Architecture of the TIM23 Inner Mitochondrial Translocon and Interactions with the Matrix Import Motor*

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**Background:** Mitochondrial import motor associates with inner membrane translocase TIM23 by undefined interactions.

**Results:** Site-specific photo-cross-linking was detected between matrix loops of TIM23 subunits Tim23 and Tim17 with Tim44 and Pam17, respectively.

**Conclusion:** Motor components are tethered to translocon via the scaffold Tim44, with regulatory protein Pam17 having independent interaction site.

**Significance:** Defined motor translocon architecture provides foundation for mechanistic understanding of efficient regulated translocation.

Translocation of proteins from the cytosol across the mitochondrial inner membrane is driven by action of the matrix-localized multi-subunit import motor, which is associated with the TIM23 translocon. The architecture of the import apparatus is not well understood. Here, we report results of site-specific in vivo photocross-linking along with genetic and coimmunoprecipitation analyses dissecting interactions between import motor subunits and the translocon. The translocon is composed of the two integral membrane proteins Tim23 and Tim17, each containing four membrane-spanning segments. We found that Tim23 having a photoactivatable cross-linker in the matrix exposed loop between transmembrane domains 1 and 2 (loop 1) cross-linked to Tim44.

Alterations in this loop destabilized interaction of Tim44 with the translocon. Analogously, Tim17 having a photoactivatable cross-linker in the matrix exposed loop between transmembrane segments 1 and 2 (loop 1) cross-linked to Pam17. Alterations in this loop caused destabilization of the interaction of Pam17 with the translocon. Substitution of individual photoactivatable residues in Tim44 and Pam17 in regions we previously identified as important for translocon association resulted in cross-linking to Tim23 and Tim17, respectively. Our results are consistent with a model in which motor association is achieved via interaction of Tim23 with Tim44, which serves as a scaffold for association of other motor components, and of Tim17 with Pam17. As both Tim44 and Pam17 have been implicated as regulatory subunits of the motor, this positioning is conducive for responding to conformational changes in the translocon upon a translocating polypeptide entering the channel.

The majority of mitochondrial proteins are encoded by nuclear DNA. Therefore, active mitochondrial function and biogenesis depend on efficient import of proteins translated on cytosolic ribosomes (1). Preproteins destined for the mitochondrial matrix initially enter mitochondria via the TOM complex of the outer membrane and are translocated across the inner membrane into the matrix through the TIM23 complex (2, 3). The TIM23 complex is heterotetrameric (4, 5), composed of two copies each of two related integral membrane proteins, Tim23 and Tim17. Both Tim23 and Tim17 have four membrane-spanning domains and expose to the matrix a relatively large (~24 residues) hydrophilic loop between transmembrane segments 1 and 2 (loop 1). Tim23 forms a protein-conducting pore, whereas evidence suggests that Tim17 regulates the TIM23 channel (5–9). Most proteins destined for the matrix have an amino-terminal targeting sequence (presequence) whose translocation across the inner membrane is driven by the membrane potential. For complete translocation, the TIM23 complex cooperates with a heat shock protein 70 (Hsp70)-based, PAM (presequence translocase-associated motor) complex, which resides on the matrix side of the inner membrane (10, 11).

The Hsp70 (called Ssc1 in Saccharomyces cerevisiae) component of PAM directly interacts with translocating polypeptides. Similar to other Hsp70s (12, 13), Ssc1 contains a peptide binding domain that interacts with short segments of unfolded polypeptide in its ATP-bound state. This interaction is stabilized by ATP hydrolysis, which is stimulated by its J domain protein partner, Pam18, an essential component of PAM (14–16). Pam16 and Tim44 are essential PAM accessory proteins, which have been proposed to play both structural and regulatory roles (17–20). Pam18 forms a stable heterodimer with Pam16 (17, 21, 22), which has been suggested to serve as a bridge between Pam18 and the translocon through its interaction with Tim44, which acts as a scaffold (17–20, 23–25). Pam18 also interacts on the intermembrane space side of the inner membrane with Tim17. Thus, the Pam16:Pam18 heterodimer has two modes of interaction with the translocon (19, 26). PAM also contains the nonessential protein called Pam17, which is anchored in the

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3 The abbreviations used are: TIM, transporter of inner membrane; Bpa, p-benzoyl-l-phenylalanine.
inner membrane through two transmembrane helices, exposing both terminal segments to the matrix (27).

Evidence indicates that interaction of the motor with the translocon is essential for preprotein translocation, but solid information as to the overall architecture of the presequence translocation apparatus of the inner membrane, particularly how PAM interacts with the TIM23 complex, is minimal (17, 19, 21, 22, 26). Here, we report the results of genetic and co-immunoprecipitation approaches, coupled with site-specific in vivo cross-linking experiments. Cross-linking was performed by engineering the incorporation of a photo-activatable non-natural amino acid p-benzoyl-l-phenylalanine (Bpa) in place of specific residues of components of the TIM23 and PAM complexes (28, 29). This cross-linking approach has several important advantages. In addition to the ability to incorporate Bpa at a single site within a single protein, the size of Bpa is only modestly more bulky than the natural amino acid phenylalanine, having only an additional benzoyl group, and thus increasing the odds of only detecting direct interactions. Together, our results strongly support the conclusion that loop 1 of Tim23 directly interacts with the motor subunit Tim44, which itself serves as a scaffold for the PAM subunits Pam16, Pam18, and Scc1, whereas loop 1 of Tim17 interacts with Pam17. Our work establishes a foundation for future understanding of the regulation of the activity of the import motor.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Plasmid, and Genetic Techniques—**All in vivo experiments were carried out in the W303 genetic background with derivatives of PJ53 (30). tim17−Δ and pam17−Δ haploids were described previously (31, 32). To create tim23−Δ haploids, a heterozygous TIM23/tim23:HIS3 diploid was constructed by replacing the ORF on one chromosome with the HIS3 gene and then transforming with pRS316-TIM23. tim23−Δ haploids carrying pRS316-TIM23 were isolated after sporulation by dissection of tetrads. Mutagenic analysis of the loop regions of TIM23 and TIM17 was performed by using the QuikChange method (Stratagene), starting with pRS315-TIM23 and pRS315-TIM17 as templates.

Plasmid systems were set up for the incorporation of Bpa into four proteins: Tim23, Tim44, Tim17, and Pam17. Tim23, Tim17, and Pam17 were expressed from the TEF1 promoter. Tim17 and Pam17 were expressed from pRS414, whereas Tim23 was expressed from pRS415 (33). The levels of Bpa-containing proteins did not exceed the normal levels of protein by >2-fold.

Tim44 was expressed from its endogenous promoter in pRS314. For each gene, codons for a hexahistidine tag were inserted using QuikChange. For Tim23, the tag was placed at the C terminus. For the other three genes, the codons were placed such that the tag was fused to the N terminus of the mature protein. Tim44 and Pam17 have N-terminal cleavable presequences. The His-encoding codons were inserted between the codons for the presequence and the first residue of the mature protein that is between residues 37 and 38 for Pam17 and between residues 43 and 44 for Tim44. QuikChange was used to substitute codons in the ORF with the amber codon TAG to create site-specific mutations, which were subsequently confirmed by sequencing. ptRNA-Bpa containing the nonsense suppressor tRNA/tRNA synthetase system was a gift from Anna Mapp (34).

Because Pam17 is not an essential protein, the PAM17-UAG plasmid (pRS414-PAM17-168UAG) and ptRNA-Bpa were cotransformed into pam17−Δ haploids to obtain the UAG strains. For the essential proteins Tim23, Tim17, and Tim44, more complicated strategies were used to create the UAG strains. In the case of Tim23 and Tim17, ptRNA-Bpa was cotransformed with the UAG plasmid (pRS415-TIM23-UAG and pRS414-TIM17-UAG, respectively) into their respective plasmid shuffle strains, followed by selection on medium containing 10 mM 5-fluoro-orotic acid and 2 mM Bpa. In the case of Tim44, ptRNA-Bpa was cotransformed with the TIM44-UAG plasmid (pRS314-TIM44-UAG) into a GAL1-TIM44 strain, in which the endogenous TIM44 promoter was replaced by the GAL1 promoter. The transformants were grown in glucose-based minimal medium containing 2 mM Bpa to repress expression of endogenous Tim44 while inducing expression of Tim44Bpa.

**Co-immunoprecipitation from Mitochondrial Lysates—**Association of Pam16, Pam17, Pam18, and Tim44 with the TIM23 complex was assessed by co-immunoprecipitation as described previously (32). To ensure low background, antibodies against Tim23 were affinity-purified prior to cross-linking to protein A Sepharose beads (32). Mitochondria were solubilized at 1 mg/ml in mitochondrial lysis buffer (25 mM Tris–HCl, pH 7.5, 10% glycerol, 80 mM KCl, 5 mM EDTA, and 1 mM PMSF) containing 1% digitonin (Acros Organics) on ice for 40 min with gentle mixing (15). After spinning at 14,000 rpm at 4 °C for 15 min, the lysates were added to 20 μl (bed volume) of Tim23 antibody beads and incubated 1.5 h with mixing at 4 °C. The beads were washed three times with lysis buffer containing 0.1% digitonin before boiling in sample buffer. The proteins were separated on SDS-PAGE and detected by immunoblotting. Image quantification was performed using ImageJ software and analyzed using GraphPad Prism software (version 6.0).

**Antibodies—**Immunoblot analysis was carried out using standard techniques with protein detection by enhanced chemiluminescence (GE Healthcare), using polyclonal antibodies to Tim23 (19), Tim44 (35), Pam16 (22), Pam18 (14), Pam17 (32), and Tim17 (gift from Nikolaus Pfanner) (26).

**In Vivo Photo-cross-linking—**Bpa (Bachem) was added to media from a 200 mM stock solution in 1 N NaOH, with an equal volume of 1 N HCl added to media for neutralization. During cultivation, cells were kept in the dark to prevent cross-linking reactions. Yeast cells were harvested, resuspended, and divided in two. One-half was UV-irradiated for 1 h at 4 °C at a distance of 2 cm from a 365-nm UV-A lamp (Stratalinker 1800 UV irradiator); the other half was kept on ice, as a control for no cross-linking. 25 A (opt) equivalent of yeast culture were resuspended in 0.5 ml of whole cell lysis buffer (20 mM HEPES–KOH, pH 8.0, 10% glycerol, 500 mM NaCl, 20 mM imidazole, 0.2% Triton X-100, 1 mM DTT, and 1 mM PMSF), and cell lysates were prepared using 0.5-mm glass beads with a vortex mixer. Ten cycles of agitation (40 s) were interspersed with cycles of cooling on ice to avoid overheating of the cell suspension. An additional 0.5 ml of lysis buffer was added prior to centrifugation for 15 min at 20,000 × g. Covalent protein adducts were isolated.
Terminus.

Tim23Bpa variants supported growth if Bpa was included in the growth medium, making them amenable to analysis. To covalently cross-link binding partners, suspensions of cells expressing Tim23Bpa were exposed to UV irradiation. The C-terminal hexahistidine tag was used to pull down Tim23 and associated proteins, which were then separated by electrophoresis, and proteins were detected by immunoblotting. In five cases, we observed a cross-linked product migrating at ~56 kDa that reacted with Tim23 antibody. However, antibodies specific for Tim17, Tim44, Pam16, Pam18, or Pam17 did not detect this Tim23 adduct (for examples see Fig. 1, B and C). As Tim23 is known to dimerize (4, 5), the adduct may be a Tim23 homodimer. In one case, that of Tim23N127Bpa, an additional species, migrating at 70 kDa was observed. This 70 kDa species was also detected with antibodies against Tim44 (Fig. 1D), indicating that loop 1 of Tim23 is in very close proximity to Tim44.

Loop 1 of Tim23 Is Important for Association of the PAM Subcomplex with the Translocon—We carried out genetic analyses to assess the in vivo importance of residues in the loop region. We used an unbiased approach, carrying out alanine-scanning mutagenesis, substituting three residues at a time, to test this idea, we carried out in vivo site-specific cross-linking using enhanced nonsense suppression (29, 38, 39) to incorporate the photo-reactive amino acid Bpa in place of the endogenous amino acid. Focusing first on Tim23, we incorporated Bpa at seven distinct sites (Ile-124, Asn-127, Leu-134, Val-137, Leu-138, Asn-139, and Ile-141) loop 1 (residues 122–145) (Fig. 1A). In all but two cases (Leu-138 and Asn-139 Bpa), the Tim23Bpa variants supported growth if Bpa was included in the growth medium, making them amenable to analysis. To covalently cross-link binding partners, suspensions of cells expressing Tim23Bpa were exposed to UV irradiation. The C-terminal hexahistidine tag was used to pull down Tim23 and associated proteins, which were then separated by electrophoresis, and proteins were detected by immunoblotting. In five cases, we observed a cross-linked product migrating at ~56 kDa that reacted with Tim23 antibody. However, antibodies specific for Tim17, Tim44, Pam16, Pam18, or Pam17 did not detect this Tim23 adduct (for examples see Fig. 1, B and C). As Tim23 is known to dimerize (4, 5), the adduct may be a Tim23 homodimer. In one case, that of Tim23N127Bpa, an additional species, migrating at 70 kDa was observed. This 70 kDa species was also detected with antibodies against Tim44 (Fig. 1D), indicating that loop 1 of Tim23 is in very close proximity to Tim44.

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substituted proline for the asparagine at position 139 with the goal of disrupting the helix. \textit{tim23N139P}, similar to \textit{tim23137AAA}, was temperature-sensitive for growth (Fig. 2A).

To test whether alterations in loop 1 that resulted in growth defects also affected interaction of PAM with the TIM23 complex, we carried out co-immunoprecipitation experiments using mitochondrial lysates and affinity-purified antibodies specific for Tim23. As expected (40), the PAM components Tim44, Pam16, and Pam18 co-precipitated with Tim23 in WT lysates. However, these PAM components, particularly Tim44, were reduced in co-precipitates from \textit{tim23137AAA} and \textit{tim23N139P} lysates, even though the amounts of Tim23 precipitated from the three extracts were similar (Fig. 2B). That Pam16 and Pam18 are reduced less than Tim44 may be due to the interaction between the N-terminal domain of Pam18 and Tim17 in the intermembrane space (19, 26). Overall, these results indicate that the alterations in loop 1 of Tim23 that compromise growth also compromise association of PAM components with the translocon.

The \textit{N} \textbf{Terminus} of \textit{Tim44} Cross-linked to \textit{Tim23} \textbf{in Vivo}—The results described above strongly suggest that Tim44 directly interacts with loop 1 of Tim23. With a goal of testing the idea that Tim44 and Tim23 interact, we carried out cross-linking using cells expressing Bpa-containing Tim44. We took advantage of our previous results, indicating that alterations in the N terminus of Tim44 between residues 154 and 180 destabilize its association with the TIM23 complex (40). We successfully incorporated Bpa at three Tim44 residues: Ser-160, Ile-163, and Asp-165. After utilizing the N-terminal hexahistidine tag to pull down Tim44, we observed a cross-linked product that migrated at 70 kDa in the strains having Bpa incorporated at positions 160, 163, or 165 (Fig. 3, A–C). The 70 kDa cross-linked products were identified as Tim44-Tim23 adducts by immunoblotting with Tim23-specific antibodies (Fig. 3, A–C). Taken together, these results indicate that the N terminus of Tim44 and Tim23 loop 1 directly interact and that this interaction is important for Tim44 association with the translocon.

Loop 1 of \textit{Tim17} Cross-linked to \textit{Pam17} \textbf{in Vivo}—We next turned our attention to Tim17. We asked whether the matrix-exposed loop 1 of Tim17 could be cross-linked to PAM components \textit{in vivo}. We examined five Tim17 sites (Ile-35, Phe-38, Leu-43, Ser-48, and Ile-54). After UV treatment we observed a Tim17 cross-reactive band migrating at 37 kDa in lysates from cells expressing Tim17\textit{I35Bpa} and Tim17\textit{L43Bpa} (Fig. 4, B and C). After stripping, the blot was retested with our set of PAM antibodies. The 37-kDa product was identified as a Tim17-Pam17 adduct based on reactivity with Pam17-specific antibodies (Fig. 4, B and C). These results indicate that Pam17 and loop 1 of Tim17 are in a close proximity within the TIM23-PAM complex.

\textit{Pam17} C \textbf{Terminus} Cross-linked to \textit{Tim17} \textbf{in Vivo}—Taking advantage of our previous result indicating that residues 167–169 (DYY) of the C terminus of Pam17 are important for its association with the translocon (32), we made constructs to incorporate Bpa into this region. Bpa was successfully incorporated at position Tyr-168. Pam17 and its cross-linked adducts were pulled down using the N-terminal hexahistidine tag. Using Pam17-specific antibodies, we detected a cross-linked product that migrated at 37 kDa. This adduct was also detected using antibodies specific for Tim17 (Fig. 5). Taken together, these results provide evidence for a direct interaction between Pam17 and Tim17.

Loop 1 of \textit{Tim23} \textbf{Is Important for Association of the PAM Subcomplex with Translocon}—To test the importance of the residues constituting loop 1 of Tim23 in Pam17 association, we generated \textit{TIM17} triple alanine substitution mutations, as described above for \textit{TIM23}. The eight variants grew indistinguishably from WT cells at a variety of temperatures (Fig. 6A). Because the phenotypic affect of a deletion of \textit{PAM17} is very mild under laboratory conditions, the absence of a growth phenotype of the mutations that alter loop 1 of Tim17 is not surprising if the primary function of the loop is to interact with Pam17. To better assess the importance of the loop, we took
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FIGURE 3. Site-specific cross-linking of Tim44 to Tim23. Yeast strains express Tim44-S160Bpa (A), Tim44-I163Bpa (B), and Tim44-D165Bpa (C) were grown in the presence of Bpa. After UV irradiation, Tim44 was affinity purified via its N-terminal His6 tag, followed by SDS-PAGE and immunoblotting with the indicated antibodies. Migration of size standards are indicated (left); Tim44 (44) and Tim23 (23) reactive bands are indicated. Cross-linking experiments were repeated a minimum of two times.

FIGURE 4. Site-specific cross-linking of Tim17 to Pam17. A, diagram of Tim17 showing loop 1 protein sequence with the end point residue numbers indicated. Yeast strains express Tim17-I35Bpa (B) and Tim17-L43Bpa (C) were grown in the presence of Bpa. After UV irradiation, Tim17 was affinity purified via its N-terminal His6 tag and followed by SDS-PAGE and immunoblotting with the indicated antibodies. Migration of size standards are indicated (left); Tim17 protein, Pam17 protein, and adduct are indicated. Cross-linking experiments were repeated a minimum of two times. N/N terminus; C, C terminus. IMS, intermembrane space.

FIGURE 5. Site-specific cross-linking of Tim17 to Pam17. A yeast strain express Pam17-Y168Bpa was grown in the presence of Bpa. After UV irradiation, Pam17 was affinity purified via its N-terminal His6 tag and followed by SDS-PAGE and immunoblotting with the indicated antibodies. Migration of size standards, in kDa, are indicated (left); Pam17 protein and adduct are indicated. Cross-linking experiments were repeated a minimum of two times.

two additional approaches: assessing co-immunoprecipitation of Pam17 and carrying out a genetic interaction test.

To test whether alterations in Tim17 loop 1 affected interaction of Pam17 with the TIM23 complex, co-immunoprecipitation experiments using affinity-purified antibodies against Tim23 were carried out as described above. The amount of Pam17, but not Pam16, Pam18, or Tim44, co-precipitating was substantially reduced in tim17_33AAA, tim17_36AAA, tim17_39AAA, and tim17_42AAA lysates compared with WT, even though the amounts of Tim17 precipitated from all mutant extracts were similar to that precipitated from WT lysates (Fig. 6B).

Second, we took advantage of our previous finding that cells having the mutation tim44_ F54S grew extremely poorly in the absence of PAM17 (32). We reasoned that mutations that affect the association of Pam17 with the translocon might also exhibit genetic interaction with tim44_ F54S. Therefore, we constructed double mutants, combining tim44_ F54S with each Tim17 triple alanine mutation. tim44_ F54S does not form colonies at 37 °C, but grows robustly at 34 °C and below. Six TIM17 alanine mutants, those between residues 33 and residue 50, displayed negative genetic interactions with tim44_ F54S (i.e. 33AAA, 36AAA, 39AAA, 42AAA, 45AAA, and 48AAA) (Fig. 6C). tim17_39AAA and tim17_45AAA showed the most dramatic effects, with growth being severely affected in the double mutants at 30 °C. Surprisingly, the mutations altering residues in the most C-terminal part of the loop, tim17_54AAA, suppressed the growth defect of tim44_ F54S, allowing colony formation at 37 °C (Fig. 6C). Together, these results support the idea that loop 1 of Tim17 interacts with Pam17, tethering it to the translocon. In addition, because of the suppression of tim44_ F54S by tim17_54AAA, these results also suggest a possible unsuspected regulatory influence.

DISCUSSION

By coupling site-specific cross-linking to genetic and co-immunoprecipitation studies, we have extended the understanding of the architecture of the TIM23 presequence translocase/PAM interaction. We provide evidence of specific interaction interfaces between the subunits of the TIM23 complex, Tim23 and Tim17, and PAM subunits. More specifically, we uncovered interactions between the translocon subunits Tim23 and Tim44 and between Tim17 and Pam17. Both interactions occur via matrix-exposed loop 1 between membrane segments 1 and 2 of Tim23 and of Tim17.

The Tim23/Tim44 Interaction—Our studies using Bpa incorporated into loop 1 of Tim23 detected two different Tim23...
cross-links. The cross-linking product migrating at $\sim 56$ kDa did not react with any of the antibodies specific for components of PAM or Tim17. This lack of interaction is consistent with a cross-link to another Tim23 monomer of the TIM23 complex. An early analysis using nonspecific cross-linkers revealed a Tim23/Tim23 cross-link that also migrated at $\sim 56$ kDa, with the N-terminal intermembrane space domain being determined to be important for homo-dimerization (5). Our results suggest that loop 1 of the monomers is in close proximity as well.

Our results are also consistent with loop 1 of Tim23 serving as the major docking site of Tim44 with the translocon. First, the second cross-link adduct that we detected, which migrated at $\sim 70$ kDa, reacted with Tim44-specific antibody. Secondly, certain alterations in loop 1 had both deleterious effects on growth and resulted in destabilization of the interaction of Tim44, Pam16, and Pam18 with the translocon. The predominant importance of loop 1 is also consistent with the recent report that a Tim23 truncation lacking transmembrane segments 3 and 4, and thus loop 3, is able to support growth, as loop 1 remains intact in such a strain (41).

Pam18 and Pam16 destabilization, along with Tim44 destabilization, is consistent with the wealth of data indicating that Tim44 serves as the predominant tether for the Pam16/Pam18 heterodimer to the translocon (17–20, 32, 40). Both cross-linking data presented here, and our earlier co-immunoprecipitation analysis of tim44 mutant strains (40) is consistent with the N terminus of Tim44, more specifically, residues 154 through 180, being important for interaction with loop 1 of Tim23.

Much evidence suggests that Tim44 serves as more than a physical tether for Pam16/Pam18 to the translocon. Precedences, typically present at the N terminus of translocating polypeptides, interact with Tim44 (10), as does Ssc1 (40). This density of interactions centering around Tim44 makes it tempting to speculate that the regulation of the import motor, likely necessary for required efficiency for the import process, is driven in part by a set of conformational changes of Tim44 serving to orient the nascent chain relative to Ssc1 and Ssc1 relative to its obligatory l-protein chaperone Pam18.

**Tim17/Pam17 Interaction**—Our data strongly suggest that Pam17, via its C-terminal matrix localized domain, directly interacts with loop 1 of Tim17. Not only were cross-links to Pam17 detected upon incorporation of Bpa into loop 1 of Tim17, a Tim17 variant having Bpa incorporated at a site previously shown to be important for its association with the translocon (32) cross-linked to Tim17 and alterations in loop 1 of Tim17 disrupted association of Pam17 with the translocon. Although no cross-linking between Pam17 and Tim17 has been reported previously, a cross-link between Tim23 and Pam17 was reported using the nonspecific cross-linker disuccinimidyl glutarate (42). Disuccinimidyl glutarate, which has a spacer arm of 7.7 Å, forms irreversible amide bonds with lysine residues. We did not observe Tim23-Pam17 cross-links, even though Bpa was incorporated at five different positions in loop 1. The

### Table:

| TIM44 | TIM17 |
|-------|-------|
| WT | WT |
| 23°C | 30°C | 34°C | 37°C |
| 33AAA | 36AAA | 39AAA | 42AAA | 45AAA | 48AAA | 51AAA | 54AAA |

FIGURE 6. Alterations in loop 1 of Tim17 affect Pam17 association with the translocon. A, growth phenotype. 10-Fold serial dilutions of tim17-Δ cells expressing the indicated proteins were plated on minimal media and incubated at the indicated temperatures for 3 days. B, co-immunoprecipitation with Tim23. Mitochondria were solubilized by treatment with digitonin. Solubilized material was subjected to immunoprecipitation using Tim23-specific antibody.

Alterations in loop 1 of Tim17 affect Pam17 association with the translocon.

Our data strongly suggest that Pam17, via its C-terminal matrix localized domain, directly interacts with loop 1 of Tim17. Not only were cross-links to Pam17 detected upon incorporation of Bpa into loop 1 of Tim17, a Tim17 variant having Bpa incorporated at a site previously shown to be important for its association with the translocon (32) cross-linked to Tim17 and alterations in loop 1 of Tim17 disrupted association of Pam17 with the translocon. Although no cross-linking between Pam17 and Tim17 has been reported previously, a cross-link between Tim23 and Pam17 was reported using the nonspecific cross-linker disuccinimidyl glutarate (42). Disuccinimidyl glutarate, which has a spacer arm of 7.7 Å, forms irreversible amide bonds with lysine residues. We did not observe Tim23-Pam17 cross-links, even though Bpa was incorporated at five different positions in loop 1. The

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FIGURE 6. Alterations in loop 1 of Tim17 affect Pam17 association with the translocon. A, growth phenotype. 10-Fold serial dilutions of tim17-Δ cells expressing the indicated proteins were plated on minimal media and incubated at the indicated temperatures for 3 days. B, co-immunoprecipitation with Tim23. Mitochondria were solubilized by treatment with digitonin. Solubilized material was subjected to immunoprecipitation using Tim23-specific antibody.

Alterations in loop 1 of Tim17 affect Pam17 association with the translocon.

Our data strongly suggest that Pam17, via its C-terminal matrix localized domain, directly interacts with loop 1 of Tim17. Not only were cross-links to Pam17 detected upon incorporation of Bpa into loop 1 of Tim17, a Tim17 variant having Bpa incorporated at a site previously shown to be important for its association with the translocon (32) cross-linked to Tim17 and alterations in loop 1 of Tim17 disrupted association of Pam17 with the translocon. Although no cross-linking between Pam17 and Tim17 has been reported previously, a cross-link between Tim23 and Pam17 was reported using the nonspecific cross-linker disuccinimidyl glutarate (42). Disuccinimidyl glutarate, which has a spacer arm of 7.7 Å, forms irreversible amide bonds with lysine residues. We did not observe Tim23-Pam17 cross-links, even though Bpa was incorporated at five different positions in loop 1. The
observed cross-link with disuccinimidyl glutarate must have occurred on the matrix side of the inner membrane, because all of the lysines present in Pam17 are localized to the matrix. It is possible that a second interaction site that we did not detect occurs with Tim23. Alternatively, because Tim17 and Tim23 interact closely with each other, the observed disuccinimidyl glutarate cross-link may not reflect a direct interaction, but rather the close proximity of the two proteins. It is worth noting that the matrix-exposed loop of Tim23 are more lysine-rich than those of Tim17, five lysines in Tim23 compared with two lysines in Tim17.

The lack of an obvious phenotype of any of the Tim17 mutants we tested is consistent with the major function of loop 1 being to interact with Pam17, as the only reported growth phenotype of pam17Δ cells is somewhat slower growth on synthetic glycerol medium (27). However, our results also suggest a more complicated relationship between Tim17 and Pam17 than expected if the only role was simply tethering. There is not a strict correspondence between the affect of Tim17 alterations on the stability of the interaction with Pam17 and the genetic interactions they have with tim44ΔF54S. In particular, although the tim17Δ4AAA alteration has no obvious effect on Tim44 association with the translocon, it has a severe negative genetic interaction with tim44ΔF54S. On the other hand, tim17Δ4AAA substantially suppressed the inability of tim44ΔF54S cells to grow at 37 °C without having an obvious affect on the stability of the Tim17-Pam17 interaction. Although the precise role of Pam17 has been controversial, there is general agreement that Pam17 serves a regulatory role in the initial stages of import of a protein across the inner membrane (31, 42–45). Our results are consistent with this overall conclusion.

**Summary**—Our results presented here establish a new level of understanding of the architecture of the TIM23 import machinery of the mitochondrial inner membrane, defining the interaction of Tim23 with Tim44 and Tim17 with Pam17. Although the exact juxtaposition of neither Tim23 and Tim17 to one another nor the homodimeric subunits themselves in the tetrameric TIM23 complex is yet completely understood, our results are compatible with models in which conformational changes within the TIM23 complex upon binding the presence of the translocating polypeptide serving as a first signal to activate the import motor. Our results provide a solid foundation for future experiments dissecting these regulatory mechanisms and fundamental issues, such as whether the interaction of the PAM complex with the TIM23 complex is dynamic (42, 46).

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