues 337–387) was incubated with digitonin-lysed WT mitochondria. The C terminus of Tom40 was eluted and analyzed by SDS-PAGE and Coomassie blue staining (top panel) or immunodecoration.

(B and C) β-signal GST-fusion proteins (Tom40 or Por1) were analyzed as in (A).

(D) GST-β-signal was incubated with digitonin-lysed WT mitochondria or isolated SAM complex.

(E) Binding of Sam35 and Sam50 to GST-β-signal analyzed with WT and $Sam50_{\Delta 120}$ mitochondria.

(F) WT and $Sam50_{\Delta 120}$ mitochondria were incubated with $Porin_{G276I}$, followed by BN-PAGE.

(G) WT and Sam50-depleted mitochondria were treated with trypsin (33 μg/ml) and proteinase K (5 μg/ml) with and without Triton X-100 lysis. μg, mitochondrial protein.

(H) WT mitochondria resuspended in SM buffer or solubilized in Mega-9 were incubated with AMS and separated by SDS-PAGE.

(I) Import of $Tom40_{G354A}$ and $Porin_{G276I}$ into mitochondria. Left panels, incubation in isotonic or hypotonic buffer in the presence of proteinase K. Middle panels, incubation with antibodies. Right panels, treatment with 0.1 M Na_2CO_3. All samples were lysed in digitonin and analyzed by BN-PAGE.

(J) WT and *sam35-15* yeast were grown at 24°C. Isolated mitochondria were heat-shocked for 15 min at 37°C and incubated with precursors for 15 min at 25°C. Analysis was by BN-PAGE and autoradiography (top and middle panels) or immunodecoration (bottom panel). SAM′, smaller SAM complex.

(K) Binding of Sam50 to GST-β-signal was analyzed with WT mitochondria (25 μg protein) and Sam35-depleted mitochondria (100 μg protein).

(L) GST-β-signal constructs were incubated with $Sam35_{His}$.

replaced Phe359. The binding of Sam50 and Sam35 to the mutant form of GST-Tom40C was inhibited (Figure 7A). We then fused the segment corresponding to the Tom40 β-signal to the C terminus of GST. The resulting fusion protein (GST-β-signal) selectively bound Sam50 and Sam35 like GST-Tom40C; replacement of Phe359 or Leu357 inhibited the interaction (Figures 7B and 7C). Similarly, the porin β-signal bound Sam50 and Sam35 while replacement of Phe281 inhibited the binding (Figure 7C). Thus, the β-signal is sufficient for specific binding of Sam proteins and selectively pulls out a core complex containing Sam35 and Sam50. The purified SAM complex similarly bound to the β-signal (Figure 7D), demonstrating that no other mitochondrial proteins were needed for the interaction.

To directly test that the POTRA domain of Sam50 was not required for recognition of β-barrel proteins, we performed the GST-β-signal pulldown assay with mitochondria from $Sam50_{\Delta 120}$

yeast. The truncated Sam50, as well as Sam35, efficiently bound to GST-β-signal (Figure 7E), demonstrating that recognition of the Tom40 β-signal does not depend on the POTRA domain. Moreover, to test for the recognition of porin by the SAM complex, we used the porin precursor with replaced Gly276. The precursor accumulated at the SAM complex of $Sam50_{\Delta 120}$ mitochondria as in WT mitochondria (Figure 7F). Thus, the POTRA domain of Sam50 is not critical for recognition of the β-signal.

Since electrophysiological and biochemical analyses indicated that Sam50 does not function as β-signal receptor, we reasoned that Sam35 is crucial for signal recognition. However, it has been assumed that Sam35 is located on the cytosolic surface of the outer membrane (Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004), while β-barrel precursors are inserted into the outer membrane from the intermembrane space side (Model et al., 2001; Wiedemann et al., 2003; Hoppins and Nargang, 2004), raising a topological problem with regard to recognition of the β-signal. Sam35 does not contain a predicted transmembrane segment; its secondary structure includes predominantly α-helical elements and a small content of β-structure. Sam35 remains membrane-associated upon sonication but is extracted from the outer membrane at alkaline pH, suggesting that it is not integrated into the lipid phase but associated with the membrane by interaction with other proteins. When Sam35 was expressed with a tag at either the N or C terminus, low concentrations of protease added to isolated mitochondria digested the tags like surface-exposed Tom receptors, supporting the view that Sam35 is located on the cytosolic surface of mitochondria (Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004). We noted, however, that significantly higher concentrations of protease were needed to digest nontagged Sam35 and that Sam50 also became accessible to protease (Waizenegger et al., 2004; Habib et al., 2005). Thus the studies with tagged Sam35 only demonstrated an exposure of both termini at the cytosolic surface. It has been shown that some proteins, which are extracted at alkaline pH, are integrated into membranes by being embedded in a proteinaceous environment (Truscott et al., 2003). As our pulldown experiments indicated a close association of Sam35 and Sam50, we speculated that Sam35 may be embedded into a proteinaceous membrane environment via its tight interaction with Sam50. We used antibodies recognizing untagged Sam35 and treated mitochondria with protease under conditions that degraded the surface receptor Tom70. Sam35 was not digested by the proteases, even after lysis of the membranes with detergent (Figure 7G, middle panel). We isolated mitochondria from a yeast strain where the *SAM50* gene was placed under an inducible promoter such that the mitochondrial levels of Sam50 were reduced to ~5% of WT level. The levels of Sam35 were reduced to ~25%–30%. Under these conditions Sam35 became partially accessible to proteases and was digested upon detergent lysis (Figure 7G, lower panel). Thus, Sam35 is protected against proteolytic attack by Sam50 both in mitochondria and detergent micelles. As Sam50 does not expose domains on the cytosolic side of the membrane but consists of an outer membrane-embedded β-barrel domain and a hydrophilic domain in the intermembrane space (Habib et al., 2007), we conclude that Sam35 is embedded into the outer membrane by its close association with the membrane domains of Sam50 molecules.

To obtain independent evidence for a protected localization of Sam35, we used the membrane-impermeable cysteine-modifying reagent 4-aceto-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; 500 Da). When mitochondria were lysed with the nonionic detergent nonanoyl-N-methylglucamide (Mega-9), leading to dissociation of the SAM complex, the size of Sam35 shifted significantly in the presence of AMS, indicating that the majority of its eight cysteines were modified (Figure 7H, lanes 6–8) (Mega-9 is a detergent of choice for renaturation of membrane proteins, and mitochondrial membrane proteins were shown to retain their activity in Mega-9; Becker et al., 2005). In mitochondria, however, only a minor shift of Sam35 was observed (Figure 7H, lane 3), demonstrating that most cysteines were not accessible to AMS when the outer membrane was intact. As control we show that the cysteine-free Tom22 was not affected by AMS and the inner membrane protein Tim23 was only modified by AMS in Mega-9 (Figure 7H). Thus most cysteines of Sam35 only became accessible to AMS when the outer membrane was lysed.

To study the topology of precursors in the SAM complex, we accumulated the Gly mutants of Tom40 and porin. The precursors were protected against added proteinase K in mitochondria but became accessible upon swelling (Figure 7I, lanes 1–6 and 13–18) (swelling exposes the intermembrane space of a majority of mitochondria). Thus the precursors exposed protease-accessible sites to the intermembrane space but not to the cytosolic side in agreement with studies of WT Tom40 (Model et al., 2001). We speculated that the β-signal is bound to Sam35 and thus may span through the SAM complex and potentially be exposed at the cytosolic side. We thus searched for independent means to probe for an accessibility of the precursor from the cytosolic side and succeeded with antibody-shift BN-PAGE (Wiedemann et al., 2003). Polyclonal antibodies directed against Tom40 and porin, which were added to the intact mitochondria, efficiently shifted the SAM intermediate while control antibodies against Tom22 and Tim23 did not (Figure 7I, lanes 8–10 and 20–22). Thus the accumulated precursors exposed antigenic epitopes at the cytosolic side. According to our prediction the precursors would be bound to Sam35 and thus should traverse the outer membrane within the SAM complex in a hydrophilic environment. Indeed, the precursors were fully extracted at alkaline pH (Figure 7I, lanes 12 and 24) whereas the mature assembled proteins were resistant to extraction at pH 11.5 (Figure 3G; Court et al., 1996; Model et al., 2001).

We generated two different *sam35* yeast mutants to further test the proposed role of Sam35 in β-recognition. First, we generated the temperature-sensitive yeast mutant strain *sam35-15* that led to a partial dissociation of the 210 kDa SAM complex such that a defined Sam50 complex (~150 kDa) was detectable on BN-PAGE (Figure 7J). The mutant cells were grown at low temperature to minimize indirect effects and the isolated mitochondria were subjected to a short heat-shock treatment, leading to dissociation of SAM. The precursor of Tom40 as well as $Tom40_{G354A}$ did not accumulate in the smaller Sam50-containing complex (Figure 7J). Second, we generated a yeast strain with *SAM35* under an inducible promoter and isolated mitochondria with strongly reduced levels of Sam35 upon shift of the cells to noninducing conditions (the levels of Sam50 were partially reduced). We tested if the remaining Sam50 was able to bind

to the purified β-signal. The pulldown assay with GST-β-signal revealed that binding of Sam50 to the β-signal was blocked upon depletion of Sam35 (Figure 7K), indicating that Sam50 itself was not able to bind to the β-signal but that Sam35 was required.

To directly demonstrate β-signal binding by Sam35, we expressed a His-tagged Sam35 in *E. coli*. $Sam35_{His}$ indeed bound to the GST-β-signal (Figure 7L, lane 3). When Phe281 was replaced, the binding was inhibited (Figure 7L, lane 4), demonstrating that recombinant Sam35 specifically recognized the β-signal.

DISCUSSION

We have identified the sorting signal of mitochondrial β-barrel proteins and show that Sam35, but not Sam50, functions as a receptor that specifically recognizes the β-signal. Since the SAM complex is required for the biogenesis of Tom40, which forms the central entry gate for hundreds of different mitochondrial precursor proteins (Wiedemann et al., 2003; Dolezal et al., 2006; Neupert and Herrmann, 2007), the function of Sam35 in recognizing the Tom40 β-signal provides the molecular basis for understanding its essential role in cell viability. The β-signal is conserved in mitochondrial β-barrel proteins from all eukaryotic kingdoms and forms the most C-terminal β-strand of the proteins. By detailed mutational analysis, competition with a synthetic β-signal peptide, and pulldown experiments with purified β-signal, we demonstrate that the β-signal is necessary and sufficient for selective recognition by the SAM complex, specifically a core complex formed by Sam35 and Sam50. The channel formed by purified Sam50 does not respond to the β-signal since Sam35 is crucial for recognition of the signal. In the presence of Sam35, the β-signal induces a change in the gating properties of the channel and a significant increase in channel conductance of the SAM complex. Comparison to the conductance and size of channels formed by TOM and TIM proteins, as well as chloroplast Toc75 (Kovermann et al., 2002; Becker et al., 2005; Schleiff and Soll, 2005), indicates that the signal-induced large SAM channel can accommodate several β-strands. In the case of TOM and TIM channels, addition of the specific signals leads to stimulation of the channels but not to the induction of larger pore sizes, whereas the β-signal apparently induces a rearrangement of the SAM channel such that several β-strands together can be inserted. This fits to the view that β-barrel precursors are not inserted into membranes as individual strands but contain a considerable amount of partially folded elements (Rapaport and Neupert, 1999).

The precursors of Tom40 and porin accumulated at SAM span across the outer membrane in a hydrophilic environment since the precursors are extracted from the membranes at alkaline pH and expose protease-accessible sites to the intermembrane space and antigenic epitopes on the cytosolic surface of the outer membrane. It is currently debated both for mitochondria and bacteria whether precursor proteins are translocated by a channel formed within an individual Sam50/Omp85 β-barrel or by a central channel formed between several Sam50/Omp85 molecules in an oligomeric complex (Paschen et al., 2003; Johnson and Jensen, 2004; Ryan, 2004; Tommassen, 2007). A lateral release of a precursor, which has been inserted into a channel formed by a single β-barrel protein, into the lipid phase would be highly unlikely since an opening of the numerous hydrogen bonds that stabilize β-barrel proteins is thermodynamically very unfavorable (Johnson and Jensen, 2004; Ryan, 2004). The findings reported here provide important new information since Sam35 is essential for recognition of the last β-strand of the precursor; however, Sam35 is not integrated into the lipid phase of the outer membrane but embedded into a proteinaceous membrane environment by its close association with Sam50 molecules. We propose the hypothesis that translocation of β-barrel precursors into SAM is initiated by binding of the last β-strand (β-signal) to the hydrophilic and predominantly α-helical Sam35 located in an oligomeric ring formed by the β-barrel domains of Sam50 molecules. Signal binding induces a conformational change that leads to opening of the SAM channel, and thus several β-strands can be inserted into a hydrophilic, proteinaceous membrane environment. Subsequently, the precursor is laterally released from the SAM complex into the lipid phase of the outer membrane. Membrane integration strictly requires highly conserved glycine residues in the β-signal and further β-strands. Since glycine residues provide a high flexibility of the polypeptide chain, this step seems to involve conformational changes of the precursor to ensure correct integration into the lipid phase.

The identification of the β-signal and the essential role of Sam35 in signal recognition provide a clarification for several previous studies. (1) Numerous mutational studies of mitochondrial β-barrel proteins suggested that regions important for β-barrel biogenesis are distributed over various regions of the precursors (Court et al., 1996; Rapaport and Neupert, 1999; Rapaport et al., 2001; Taylor et al., 2003; Humphries et al., 2005; Sherman et al., 2006), leading to the suggestion that a linear consensus signal does not exist (Habib et al., 2007). The dissection of the sorting process into two steps, recognition by SAM and subsequent membrane insertion, provided the basis for identification of the β-sorting signal that is responsible for SAM binding, while the subsequent membrane insertion involves several regions of the β-precursors. (2) The N-terminal POTRA domain of Sam50 is not critical for recognition of β-barrel proteins as deletion of the complete POTRA domain did not impair cell growth and β-barrel precursors were sorted with an efficiency close to that of WT mitochondria. The reported defects in cell growth and β-barrel sorting upon partial deletion of the POTRA domain (Habib et al., 2007) are apparently caused by a negative influence of the remaining POTRA segment on precursor transport. These findings will also be interesting for the ongoing discussion on the functional importance of the five bacterial POTRA domains and the question of which domains are essential for cell viability (Bos et al., 2007; Kim et al., 2007).

A direct comparison of the mitochondrial β-signal and the C-terminal signature sequence of bacterial β-barrel proteins (Robert et al., 2006) reveals why the mitochondrial signal could not be found by homology searches although it likely evolved from the bacterial signal (Figure S2). Both signals are formed by the last predicted β-strand and include a large hydrophobic residue at the end of the β-strand. However, this hydrophobic residue forms the last C-terminal residue of the vast majority of bacterial β-barrel proteins, while in mitochondria the residue never represents the last residue. The additional conserved residues of the signals differ between mitochondria and bacteria.

We speculate, however, that the basic principle of membrane insertion has been conserved during evolution from Gram-negative bacteria to mitochondria. It is thus conceivable that the mechanism found for mitochondrial β-barrel sorting, i.e., recognition and initial insertion via a hydrophilic SAM pathway followed by integration into the lipid phase, may also be used by bacterial β-barrel proteins and the Omp85/YaeT complex.

EXPERIMENTAL PROCEDURES

Molecular Biological Methods

Porin, Mdm10, and Tom40 templates were obtained from yeast genomic DNA. Mutants were obtained using the QuickChange II Site-Directed Mutagenesis Kit. *SAM50* was amplified from genomic DNA and cloned into pFL39. $Sam50_{\Delta 29\text{-}120}$ and $Sam50_{\Delta 120}$ were generated using pFL39-*SAM50* as template and transformed into a *sam50* deletion strain (Kozjak et al., 2003) followed by plasmid shuffling on 5-FOA plates. *SAM50* was also cloned into pRS413, and $Sam50_{\Delta 120}$ and $Sam50_{\Delta 102}$ were generated by inverse PCR. WT *TOM40* was cloned into pRS314 and used as template to create pRS-$Tom40_{L357Q\ F359Q}$. Constructs were transformed into a *TOM40* deletion strain covered by a plasmid encoding WT Tom40 (Yep-*TOM40*). *sam35* mutants were generated by error-prone PCR (Milenkovic et al., 2004). For regulated expression, genes were placed under the *GAL1* promoter. For generation of GST-Tom40C the nucleotide sequence of Tom40 (amino acids 337–387) was amplified from genomic DNA, cloned into pGEX-4T-1, and used to create GST-β-signal (including N-terminal linker residues). For generation of GST-$\beta\text{-signal}_{Por}$ the Porin1 ORF was cloned into pGEX-4T-1 and used as template.

Import and Assembly of Precursor Proteins

Proteins were synthesized in reticulocyte lysate in presence of [^{35}S]methionine. Equal radiochemical amounts of precursors were incubated with isolated mitochondria in import buffer (3% (w/v) BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 5 mM L-methionine, 2 mM KH_2PO_4, 10 mM MOPS-KOH, pH 7.2, 2 mM NADH, 2–4 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase) at 25°C. Synthetic peptides, blocked N-terminally by acetylation and C-terminally by amidation, were solubilized in water.

In Vitro Binding Assays

GST fusion proteins were isolated after 2 hr induction with 1 mM IPTG in DH5α cells in LB 100 μg/ml ampicillin medium at 30°C, cell lysis (0.5 g), and glutathione sepharose 4B affinity chromatography. The sepharose-bound GST proteins were incubated for 30 min at 4°C with 1% (w/v) digitonin or Triton X-100 solubilized mitochondria (1 mg protein in 10 mM Mg-$acetate_2$, 100 mM K-acetate, 20 mM HEPES, 10% glycerol). After washing, bound mitochondrial proteins were eluted by thrombin cleavage (10 U, 16 hr, 4°C). Load was 50% for mitochondrial extracts and 20% for purified proteins; elution was 100%.

Reconstitution in Liposomes and Electrophysiological Measurements

Sam50 was expressed in *E. coli*, purified in 8 M urea, and diluted in 1% (w/v) SDS, 100 mM NaCl, 10 mM MOPS-Tris (pH 7). By addition of soybean phosphatidylcholine, mixed detergent/lipid/protein micelles with a protein:lipid ratio of 1:30 were formed. Detergent was removed by Calbiosorb Adsorbent. SAM complex was isolated from digitonin-lysed mitochondria containing Sam50 with Protein A-tag and TEV cleavage site (Kozjak et al., 2003). Purified SAM complex in a buffer with 0.1% (w/v) decylmaltoside was mixed in a 1:1 ratio with mixed detergent/lipid micelles containing a mixture of synthetic lipids corresponding to outer mitochondrial membranes and 0.5% (w/v) decylmaltoside in 100 mM KCl, 10 mM MOPS-Tris (pH 7). Planar bilayers were generated by the painting technique (Kovermann et al., 2002). Proteoliposomes were added to the *cis* chamber at asymmetrical buffer conditions. After fusion, buffers were changed to symmetrical conditions (250 mM KCl, 10 mM MOPS-Tris, pH 7.0). Where indicated, β-signal peptides were added (10 μM for Sam50, 6 μM for SAM complex).

Miscellaneous

Crosslinking was performed in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS-KOH, pH 7.2) with 600 μM DPDPB for 1 hr at 4°C and quenched by 5 mM cysteine and 50 mM Tris-HCl (pH 7.2). Mitochondria were separated by SDS-, Tris-Tricine- or BN-PAGE. For AMS modification mitochondria were resuspended in SM buffer or solubilized in 2.5% Mega-9, 20 mM Tris-Cl, pH 7,4, 0.1 mM EDTA, 50 mM NaCl, 10% (w/v) glycerol. Samples were incubated on ice for 20 min followed by 15 min at 25°C in the presence of AMS. Samples were diluted 10-fold in SM buffer, TCA precipitated, and resolved by SDS-PAGE.

SUPPLEMENTAL DATA

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/cgi/content/full/132/6/1011/DC1/.

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