

# Interaction of the J-Protein Heterodimer Pam18/Pam16 of the Mitochondrial Import Motor with the Translocon of the Inner Membrane

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**Import of proteins across the inner mitochondrial membrane through the Tim23:Tim17 translocase requires the function of an essential import motor having mitochondrial 70-kDa heat-shock protein (mtHsp70) at its core. The heterodimer composed of Pam18, the J-protein partner of mtHsp70, and the related protein Pam16 is a critical component of this motor. We report that three interactions contribute to association of the heterodimer with the translocon: the N terminus of Pam16 with the matrix side of the translocon, the inner membrane space domain of Pam18 (Pam18<sub>IMS</sub>) with Tim17, and the direct interaction of the J-domain of Pam18 with the J-like domain of Pam16. Pam16 plays a major role in translocon association, as alterations affecting the stability of the Pam18:Pam16 heterodimer dramatically affect association of Pam18, but not Pam16, with the translocon. Suppressors of the growth defects caused by alterations in the N terminus of Pam16 were isolated and found to be due to mutations in a short segment of *TIM44*, the gene encoding the peripheral membrane protein that tethers mtHsp70 to the translocon. These data suggest a model in which Tim44 serves as a scaffold for precise positioning of mtHsp70 and its cochaperone Pam18 at the translocon.**

## INTRODUCTION

The majority of proteins of the mitochondrial matrix are synthesized on cytosolic ribosomes and then transported across the mitochondrial membranes through proteinaceous channels. Those destined for the matrix use the TIM23 translocase to cross the inner membrane (Koehler, 2004; Wiedemann *et al.*, 2004; Mokranjac *et al.*, 2006; Neupert and Herrmann, 2007). The TIM23 translocase is formed by three essential proteins: two integral membrane proteins Tim17 and Tim23, which form the channel itself (Truscott *et al.*, 2001), and the peripheral membrane protein Tim50. Typically, N-terminal targeting sequences are first driven through the channel by the membrane potential that exists across the inner membrane. Translocation of the remainder of the protein requires the action of the import motor, which is associated with the translocon (Jensen and Johnson, 2001; Neupert and Brunner, 2002; Rehling *et al.*, 2004).

The import motor, which is composed of several essential components, has at its core the matrix 70-kDa heat-shock protein (Hsp70), called Ssc1 in yeast. Ssc1, which interacts directly with the translocating polypeptide, is tethered to the channel via its interaction with the peripheral membrane protein Tim44 (Rassow *et al.*, 1994; Schneider *et al.*, 1994). This interaction is destabilized upon binding a client protein that is a translocating polypeptide (Liu *et al.*, 2001; D'Silva *et al.*,

*et al.*, 2004). Like all Hsp70s, Ssc1 has J-protein partners that serve the vital function of stimulating its ATPase activity, thus stabilizing its interaction with client proteins (Bukau *et al.*, 2006; Craig *et al.*, 2006). Pam18 (sometimes referred to as Tim14) is the J-protein partner of Ssc1 at the import channel (D'Silva *et al.*, 2003; Mokranjac *et al.*, 2003; Truscott *et al.*, 2003).

The genes encoding each of these three motor components, Ssc1, Tim44, and Pam18, are essential, as is the gene *PAM16* (sometimes referred to as *TIM16*), which encodes a fourth constituent. Pam16 is related to and can interact with Pam18. Although Pam18 can homodimerize, it has a higher affinity for Pam16, and it is found at the import channel as a heterodimer with Pam16. Pam16 has been called a J-like protein, because it has a degenerate J-domain that lacks the highly conserved histidine proline, aspartic acid tripeptide (HPD) motif, and it is incapable of stimulating ATPase activity of Ssc1 (Frazier *et al.*, 2004; Kozany *et al.*, 2004; Li *et al.*, 2004; D'Silva *et al.*, 2005). The Pam18:Pam16 complex is associated with the translocon, because it can be coimmunoprecipitated with antibodies specific for either Tim17 or Tim23 (Kozany *et al.*, 2004; Mokranjac *et al.*, 2006). Pam18 and Pam16 interact via their J- and J-like domains (Truscott *et al.*, 2003; D'Silva *et al.*, 2005) (Figure 1), which have virtually identical folds and form a pseudosymmetrical arrangement (Mokranjac *et al.*, 2006). The residues forming the interface between the two proteins are highly conserved.

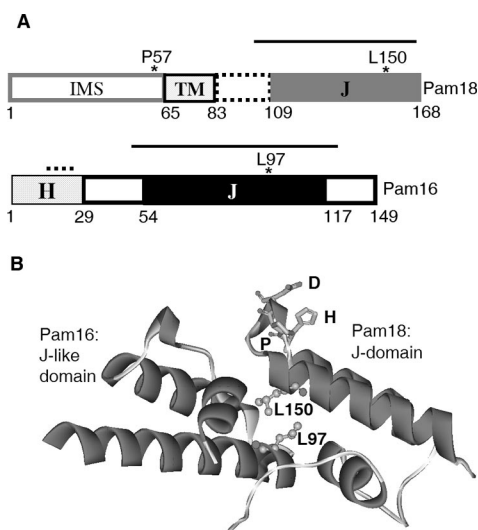
Although Pam18 and Pam16 have significant sequence similarity, particularly in their J-domain regions, significant differences exist as well (Figure 1A). Pam18 has a single transmembrane domain, with ~60 N-terminal amino acids extending into the intermembrane space (IMS) (D'Silva *et al.*, 2003; Truscott *et al.*, 2003; Chacinska *et al.*, 2005). Pam16 does not extend into the IMS, and it has been classified as a peripheral membrane protein (Frazier *et al.*, 2004). Analysis of deletion mutants, and the results of depletion experi-

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**Figure 1.** Domain organization of Pam16 and Pam18. (A) Schematic representation of Pam18 and Pam16 with amino acids corresponding to predicted domains indicated. Pam18: aa 1-60, intermembrane space (IMS); aa 65-83, transmembrane (TM); aa 101-168, J-domain (J). Pam16: aa 1-29, hydrophobic (H); aa 54-117, J-like domain (J). Amino-acid alterations analyzed in this report are indicated by asterisks or, in the N terminus of Pam16, by a dotted line, indicating K19, E23, R26, and Q27. The segment of Pam18 and Pam16 used in structural determination (Mokranjac *et al.*, 2006) is indicated by the solid line. (B) Interaction of Pam18 and Pam16 J-domains (Mokranjac *et al.*, 2006). A symmetrical set of interactions were observed between Pam16 and Pam18 in a strongly interacting hydrophobic pocket that includes: L150 of Pam18 and L97 of Pam16, indicated. The conserved HPD found in J-domains and required for stimulation of Hsp70 ATPase activity is also indicated.

ments, has implicated both Pam16 and Pam18 in the association of the heterodimer with the translocon (Frazier *et al.*, 2004; Kozany *et al.*, 2004; Mokranjac *et al.*, 2006; Neupert and Herrmann, 2007).

To understand the mechanism of action of the import motor, it is important to know how different components interact with each other and the translocon, and the contribution of these different interactions. Although it is well established that Pam18 and Pam16 interact with each other via their J-domains, neither the sequences important for translocon association nor the importance of the direct interaction between Pam18 and Pam16 in stabilizing translocon association has been established. Therefore, we undertook a combined genetic and biochemical analysis with the aim of better understanding the interaction of the Pam16: Pam18 heterodimer with the translocon. Our results indicate that three sets of interactions are important for optimal functioning of the import motor.

## MATERIALS AND METHODS

### Plasmid Construction and Genetic Methods

Yeast strains deleted for *TIM44*, *PAM18*, and *PAM16* have been described previously (Maarse *et al.*, 1992; D'Silva *et al.*, 2003; D'Silva *et al.*, 2005). Double deletion strains were created by mating single deletion haploids. The resulting diploids were sporulated, and the desired haploids isolated by dissecting tetrads.

The *PAM16*[1-52]:*PAM18*[106-168] chimera was described previously (D'Silva *et al.*, 2005). Another chimera, designated *PAM16*[1-27]:*PAM18*[81-168] was constructed by introducing a *NarI* site at the codons for amino acids 27 and 28 of *PAM16* and amino acids 80 and 81 of *PAM18*. The *NarI*-*KpnI* fragment of *PAM16* was replaced with the *NarI*-*KpnI* fragment of *PAM18* in the pRS315-*PAM16* plasmid (D'Silva *et al.*, 2005). The Su9-Pam16(51-117)

clone was constructed by PCR amplifying the codons encoding the N-terminal 66 amino acids of Su9 with an *NdeI* site at the 5' end and polymerase chain reaction (PCR) sewing to Pam16(51-117). The resulting product was cloned into pRS315-*Pam16*, replacing the wild-type (wt) open reading frame. All point mutations were created using the QuikChange protocol (Stratagene, La Jolla, CA).

To obtain suppressor mutations in *TIM44*, a library of random mutations in pRS314-*Tim44* was created through PCR amplification of the PstI-BglII 1080 base pairs fragment of *TIM44*.  $\Delta$ *pam16*  $\Delta$ *tim44* carrying pRS316-*Tim44* and pRS315-*PAM16*[1-27]:*PAM18*[81-168]*K19A* was transformed with the library and incubated at 30°C on tryptophan omission plates. Transformants were replica plated to 5-fluoroorotic acid plates to select for candidates that could grow in the absence of the wild-type copy of *TIM44* at 34°C. Colonies were resuspended from the 5-fluoroorotic acid plates, diluted, and 1500 cells were plated on tryptophan omission plates at 34°C. Plasmid DNA was recovered from transformants that could grow at 34°C and used to transform the parent strain to verify the phenotype. Correct candidates were sequenced at the University of Wisconsin Biotechnology facility.

The coding sequences of the Pam18<sub>IMS</sub> domain (amino acids [aa] 2-65) of wt *PAM18* and *pam18*<sub>A57P</sub> were cloned into the pGEX-2T expression vector (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), to create a fusion with the coding sequences of glutathione S-transferase (GST). The GST-Pam18<sub>IMS</sub> fusions (GST-Pam18<sub>IMS</sub>) were expressed in the *E. coli* strain BL21 (DE3) (Novagen, Madison, WI).

To facilitate purification of Pam16 and Pam18 proteins, six histidine codons were introduced at the 5' end of genes corresponding to wt *PAM18*, *PAM18*<sub>L150W</sub> and at the 3' end of wt *PAM16* and *PAM18*<sub>L97W</sub>. All were cloned into the plasmid pET3a. Overexpression was carried out in *E. coli* strain C41 cells (Miroux and Walker, 1996) by allowing them to grow at either 20 or 28°C to an *A*<sub>600</sub> of 0.5–0.6, followed by induction using 0.5 mM isopropyl  $\beta$ -D-thiogalactoside for 4–6 h.

### Protein Purification

The wt or mutant Pam16 and Pam18 proteins were expressed in 1.2 l of *E. coli*, as described above. Cells were resuspended in buffer A (20 mM Tris-buffer, pH 8.0, 0.5 M KCl, 10% glycerol, 1% Triton X-100, and 20 mM imidazole) containing EDTA-free protease inhibitors (Roche Diagnostics, Indianapolis, IN), and then they were subjected to treatment in the French Press twice at 4°C. Extracts were clarified by 12,000  $\times$  g centrifugation for 30 min at 4°C. The clear supernatant was subjected to batch affinity chromatography by using nickel-nitrilotriacetic acid agarose (0.5 ml of wet bead volume) by incubating at 4°C for 1 h, followed by four washes with 40 ml of buffer A to remove unbound proteins. The resin was then washed once with 40 ml of buffer B (20 mM Tris buffer, pH 8.0, 0.5 M KCl, 10% glycerol, 60 mM imidazole, 2 mM ATP, 10 mM MgCl<sub>2</sub>, and 1% Triton X-100) and two times with 40 ml of buffer C (20 mM Tris buffer, pH 8.0, 0.1 M KCl, 10% glycerol, 60 mM imidazole, and 0.2% Triton X-100). Bound proteins were eluted with 1 ml of 250 mM imidazole in buffer D (20 mM Tris buffer, pH 8.0, 0.1 M KCl, 10% glycerol, and 0.2% Triton X-100) and dialyzed against buffer appropriate for use in particular experiments.

### In Vitro Coimmunoprecipitation (CoIP) of Purified Proteins

Antibodies against the C-terminal fragments of Pam18 and Pam16, amino acids 80–168 and 48–149, respectively, were affinity purified and cross-linked to protein A-Sepharose beads at a saturated level, as described previously (D'Silva *et al.*, 2005). Then, 10  $\mu$ g of either wt or mutant Pam18 or Pam16 proteins was incubated with Pam18- or Pam16-specific antibody beads in CoIP buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid-KOH, pH 7.4, 250 mM sucrose, 80 mM KCl, 0.2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] at 4°C for 1 h. Under these conditions, ~90% of Pam18 or Pam16 was immunoprecipitated. The Pam16- and Pam18-bound beads were washed three times in CoIP buffer, and then they were blocked with 0.1% bovine serum albumin at 23°C for 20 min. Ten micrograms of purified protein was added, and the mixture was incubated in a final volume of 200  $\mu$ l at 23°C for 30 min. The beads were washed four times with CoIP buffer and analyzed on SDS-polyacrylamide gel electrophoresis (PAGE), followed by Coomassie staining.

### CoIP from Mitochondrial Lysates

For analysis of the interaction between Pam16 and Pam18 under conditions where they are separated from the translocon, 1.25–2 mg of wt or mutant mitochondria was lysed in lysis buffer (20 mM MOPS-KOH, pH 7.4, 250 mM sucrose, 80 mM KCl, 5 mM EDTA, and 1 mM PMSF) in the presence of 1% Triton X-100 on ice for 30 min by gentle vortexing for 5 s at 5-min intervals. Lysates were centrifuged for 10 min at 14,000 rpm at 4°C. Ten microliters (bed volume) of saturated Pam18 or Pam16 cross-linked antibody beads was incubated with the supernatants of the lysates for 1 h at 4°C. The beads were washed with mitochondrial lysis buffer containing 1% Triton X-100 four times. Samples were separated by SDS-PAGE, followed by immunoblot analysis by using antibodies against Pam16 and Pam18.

For analysis of the interaction of Pam16 and Pam18 with the translocon, 2–3 mg of wt or mutant mitochondria was gently lysed and solubilized in 1 ml of lysis buffer (25 mM Tris, pH 7.5, 10% glycerol, 80 mM KCl, 5 mM EDTA, and 1 mM PMSF) in the presence of 1% digitonin at 4°C for 45 min by using end-to-end rotating shaker. Lysates were centrifuged for 20 min at 14,000 rpm at 4°C. A 20- $\mu$ l (bed volume) of saturated cross-linked Tim23 antibody beads was incubated with the supernatants of the lysates for 1.5 h at 4°C. The beads were washed four times with the corresponding mitochondrial lysis buffer containing 0.1% digitonin. Samples were separated on SDS-PAGE, followed by immunoblot analysis using antibodies against Pam16, Pam18, Tim17, Tim50, and Tim23.

### Pull-Down Assays Using GST-Pam18<sub>IMS</sub> Fusions

GST-Pam18<sub>IMS</sub> fusions were purified from the bacterial lysates by binding to glutathione-agarose beads (Sigma-Aldrich, St. Louis, MO). After washing with phosphate-buffered saline, the beads having immobilized GST-Pam18<sub>IMS</sub> were equilibrated in PDG buffer [20 mM HEPES, pH 7.5, 100 mM KOAc, 10 mM Mg(OAc)<sub>2</sub>, and 10% glycerol] for the subsequent pull-down assays, which were performed as described previously (Chacinska *et al.*, 2005). Isolated mitochondria were solubilized in PDG buffer containing 2 mM PMSF and Triton X-100 at a detergent/protein ratio of 5 (wt/wt). Lysates were centrifuged for 10 min at 20,800  $\times$  g at 4°C to remove the insoluble fraction. The cleared lysates were then diluted with PDG buffer to 0.25% Triton X-100. The glutathione-agarose beads bound with GST or GST-Pam18<sub>IMS</sub> were incubated for 2 h at 4°C in the mitochondrial lysates. After the beads were washed with PDG buffer four times, bound proteins were eluted in SDS sample buffer, separated on SDS-PAGE and detected by immunoblotting.

### Miscellaneous

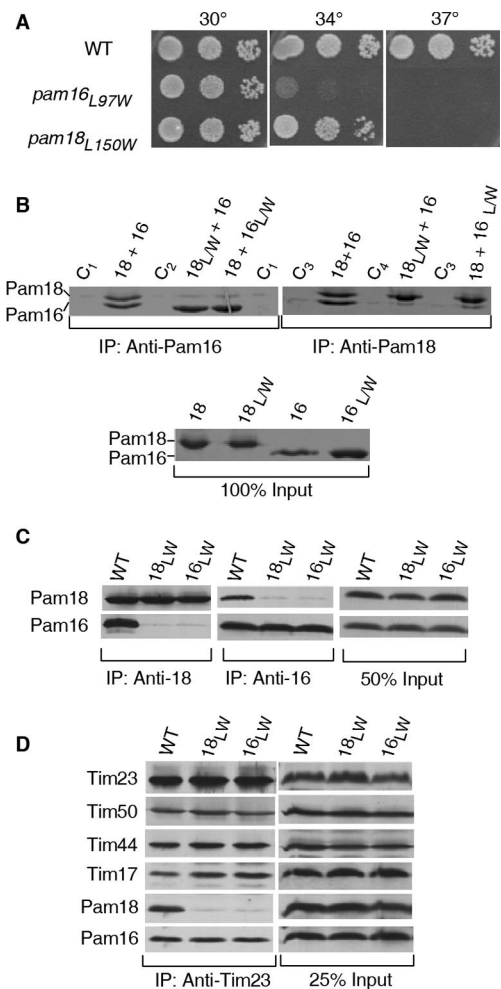
Purification of mitochondria and affinity-purification of antibodies against Pam18, Pam16, and Tim23 were carried out as described previously (Liu *et al.*, 2001). Separation of mitochondria into soluble and membrane fractions was performed essentially as described previously (Truscott *et al.*, 2003). Briefly, mitochondria (1 mg protein ml<sup>-1</sup>) were sonicated on ice in the presence of 1 mM PMSF in 20 mM HEPES, pH 7.5, and 500 mM NaCl. Samples were subjected to centrifugation at 100,000  $\times$  g for 45 min. After trichloroacetic acid precipitation, supernatant and pellet fractions were analyzed by SDS-PAGE and immunoblotted with Pam16-, Pam18-, and Mge1-specific antibodies. Whole cell extracts were prepared by treatment of cells with 0.1M NaOH followed by heating to 100°C for 5 min in SDS sample buffer. Immunoblot analysis was carried out using the ECL system (GE Healthcare) according to the manufacturer's suggestion, by using polyclonal antibodies specific for Pam16 (D'Silva *et al.*, 2005), Pam18 (D'Silva *et al.*, 2003), Tim44 and Mge1 (Liu *et al.*, 2001), and Tim17 (Ryan *et al.*, 1998). In addition, antibodies were generated in rabbits to amino acids 1–100 of Tim23 having a His tag and the C-terminal 450 amino acids of Tim50 having a His tag.

## RESULTS

### The Pam16:18 Interaction Is Important for the Association of Pam18 with Translocon

Pam16 and Pam18 interact via their related J-domains (Figure 1B) (Mokranjac *et al.*, 2006). To ask whether this interaction is important for the association of either protein with the translocon, we took advantage of a temperature-sensitive PAM18 mutant that we isolated previously, *pam18*<sub>L150W</sub>, encoding a tryptophan, rather than a leucine, at position 150 (D'Silva *et al.*, 2005). Not surprisingly, this alteration reduces the stability of the Pam16:Pam18 complex (D'Silva *et al.*, 2005), as L150 forms a direct contact with Pam16. Based on the sequence similarity between the J-domain of Pam18 and the J-like domain of Pam16, we constructed the corresponding mutation in PAM16, which resulted in conversion of leucine to tryptophan at position 97. Because of the twofold pseudosymmetrical arrangement of the Pam18:Pam16 complex, leucine 97 interacts with I116, I136, F149, and L150 of Pam18 (Figure 1B). We tested the ability of Pam16<sub>L97W</sub> to substitute for the wt protein. Like *pam18*<sub>L150W</sub> cells, *pam16*<sub>L97W</sub> cells grew like wt cells at 30°C, and they were temperature sensitive for growth (Figure 2A). In contrast, *pam18*<sub>L150W</sub> formed colonies at 34°C, but not 37°C, whereas *pam16*<sub>L97W</sub> was unable to grow at either 34 or 37°C.

The effect of the alteration in Pam16 on the stability of the Pam16:Pam18 heterodimer was assessed in two ways. First,



**Figure 2.** Analysis of mutations affecting stability of the Pam16:Pam18 heterodimer. (A) Growth phenotypes. Tenfold serial dilutions of wt, *pam18*<sub>L150W</sub>, and *pam16*<sub>L97W</sub> cells were spotted onto rich glucose-based media, followed by incubation at the indicated temperatures for 2 d. (B) Interaction of purified Pam16 and Pam18. Purified proteins (3.1  $\mu$ M) were incubated together for 30 min at 23°C: wt Pam18 (18), wt Pam16 (16), Pam16<sub>L97W</sub> (16<sub>L/W</sub>), or Pam18<sub>L150W</sub> (18<sub>L/W</sub>). CoIPs was carried out using Pam18- and Pam16-specific antibodies. The samples were analyzed by SDS-PAGE and stained with Coomassie Blue. Samples containing 100% of the input served as a control for immunoprecipitation efficiency (bottom). Controls (C) were Pam18 (C<sub>1</sub>), Pam18<sub>L150W</sub> (C<sub>2</sub>), Pam16 (C<sub>3</sub>), and Pam16<sub>L97W</sub> (C<sub>4</sub>). (C and D) In organelle analysis of Pam18 and Pam16. wt, *pam18*<sub>L150W</sub> (18<sub>L/W</sub>), or *pam16*<sub>L97W</sub> (16<sub>L/W</sub>). (C) Mitochondria were incubated in 1% Triton X-100 and then subjected to immunoprecipitation by using Pam18- and Pam16-specific antibodies, as indicated by brackets, followed by SDS-PAGE and immunoblotting by using Pam18 and Pam16-specific antibodies. Fifty percent of soluble material after lysis was used as a loading control (50% input). (D) Coimmunoprecipitation of the Pam18:Pam16 complex with the core Tim23 translocon complex. Mitochondria were solubilized with buffer containing 1% digitonin, and the supernatants were subjected to immunoprecipitation using Tim23-specific antibodies. The samples were analyzed by SDS-PAGE and immunoblotted against Tim23-, Tim50-, Tim44-, Tim17-, Pam16- and Pam18-specific antibodies. Twenty-five percent of total soluble material after lysis was used as a loading control (25% input).

mutant and wt proteins were purified, mixed together in equimolar amounts, and immunoprecipitated using either Pam18- or Pam16-specific antibodies. As expected, wt Pam16

and Pam18 were efficiently coprecipitated. However, Pam16<sub>L97W</sub> was not efficiently coprecipitated with wt Pam18, nor was wt Pam16 efficiently coprecipitated with Pam18<sub>L150W</sub> (Figure 2B), regardless of the antibody used. Second, the Pam16:Pam18 interaction was assessed in mitochondrial extracts. To dissociate the import motor from the translocon, mitochondria were disrupted by the addition of Triton X-100. As expected, Pam16 and Pam18 coprecipitated from extracts of wt mitochondria (Figure 2C). But, consistent with the results of the analysis of purified proteins, reduced coprecipitation was observed from *pam16*<sub>L97W</sub> or *pam18*<sub>L150W</sub> extracts.

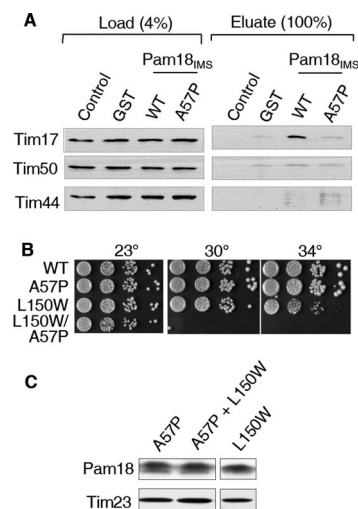
The availability of analogous single amino-acid alterations in Pam16 and Pam18, both of which destabilized the Pam18:Pam16 heterodimer, allowed us to assess the effect of this interaction on the association of each component with the translocon. Mitochondria isolated from wt and mutant strains were lysed by addition of digitonin, a treatment that leaves the complex of the translocon and associated import motor substantially intact. Immunoprecipitations were carried out with antibodies specific for Tim23, an integral membrane component of the translocon. As expected from previously reported results (Mokranjac *et al.*, 2003; Frazier *et al.*, 2004; Kozany *et al.*, 2004; Chacinska *et al.*, 2005), both Pam18 and Pam16 were coprecipitated from wt extracts, along with other components of the translocon, including Tim17, Tim50, and Tim44. However, Pam18 coprecipitation from extracts derived from both *pam18*<sub>L150W</sub> and *pam16*<sub>L97W</sub> was greatly reduced. In contrast, Pam16 was efficiently pulled-down from all three extracts (Figure 2D), as were the other components tested.

The reduced coprecipitation of Pam18, but not Pam16, with the translocon when Pam16:Pam18 heterodimer formation was compromised is consistent with an important role for Pam16 in tethering Pam18 to the translocon. In addition, the isolation of point mutations that resulted in destabilization of the Pam16:Pam18 heterodimer provided tools to facilitate identification and analysis of regions of Pam18 and Pam16 involved in interaction with the translocon, as described below.

#### Interaction of the IMS Domain of Pam18 with Tim17

It has been reported previously (Chacinska *et al.*, 2005) that the N-terminal 60 amino acids of Pam18 interacts directly with Tim17, a core component of the translocon, suggesting a role of Pam18<sub>IMS</sub> in tethering Pam18 to the import channel. However, the absence of these N-terminal 60 amino acids had no obvious effect on growth, nor on the stability of the interaction of Pam18 with the translocon, as indicated by similar abilities of Pam18 and Pam18<sub>Δ1-60</sub> to be coprecipitated using Tim23-specific antibodies (Mokranjac *et al.*, 2007; data not shown). To test whether an interaction between Pam18<sub>IMS</sub> and Tim17 plays any role in the interaction of Pam18 with the translocon, we combined *pam18*<sub>L150W</sub>, the mutation that causes a destabilization of the interaction between the J-domains of Pam16 and Pam18, and the N-terminal deletion mutation *pam18*<sub>Δ1-60</sub>. *pam18*<sub>Δ1-60/L150W</sub> cells were inviable (data not shown), demonstrating a very strong genetic interaction between these two mutations.

To more directly assess whether this genetic interaction was due to the disruption of the interaction of the IMS domain of Pam18 with Tim17 or some other reason, we attempted to isolate a point mutation causing an amino acid change in Pam18<sub>IMS</sub> that diminished the interaction with Tim17. A secondary structure prediction of the 60 amino acid N terminus of Pam18 revealed a possible  $\alpha$  helix between amino acids 51 and 61, which we reasoned might be a site of interaction between Pam18 and Tim17. To poten-

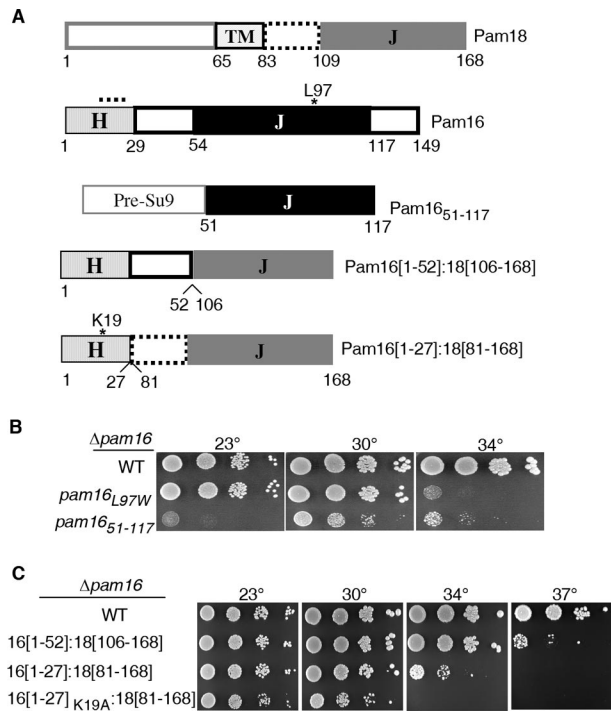


**Figure 3.** Functional importance of IMS domain of Pam18. (A) Interaction of Pam18<sub>IMS</sub> with Tim17. Fusions between GST and Pam18<sub>IMS</sub> domains (Pam18 [WT] and Pam18<sub>A57P</sub> [A57P]), and GST alone as a control) were immobilized on glutathione-agarose beads, and then they were incubated in wt mitochondrial lysates. Bound proteins were subjected to SDS-PAGE and immunoblotted with antibodies specific for the indicated proteins. Untreated glutathione-agarose beads (control) were used as a negative control. (B) Effect of combination of the A57P and L150W alterations in Pam18 on cell growth. Tenfold serial dilutions of  $\Delta$ pam18 cells carrying a plasmid expressing either wt or indicated PAM18 mutant gene were plated on rich glucose-based media and grown at 23°C for 3 d and at 30 or 34°C for 2 d. (C) Expression levels of Pam18 mutant proteins. Whole cell extracts from *pam18*<sub>A57P</sub>, *pam18*<sub>L150W</sub>, and *pam18*<sub>A57P/L150W</sub> were separated by SDS-PAGE and subjected to immunoblot analysis by using antibodies specific for Pam18, and as a loading control Tim23. All samples were run on the same gel, but lanes unrelated to the experiment presented here were removed.

tially disrupt this helix, the codon for alanine at position 57 was changed to encode proline, thus generating *pam18*<sub>A57P</sub>.

To assess whether this amino acid change affected the interaction with Tim17, we used a binding assay, between Pam18<sub>IMS</sub> tethered to beads and lysates of mitochondria generated by treatment with Triton X-100, as described previously (Chacinska *et al.*, 2005). Fusions between the N-terminal 60 amino-acid segments of wt Pam18 or Pam18<sub>A57P</sub> and GST were bound to glutathione beads, and their ability to interact with Tim17 was assessed. As expected (Chacinska *et al.*, 2005), interaction between the fusion having the wt sequence and Tim17, but not Tim50 or Tim44, was observed (Figure 3A). This interaction with Tim17 was specific, because no strong interaction with GST lacking the Pam18 segment was detected. The amount of Tim17 pulled down by the Pam18<sub>A57P</sub> fragment was reduced compared with that of the wt fusion, indicating that the amino acid alteration affected the Tim17:Pam18<sub>IMS</sub> interaction.

Next, we tested for genetic interactions between the mutations at codon 57 and codon 150. As expected, cells having *pam18*<sub>A57P</sub> cells grew well, even at 37°C. However, Pam18<sub>A57P/L150W</sub> was unable to form colonies at 30°C, a temperature at which the single mutants grew as well as wt cells (Figure 3B). This synthetic growth defect was not due to expression levels, because Pam18<sub>A57P/L150W</sub> was expressed at levels similar to those of Pam18<sub>A57P</sub> and Pam18<sub>L150W</sub> (Figure 3C). These results are consistent with the idea that the interaction between Pam18<sub>IMS</sub> and Tim17 serves to stabilize the interaction of Pam18 with the translocon.



**Figure 4.** Analysis of the functional importance of N terminus of Pam16 by using Pam16:Pam18 chimeras. (A) Schematic representation of Pam16 deletion construct and Pam16:Pam18 chimeras used. Because Pam16<sub>51-117</sub> lacks a mitochondrial targeting sequence, it was expressed with the cleavable Su9 targeting sequence (pre) at the N terminus as reported previously (Mokranjac *et al.*, 2006). Other abbreviations are as in the legend of Figure 1. (B and C) Tenfold serial dilutions of  $\Delta$ pam16 cells carrying a centromeric plasmid expressing indicated protein were plated on rich media and incubated at the indicated temperatures for 3 d.

#### Sufficiency of the N Terminus of Pam16 in Pam16:Pam18 Chimeras

Next, we sought to better understand the means by which Pam16 associated with the translocon, and the importance of this interaction to the translocation process. It has been proposed that the N-terminal region of Pam16 is important for membrane association. However, in apparent contradiction to this idea, the J-like domain has been reported to be the only essential part of Pam16 (Mokranjac *et al.*, 2006). To better understand the contribution of the different regions of Pam16 to its biological function, we directly compared the growth of cells expressing full-length Pam16, Pam16<sub>L97W</sub>, or the Pam16 J-like domain Pam16<sub>51-117</sub> (Figure 4A). Viable pam16<sub>51-117</sub> cells were obtained. However, the Pam16 J-like domain supported growth only very poorly even when expressed at levels similar to full-length protein, because only very small colonies were formed at 30 and 34°C; no colony formation occurred at either 23 or 37°C (Figure 4B). In addition, pam16<sub>51-117</sub> cells were respiration deficient, as indicated by their failure to grow on medium containing the nonfermentable carbon source glycerol (data not shown). Thus, sequences outside the J-like domain are important for Pam16 function.

To aid in the identification of residues outside the J-like domain important for function, we used chimeras composed of segments of Pam16 and Pam18. We had previously determined that the Pam18 J-domain could substitute for the Pam16 J-like domain, because a chimera containing the N-terminal 52 amino acids of Pam16 and the Pam18 J-domain,

aa 106-168 (Pam16[1-52]:Pam18[106-168]), allowed robust growth of  $\Delta$ pam16 cells at 30 and 34°C, but not 37°C (D'Silva *et al.*, 2005) (Figure 4C). We proceeded to test whether a smaller portion of the N terminus of Pam16 was sufficient for function in the context of Pam18. A chimera containing the first 27 amino acids of Pam16 fused to the last 88 aa of Pam18 (Pam16[1-27]:Pam18[81-168]), hereafter called Pam16[1-27]:18 (Figure 4A), was sufficient to allow robust growth at 30°C and slow growth at 34°C (Figure 4C).

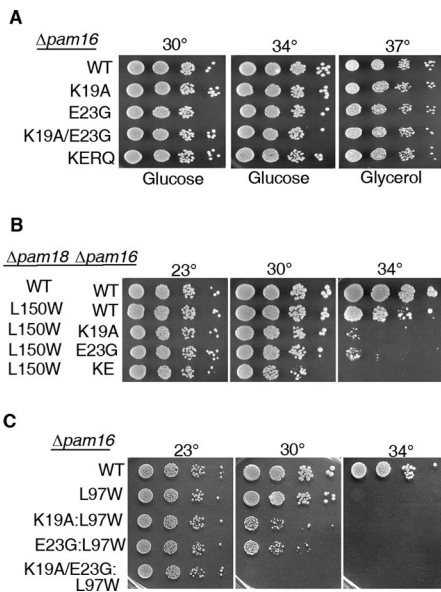
The ability of Pam16[1-27]:18 to suffice at optimal growth temperatures indicated that the N-terminal 27 amino acids of Pam16 were functionally important. The results of an analysis of this sequence suggested an amphipathic character. To first assess whether the hydrophilic residues were important for the functionality of the chimera, codons encoding two positively charged residues, K19 and E23, were altered to encode A and G, respectively, in the Pam16[1-27]:18, yielding Pam16[1-27]<sub>K19A</sub>:18 and Pam16[1-27]<sub>E23G</sub>:18. pam16[1-27]<sub>E23G</sub>:18 cells were inviable (data not shown); pam16[1-27]<sub>K19A</sub>:18 cells were viable but compromised for growth compared with pam16[1-27]:18 cells, being unable to form colonies at 34°C (Figure 4C).

#### Genetic Interactions Involving the N Terminus of Full-Length Pam16

We next tested the effect of the K19A and E23G alterations in the context of native Pam16, by constructing full-length PAM16 having these alterations alone and in combination, and then testing their ability to substitute for wt PAM16. Neither single nor double alterations had an obvious effect on Pam16 function (Figure 5A), even though in the chimera these alterations had severe consequences. Even when four residues predicted to form the hydrophilic face of the N-terminus of Pam16, K19, E23, R26, and Q27 were altered, little or no phenotype was observed.

This lack of phenotypic effect of alterations in the N terminus of Pam16 when in the context of the full-length protein, compared with the severe effect when in the context of the Pam16:Pam18 chimera led us to consider possible overlap in function of the N- and C-terminal regions of Pam16. In particular, we considered the fact that alterations in the J-domain of Pam18 or J-like domain of Pam16 that destabilized the interaction between Pam16 and Pam18 also destabilized the association of Pam18 with the translocon and adversely affected cell growth (Figure 2A). This idea was based on the fact that purified Pam18 forms a homodimer, but the affinity of the Pam18 J-domains for itself is less than for the J-like domain of Pam16 (D'Silva *et al.*, 2005). Thus, a chimera that has the Pam18 J-domain would be expected to form a dimer, but it would be less stable than the native Pam16:Pam18 heterodimer. We reasoned that if the strong effect on growth of the N-terminal alterations in Pam16[1-27]:18 was due to an effect on tethering Pam18 to the translocon, similar synthetic genetic interactions would occur when mutations encoding alterations in the N terminus of full-length Pam16 were combined with alterations in either the J-domains of full-length Pam16 itself or in Pam18.

To test for such genetic interactions, we compared the growth of pam16<sub>K19A,E23G</sub> pam18<sub>L150W</sub> with cells having only mutations in either PAM16 or PAM18, that is, pam16<sub>K19A,E23G</sub> or pam18<sub>L150W</sub>. As shown above, pam16<sub>K19A,E23G</sub> cells had no obvious growth phenotypes, whereas pam18<sub>L150W</sub> cells grew as wt up to 34°C. pam16<sub>K19A,E23G</sub> pam18<sub>L150W</sub> cells were inviable at 34°C, and although they formed colonies at 30°C, growth was slower than that of pam18<sub>L150W</sub> (Figure 5B). Cells having either single PAM16 mutation in combination with pam18<sub>L150W</sub> displayed growth defects as well. Although



**Figure 5.** Analysis of the functional importance of the N terminus of Pam16 by using full-length protein. (A) Growth phenotypes of  $\Delta pam16$  cells expressing the indicated N-terminal amino-acid alterations in full-length Pam16 (KERQ indicates K19A/E23G/R26A/Q27A). Tenfold serial dilutions were plated on rich glucose-based medium and grown at 30 or 37°C for 2 d or on rich glycerol-based medium and incubated at 37°C for 5 d. (B) Growth phenotypes of  $\Delta pam18 \Delta pam16$  cells expressing wt or the indicated N-terminal amino-acid alterations in full-length Pam16 and either wt Pam18 or Pam18<sub>L150W</sub>. KE indicates K19A/E23G. Tenfold serial dilutions were plated on rich glucose-based medium at the indicated temperatures for 3 d. (C) Growth phenotypes of  $\Delta pam16$  cells expressing either full-length wt Pam16 or Pam16 either with N-terminal amino-acid alterations and/or the L97W alteration in the J-like domain. Tenfold serial dilutions were plated on rich glucose-based medium and incubated at the indicated temperatures for 3 d.

these mutants grew as well as  $pam18_{L150W}$  at 30°C, no colonies were formed at 34°C. Synthetic genetic interactions were also observed between mutations causing alterations in the N and C termini of Pam16. Cells having only the alteration in the J-like domain, that is, Pam16<sub>L97W</sub>, could grow well at 30°C, whereas the triple mutant did not form colonies (Figure 5C).

#### N Terminus of Pam16 Is Involved in Association with the Translocon

The synthetic genetic interactions are consistent with a role for the N terminus of Pam16 in the association of the Pam16 with the translocon. To test whether alterations in the N terminus of Pam16 alone affect interaction of Pam16 or Pam18 with the translocon, we prepared mitochondria from the strains described above. First as a control, we asked whether the alterations in the N terminus of Pam16 alone affected the Pam16:Pam18 interaction, by using lysates obtained by incubation in buffer containing Triton X-100, because such treatment results in dissociation of the import motor components from the core translocon (D'Silva *et al.*, 2003; Kozany *et al.*, 2004). Lysates were subjected to immunoprecipitation with Pam18-specific antibodies. Pam16 was coimmunoprecipitated efficiently in all mutants tested (Figure 6A), even  $pam16_{KERQ}$  having alterations of K19A, E23G, R26A, and Q27A, indicating that such alterations did not affect the stability of the Pam16:Pam18 heterodimer. Extracts were also prepared in the presence of digitonin and precip-

itations carried out with Tim23-specific antibodies. The alterations had no discernible effect on association of Pam16 or Pam18 with the translocon (Figure 6B).

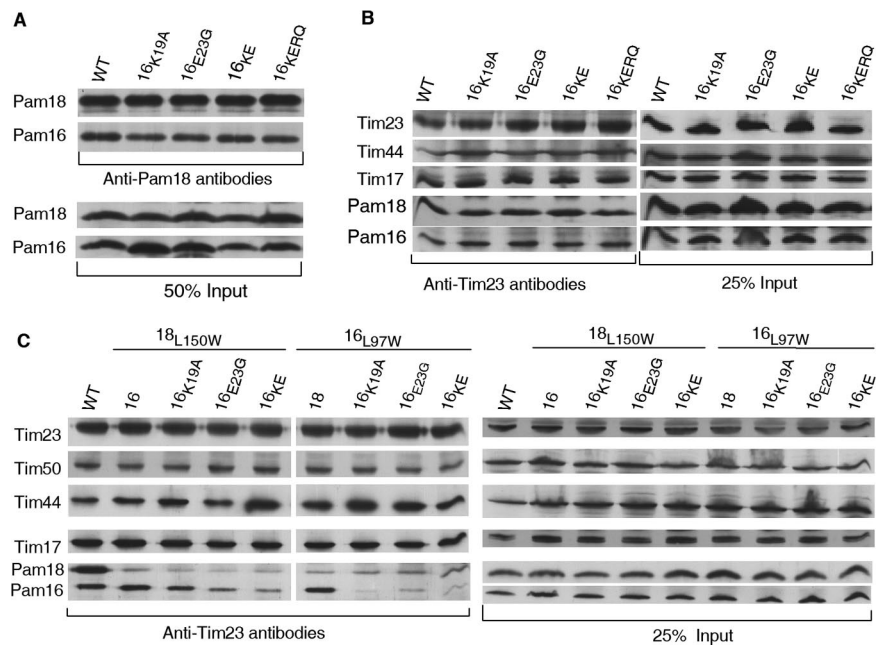
Because this biochemical test failed to reveal deleterious effects of alterations of the N terminus of Pam16 on translocon association, we next tested the effect of such alterations in combination with the L150W alteration in Pam18 and the L97W alteration in Pam16, which cause a reduction in the stability of the heterodimer and of the association of Pam18 with the translocon (Figures 2C and 6C). Although the association of Pam16<sub>K19A</sub> in the context of Pam18<sub>L150W</sub> was not obviously affected, coprecipitation of Pam16<sub>E23G</sub> and Pam16<sub>K19A/E23G</sub> was diminished. The effects of the N-terminal alterations in the context of the L97W alteration in Pam16 itself were more severe. Neither Pam16<sub>K19A,L97W</sub> nor Pam16<sub>E23G,L97W</sub> were coprecipitated with the translocon (Figure 6C). The destabilization of Pam16 having N-terminal amino acid alterations, when in combination with either Pam16 or Pam18 alterations that destabilize the Pam16:18 heterodimer, are consistent with the idea that the N terminus of Pam16 plays a direct role in association of Pam16 with the translocon and therefore an indirect role in Pam18's association.

#### Mutations in TIM44 Can Suppress the Growth Defect Caused by Alteration of the N Terminus of Pam16

The deleterious effects caused by alterations of the charged residues in the N terminus of Pam16 are consistent with the notion that this region plays a role in the association of Pam16 with the translocon. The site of tethering of Pam16 to the translocon is unknown. However, Pam16 and Tim44 are apparently in proximity, because they can be cross-linked together in mitochondria (Kozany *et al.*, 2004; Mokranjac *et al.*, 2007). Therefore, we asked whether mutations in *TIM44* that suppressed the temperature growth phenotype caused by the alterations of the K19 residue could be isolated. This approach was based on the idea that amino acid alterations in Tim44 might be able to either directly or indirectly compensate for binding defects caused by alterations in the N-terminus of Pam16. Suppressors encoding single amino-acid alterations in Tim44 were isolated that allowed growth of cells carrying the  $pam16[1-27_{K19A}]:18$  chimera instead of *PAM16*. All changes were between residues 76 and 99: Q76R, S79A, G80R, G83C, and Q99R of Tim44 (Figure 7A).

To begin to determine whether the suppressor alterations in Tim44 had an effect on the localization of the Pam16[1-27<sub>K19A</sub>]:18 chimera, we assessed its association with the membrane. Mitochondria were subjected to sonication, and fractions were separated by centrifugation. As a control, mitochondria expressing Pam16, that is, wt mitochondria, were tested (Figure 7B). As expected, Pam16 and Pam18 were predominantly in the membrane fraction, whereas Mge1, the nucleotide release factor for Ssc1, was in the soluble fraction. Mitochondria from cells expressing the Pam16 [1-27]:18 chimera were also tested. The majority of the chimera was in the membrane fraction, but a portion remained in the supernatant, likely because sequences adjacent to the N-terminal 27 amino acids of Pam16 play a role in tethering to the translocon. The K19A alteration resulted in the majority of the chimera to be released into the supernatant under these conditions. We tested the effect of two of the strongest *TIM44* suppressors,  $tim44_{S79L}$  and  $tim44_{G80R}$ , on the association of the Pam16[1-27<sub>K19A</sub>]:18 chimera. In both cases, the majority of the chimera was membrane associated (Figure 7B). These data are consistent with the idea that the alterations in Tim44 compensate for the translocon binding defect caused by alterations in the N terminus of Pam16.

**Figure 6.** Association of Pam16 having alterations in the N terminus with the translocon. (A) Pam16-Pam18 association. Equivalent amounts of wt and *pam16* (16) mutant mitochondria were lysed by incubation with mitochondrial lysis buffer containing 1% Triton X-100. Lysates were subjected to immunoprecipitation by using Pam18-specific antibodies. The samples were analyzed by SDS-PAGE and immunoblotted against antibodies specific for Pam18 and Pam16. Fifty percent of soluble material after lysis was used as a loading control (50% load). *pam16*<sub>K19A/E23G</sub>, 16<sub>KE</sub>, *pam16*<sub>K19A/E23G/R26A/Q27A</sub>, 16<sub>KERQ</sub>. (B and C) Association of Pam18 and Pam16 mutants with the Tim23-core complex. Equivalent amount of mitochondria from cells expressing the indicated combination of Pam16 and/or Pam18 mutant proteins were solubilized by incubation in buffer containing 1% digitonin. Supernatants were subjected to immunoprecipitation using Tim23-specific antibodies. The samples were analyzed by SDS-PAGE and immunoblotted with Tim23-, Tim17-, Tim44-, Pam16-, and Pam18-specific antibodies. Twenty-five percent of total soluble material after lysis was used as a loading control (25% I).



The isolation of the suppressors described above indicate that mutations in *TIM44* are able to suppress the growth defect caused by alterations in the N terminus of Pam16 when in the context of the Pam16:Pam18 chimera. We next tested the three strongest *TIM44* suppressor mutations, S79L, G80R, and G83C, for their ability to suppress the growth phenotype of *PAM16* having the K19A and L97W mutations to determine whether these mutations could also suppress the growth defects when in the context of full-length Pam16 rather than the Pam16:Pam18 chimera. Cells having the *TIM44* mutations grew as well as those having only the L97W mutation in *PAM16*, allowing growth at 30°C (Figure 7C).

## DISCUSSION

At least three different interactions are formed among Pam16, Pam18, and other components of the translocation apparatus of the inner mitochondrial membrane. The results of our genetic and biochemical experiments reported here begin to parse the relative importance of these interactions. A predominant role is played by the N terminus of Pam16, likely because of its interaction with Tim44, which also serves as a tether for mtHsp70 to the translocon.

### The Specificity of Pam16 and Pam18

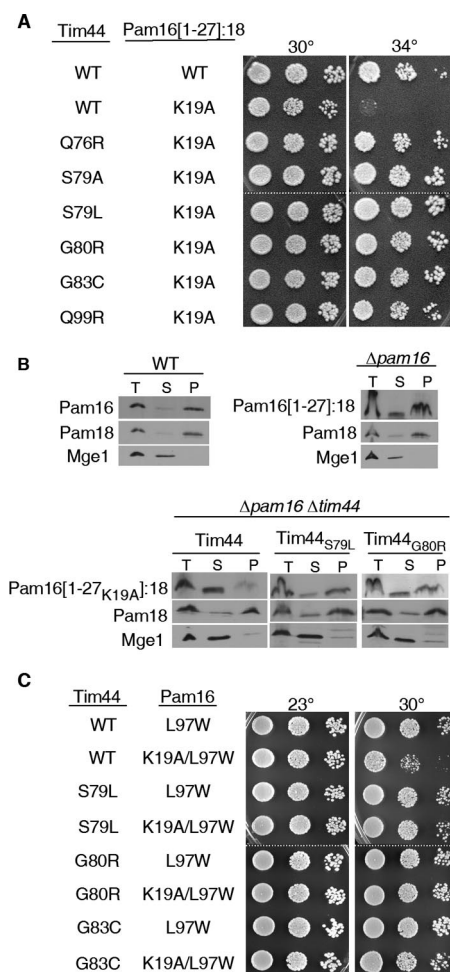
Both Pam16 and Pam18, homologous proteins present in virtually all eukaryotes, are essential and thus must have evolved a degree of specificity, as one cannot substitute for another. The required specificity of Pam18 function is unambiguous. Previous experiments demonstrated that Pam16 cannot stimulate the ATPase activity of Ssc1 (Li *et al.*, 2004; D'Silva *et al.*, 2005), whereas Pam18 is very active in this regard (D'Silva *et al.*, 2003; Truscott *et al.*, 2003; Mokranjac *et al.*, 2006). The importance of this activity is illustrated by the fact that single amino acid alterations in the highly conserved HPD motif that severely compromise this activity cannot support growth (D'Silva *et al.*, 2003; Mokranjac *et al.*, 2003). The basis of the specificity of Pam16 is not as clear. The results reported here indicate that the specificity of

Pam16 resides, at least in part, within its N-terminal 27 amino acids. This segment fused to the C-terminal 87 amino acids of Pam18 is capable of supporting robust growth of a  $\Delta$ *pam16* strain at optimal growth temperatures. Single amino acid changes within this 27 amino acid stretch have severe consequences for the ability of the chimera to substitute for Pam16.

### A Central Role for Tim44?

Several data presented here and elsewhere are consistent with a direct interaction between the N-terminus of Pam16 and Tim44, the peripheral membrane protein known to be critical for tethering of mtHsp70 to the translocon. First, as reported here, several mutations causing single amino-acid alterations in Tim44 suppressed the additional growth defect caused by changes in the 27 amino acids of the Pam16:Pam18 chimera. Second, Pam16 can be cross-linked to Tim44 (Kozany *et al.*, 2004; Mokranjac *et al.*, 2007), consistent with the idea that the two proteins are physically in proximity. Third, depletion of Tim44 results in dissociation of both Pam16 and Pam18 from the translocon (Kozany *et al.*, 2004).

The idea that Pam16 and Tim44 directly interact is appealing. One can envision Tim44 acting as a tether for the critical components of the import motor, directly for mtHsp70 and indirectly for the J-protein cochaperone Pam18, via interaction with Pam16. Such an arrangement could allow precise positioning of 1) Hsp70 to interact with translocating polypeptides as they enter the matrix through the Tim23 channel and 2) Pam18 such that it efficiently stimulates the ATPase activity of Hsp70. The data for interaction with mtHsp70 are compelling, because the interaction is stable enough to be analyzed in vitro using purified components (Liu *et al.*, 2001). However, demonstration of a direct interaction between Pam16 and Tim44 will require further analysis. If the interaction between Pam16 and Tim44 is indeed direct, it is possible that the suppressors whose isolation is reported here identify the binding site between Pam16 and Tim44. However, it is perhaps more likely that these alterations cause a change in conformation of Tim44 that permits a more productive interaction between it and Pam16.



**Figure 7.** Suppression of defects caused by alterations in the N terminus of Pam16 by alterations in Tim44. (A) Tenfold serial dilutions of  $\Delta tim44 \Delta pam16$  cells expressing wt or mutant *TIM44* genes and Pam16[1-27]:Pam18 chimeras having the indicated amino acid alterations in the N terminus of the Pam16 segment were spotted on rich glucose medium and incubated at the indicated temperatures for 3 d. Dotted lines separate individual plates, which were all spotted at the same time. (B) Mitochondrial membrane association analysis of chimeric mutant proteins and suppressors. Sonicated mitochondria were subjected to centrifugation and equivalent samples of supernatant (S) and pellet (P) fractions, and unfractionated extract (T), were analyzed by SDS-PAGE and immunoblotted with Pam16-, Pam18-, and Mge1-specific antibodies. (C) Ten-fold serial dilutions of  $\Delta tim44 \Delta pam16$  cells expressing wt or mutant *TIM44* genes and full-length Pam16 with various amino-acid alterations were spotted on rich glucose medium and incubated at the indicated for 3 d at 23°C or 2 d at 30°C. Dotted lines separate individual plates, which were all spotted at the same time.

### The Relative Roles of Pam16 and Pam18 in Tethering the Heterodimer to the Translocon

A picture emerges of a complex interaction of Pam16 and Pam18 with the translocon. We propose that the predominant player in the stability of the association of Pam16: Pam18 to the translocon is Pam16. The N-terminal 27 amino acids are important in this regard as indicated by the effect of alterations of these amino acids on the stability of the association of Pam16 with the translocon. However, we do not mean to imply that these amino acids of Pam16 are the only amino acids involved in such interactions. It is possible that the adjacent segment of Pam16 is important as well.

Consistent with this idea, the effect of alterations in the context of the chimera are much more severe than in the context of the full-length Pam16, and the chimera containing 52 rather than 27 amino acids of Pam16 permits robust growth at higher temperatures.

The only known direct interaction of Pam18 with the translocon is between the IMS domain of Pam18 and Tim17. This interaction is dispensable under typical growth conditions in the laboratory, because no obvious phenotypic effect was observed. However, this interaction does play a role as demonstrated by the additive effect of additional mutations. We predict that there are environmental conditions under which this interaction makes a significant difference in growth, perhaps when import rates into mitochondria need to be maximal.

Because of the strength of the Pam16 interaction with the translocon, compared with that of Pam18, the interaction between Pam16 and Pam18 themselves plays a crucial role in the tethering Pam18. This effect is very apparent in the presence of single amino acid alterations that weaken the stability of the heterodimer. Using digitonin-treated mitochondria, which solubilizes the translocon, Pam16, but not Pam18, can be coprecipitated with the core translocon complex.

### Summary

In sum, we propose that the strength of the association of Pam16 and Pam18 with the translocon is the cumulative effect of at least three interactions: Pam18<sub>IMS</sub> with Tim17 on the intermembrane space side of the inner membrane, the Pam18 J-domain with the Pam16 J-like domain, and the N terminus of Pam16 with the peripheral membrane protein Tim44. In addition, the transmembrane domain of Pam18 likely serves to stabilize the interaction, by maintaining the complex in the two-dimensional space of the inner membrane.

In a broader sense, our results are consistent with the idea that Tim44 serves as a "molecular platform" on which both mtHsp70 and the Pam16:Pam18 heterodimer are positioned to allow Pam18 to efficiently interact with mtHsp70, stimulating its ATPase activity, thus driving the import process. This interaction may well be regulated through interaction of Tim44 with the channel, with a change in conformation occurring when a translocating polypeptide is in the channel, perhaps aligning Pam18 to stimulate mtHsp70s ATPase activity and subsequent release of mtHsp70 from the translocon.

### ACKNOWLEDGMENTS

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