Two Intermembrane Space TIM Complexes Interact with Different Domains of Tim23p during Its Import into Mitochondria

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Abstract. Tim23p (translocase of the inner membrane) is an essential import component located in the mitochondrial inner membrane. To determine how the Tim23 protein itself is transported into mitochondria, we used chemical cross-linking to identify proteins adjacent to Tim23p during its biogenesis. In the absence of an inner membrane potential, Tim23p is translocated across the mitochondrial outer membrane, but not inserted into the inner membrane. At this intermediate stage, we find that Tim23p forms cross-linked products with two distinct protein complexes of the intermembrane space, Tim8p–Tim13p and Tim9p–Tim10p. Tim9p and Tim10p cross-link to the COOH-terminal domain of the Tim23 protein, which carries all of the targeting signals for Tim23p. Therefore, our results suggest that the Tim9p–Tim10p complex plays a key role in Tim23p import. In contrast, Tim8p and Tim13p cross-link to the hydrophilic NH_2-terminal segment of Tim23p, which does not carry essential import information and, thus, the role of Tim8p–Tim13p is unclear. Tim23p contains two matrix-facing, positively charged loops that are essential for its insertion into the inner membrane. The positive charges are not required for interaction with the Tim9p–Tim10p complex, but are essential for cross-linking of Tim23p to components of the inner membrane insertion machinery, including Tim54p, Tim22p, and Tim12p.

Key words: protein translocation • cross-linking

Introduction

Although much is known about how proteins are imported into the mitochondrial matrix, very little is known about how proteins are sorted to the mitochondrial membranes (for reviews see Lithgow et al., 1997; Pfanner and Meijer, 1997; Bauer et al., 2000; Ryan et al., 2000). Most mitochondrial proteins, whether soluble or membrane-bound, are encoded in the nucleus, translated on cytosolic ribosomes, and imported posttranslationally into the mitochondria. Mitochondrial precursor proteins are bound by cytosolic chaperones and targeted to specific receptors on the outer membrane (OM;[1] Ryan et al., 1997). In yeast, there are at least two major receptors on the OM, Tom20p and Tom70p (translocase of the outer membrane), and each receptor has a preference for different precursor proteins (Hase et al., 1983; Hines et al., 1990; Steger et al., 1990; Moczko et al., 1993; Ramage et al., 1993; Gratzer et al., 1995; Honlinger et al., 1996). For example, Tom20p interacts primarily with presequence-bearing proteins, whereas Tom70p prefers membrane proteins with internal targeting signals. After interaction with the Tom20p or Tom70p receptors, the precursor is transferred to the TOM complex, which mediates the transport of proteins through the mitochondrial OM (Kiebler et al., 1990; Söllner et al., 1992). TOM consists of five proteins (Tom40p, Tom22p, Tom7p, Tom6p, and Tom5p) that form a protein-translocating pore in the OM (Hill et al., 1998; Kunkele et al., 1998a,b). The TOM complex is used by all substrates, regardless of whether their final destination is the outer membrane, the intermembrane space (IMS), the inner membrane (IM), or the matrix (Ryan et al., 1999).

Unlike the OM, the mitochondrial inner membrane contains at least two separate translocons. One translocon, called the TIM23 complex, consists of the integral membrane proteins Tim23p and Tim17p (translocase of the inner membrane; Dekker et al., 1993; Emtage and Jensen, 1993), which appear to form a protein-translocating pore

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[1]*Abbreviations used in this paper:* CCCP, carbonyl cyanide m-chlorophenylhydrazone; DSP, dithiobis(succinimidyl propionate); IM, inner membrane; IMS, inner membrane space; OM, outer membrane; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate; Tim, translocase of the inner membrane; Tom, translocase of the outer membrane; *WT*, wild-type.

in the IM (Lohret et al., 1997). The TIM23 complex is required for the transport of presequence-containing proteins across the IM into the matrix, although a few IM membrane proteins are also inserted by the TIM23 machinery (Stuart and Neupert, 1996; Herrmann et al., 1997; Folsch et al., 1998; Kurz et al., 1999). The matrix-localized Tim44 and mtHsp70 proteins function to drive the translocation of substrates through the Tim23p-Tim17p translocon (Scherer et al., 1992; Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994; Ungermann et al., 1994; Blom et al., 1995). A second translocon, named the TIM22 complex, mediates the insertion of polytopic membrane proteins into the IM. The TIM22 complex consists of three integral membrane proteins: Tim54p, Tim22p, and Tim18p (Kerscher et al., 1997, 2000; Sirrenberg et al., 1998; Koehler et al., 2000). Proteins that require the TIM22 complex for insertion include the mitochondrial carrier family proteins, such as the ADP-ATP carrier, Aac2p, as well as some of the TIM components themselves, such as Tim23p, Tim22p, and Tim17p (Sirrenberg et al., 1996; Kerscher et al., 1997; Koehler et al., 2000).

The TIM22 complex works coordinately with three homologous proteins located in the intermembrane space: Tim12p, Tim10p, and Tim9p (Koehler et al., 1998a, 1998b; Sirrenberg et al., 1998; Adam et al., 1999; Endres et al., 1999). All of Tim12p is associated with an ~300-kD complex in the IM containing Tim54p, Tim22p, and Tim18p, whereas Tim10p and Tim9p are found in two locations (Koehler et al., 1998a,b; Sirrenberg et al., 1998; Adam et al., 1999; Endres et al., 1999). A fraction of the pool of Tim9p and Tim10p associates with the 300-kD Tim54p-Tim22p-Tim18p-Tim12p complex, whereas the remaining Tim9p and Tim10p combine to form an ~70-kD complex that is soluble in the IMS. The 70-kD Tim9p–Tim10p complex is thought to play a role in shuttling imported proteins from the TOM complex in the outer membrane to the TIM complex in the IM.

Recently, two new proteins, Tim13p and Tim8p, which are homologous to Tim12p, Tim10p, and Tim9p, have been identified (Koehler et al., 1999). Tim13p and Tim8p are mitochondrial proteins and appear to form an ~70-kD complex in the IMS distinct from the Tim10p–Tim9p complex (Koehler et al., 1999). Whereas Tim12p, Tim10p, Tim9p, Tim54p, and Tim22p are required for yeast cell viability (Koehler et al., 1998a,b; Sirrenberg et al., 1998; Adam et al., 1999); Tim13p and Tim8p are not essential (Koehler et al., 1999). Yeast cells disrupted for either *TIM8*, or *TIM13*, or both genes are completely viable. Moreover, it is not clear if Tim13p or Tim8p plays a role in mitochondrial protein import.

The Tim23 protein, like other IM proteins such as Aac2p, lacks an NH_2-terminal, cleavable targeting signal (Emtage and Jensen, 1993), and relies on the Tim54p–Tim22p complex for insertion into the IM (Kerscher et al., 1997). Tim23p has a hydrophilic NH_2-terminal domain facing the IMS, and four transmembrane segments in its COOH-terminal domain (Bauer et al., 1996; Davis et al., 1998; Ryan et al., 1998). Recently, we found that the NH_2-terminal half of Tim23p does not have essential targeting information, whereas the COOH-terminal domain contains at least two separate and distinct import signals (Davis et al., 1998; Ryan et al., 1998). Sequences within the first and fourth transmembrane segments are required to target Tim23p to mitochondria and for complete translocation across the OM. In addition, two positively charged, matrix-facing loops located between the transmembrane spans are necessary for the insertion of Tim23p into the IM.

To determine which TIM components recognize the different signals in Tim23p, we used chemical cross-linking to identify proteins adjacent to Tim23p during its import into mitochondria. When Tim23p is arrested at an intermediate step in its import, it is efficiently cross-linked to the Tim8 and Tim13 proteins. Tim23p also forms significant, but less abundant cross-links to Tim9p and Tim10p. In contrast, Aac2p, when arrested at the same stage, does not interact with the Tim8p–Tim13p complex, but instead cross-links only to Tim9p and Tim10p. We find that Tim8p and Tim13p cross-link to the Tim23p NH_2-terminal segment, whereas the Tim9p–Tim10p complex binds to the Tim23p COOH-terminal domain. The positively charged loops of Tim23p are not required for interaction with Tim8p, Tim9p, Tim10p, and Tim13p, but are essential for cross-linking to Tim54p, Tim22p, and Tim12p import components acting at a later step in the import pathway.

Materials and Methods

Strains

Wild-type strain D273-10B (Sherman, 1964), and KRR146 (Ryan et al., 1998), a *tim23::URA3 leu2 trp1 cyh2* strain carrying *TIM23* on a *TRP1-CYH2* plasmid, pKR1, have been described. The *tim8*Δ strain YRJ1203 and *tim13*Δ strain YRJ1204, in which the *TIM8* or *TIM13* open reading frame was replaced by the yeast *HIS3* gene, were constructed in strain FY833 (Brachmann et al., 1998) using the procedure of Lorenz et al. (1995). *tim8*Δ *tim13*Δ strain YRJ1205 and *TIM8 TIM13* strain YRJ1206 were constructed by crossing strain YRJ1203 to YRJ1204. Standard yeast media and genetic techniques were used (Kaiser et al., 1994).

Plasmids

SP6-TIM23 plasmid pJE29 (Ryan et al., 1998), which expresses Tim23pg, pAD67 (Davis et al., 1998), which expresses *L1L3neut* behind the SP6 promoter, pKR35 (Ryan et al., 1998), which expresses the Tim23Δ2-94 protein from the SP6 promoter, pJE50 (Emtage and Jensen, 1993), which expresses wild-type Tim23p in yeast, and pKR15 (Ryan et al., 1998), which expresses Tim23Δ2-94 in yeast, have been described. pGEM4Z-AAC2, a plasmid which expresses the *Saccharomyces cerevisiae* Aac2 protein from the SP6 promoter, was a gift from N. Pfanner (University of Freiburg, Freiburg, Germany).

pAD103, a plasmid which expresses the Tim23Δ2-50 protein from the SP6 promoter, was created using PCR and oligos 98 (5′-AACAGCTATGACCATG-3′) and 387 (5′-GGAGCGGCCGCCATGTCGACACCGC-3′) from the *TIM23* plasmid pJK2. The PCR fragment was digested with NotI and SacII and inserted into pKR35, creating pAD103. Tim23Δ2-50p consists of MGGR, followed by amino acids 52–222 of Tim23p. pAD105, a *CEN6-LEU2* plasmid which expresses Tim23Δ2-50p in yeast, was made by inserting a NotI-NcoI fragment from pAD103 into pJE5.

pAD104, which contains the Tim23Δ51-94 protein behind the SP6 promoter, was created using template pJK2 and oligos 11 (5′-CGATTTAGGTGACACTATAG-3′) and 388 (5′-CCGCGGCCGCCTCCACCAGGACCTG-3′). The PCR fragment was digested with NotI and HindIII and inserted into pKR35, creating pAD104. Tim23Δ51-94p consists of amino acids 1–50, followed by GGR, followed by amino acids 95–222 of Tim23p. pAD106, which contains Tim23Δ51-94, was made by inserting a Bsu36I-NcoI–digested fragment of pAD104 into pAD74 (Davis et al., 1998).

pAD108, which contains the Tim23Δ2-24 protein behind the SP6 promoter, was created using oligos 98 and 396 (5′-GGGGGCGGCCG-

CAAGCCTAAGGAACTATCG-3′) and pJK2. The PCR fragment was digested with NotI and SacII and inserted into pKR35, creating pAD108. Tim23Δ2-24p consists of Met, followed by GGR, followed by amino acids 25–222 of Tim23p. pAD109, which contains Tim23Δ2-24, was made by inserting a NotI-NcoI fragment from pAD108 into pJE5.

pAD110, which expresses the Tim23Δ2-24,Δ75-94 protein from the SP6 promoter, was created using oligos 399 (5′-GGGGCGGCCGCCTTCT-TCCAGATCTAAATAC-3′) and 11 and pAD108. The PCR fragment was digested with NotI and inserted into pKR35, creating pAD110. The Tim23Δ2-24,Δ75-94 protein consists of Met, followed by GGR, followed by amino acids 25–75, followed by GGR, followed by amino acids 95–222 of Tim23p. pAD111, which contains the Tim23Δ2-24,Δ75-94 protein, was created by inserting an XbaI-NcoI fragment from pAD110 into pAD109.

pAD112, which expresses the Tim23N-Aac2 protein from the SP6 promoter, was created by first engineering a NotI site in front of the second codon of Aac2p using oligos 99 (5′-AATACGACTCACTATAG-3′) and 400 (5′-CCCGGCGGCCGCTCTTCCAACGCCCAAGTC-3′) and pGEM4Z-AAC2. The PCR product was digested with NotI and the NotI blunt fragment was inserted into the NotI-PvuII–digested pKR14 (Ryan et al., 1998), an SP6 plasmid which encodes the Tim23p NH_2 terminus followed by an NotI site. The Tim23N-Aac2 fusion protein contains the first 96 amino acids of Tim23p, followed by GGR, and then followed by residues 2–318 of the Aac2 protein.

Imports into Isolated Mitochondria

Mitochondria were isolated as previously described (Daum et al., 1982). Precursor proteins were synthesized in reticulocyte lysate (Promega) using 1.5 mCi/ml (1,000 Ci/mmol) [^{35}S]methionine. 5 μl of precursor protein was incubated with 100 μg of mitochondria in 100 μl import buffer (0.6 M sorbitol, 50 mM Hepes-KOH, 25 mM KCl, 10 mM magnesium chloride, 2 mM potassium phosphate, 0.5 mM EDTA, 2 mM ATP, 2 mM NADH, and 1 mg/ml BSA, pH 7.4) at 30°C for 20 min and stopped on ice as previously described (Davis et al., 1998). As indicated, intact mitochondria, or mitochondria whose outer membrane was disrupted by osmotic shock (mitoplasts; Davis et al., 1998), were treated with 50 μg/ml proteinase K (Sigma Chemical Co.) for 20 min on ice, followed by the addition of 1 mM PMSF (Sigma Chemical Co.). To generate translocation intermediates, the mitochondrial inner membrane potential was dissipated by the addition of 40 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma Chemical Co.) to mitochondria in import buffer lacking NADH before the addition of precursors. To chase translocation intermediates, CCCP was removed from reactions by washing mitochondria twice in import buffer containing 20 mg/ml BSA as previously described (Hines et al., 1990). After SDS-PAGE, ^{35}S-labeled proteins were detected by fluorography (Bonner and Laskey, 1974) or phosphorimaging (model Storm 860; Molecular Dynamics). Quantification was performed using ImageQuant software version 1.2 (Molecular Dynamics Corp.).

Chemical Cross-linking and Immunoprecipitation

After import, mitochondria were treated with proteinase K, reisolated by centrifugation through a 1-ml sucrose cushion (0.625 M sucrose, 20 mM Hepes-KOH, pH 7.4) and treated with succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB; Pierce Chemical Co.) or dithiobis(succinimidyl propionate) (DSP; Pierce Chemical Co.) as previously described (Ryan et al., 1998), except that the final cross-linker concentrations were 400 μM. Mitochondrial pellets were analyzed by SDS-PAGE. Alternatively, after cross-linking reactions, mitochondrial pellets were solubilized in SDS-containing buffer and immunoprecipitated as previously described (Ryan et al., 1998), with the following modifications. Solubilized mitochondria (100 μl) were diluted with 1 ml TNET (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% [vol/vol] Triton X-100), centrifuged at 14,000 *g* for 10 min, and then 40 μl of a 1:1 slurry of protein A–Sepharose beads (Sigma Chemical Co.) in TNET were added to the supernatant. After 30 min at 4°C with rocking, beads were removed by centrifugation for 2 min at 14,000 *g*. Supernatants were transferred to new tubes containing either 10 μl of antiserum to Tim10p, or 20 μl of antiserum to Tim8p, Tim9p, Tim12p, Tim13p, Tim22p (Kerscher et al., 1997), and Tim54p (Kerscher et al., 1997).

Antibody Production

To raise antiserum to Tim10p and Tim12p, the complete open reading frame of each gene was PCR amplified from yeast genomic DNA and inserted into pMAL-cRI (New England Biolabs) as described previously (Sirrenberg et al., 1998). The MBP-Tim10p or MBP-Tim12p fusion proteins were expressed in DH5α bacteria, and crude protein homogenates were isolated as per the manufacturer's instructions. For Tim8p, Tim9p, and Tim13p, each open reading frame was PCR-amplified and inserted into the SapI-EcoRI sites of pTYB11 (New England Biolabs). Intein-Tim8p, Intein-Tim9p, and Intein-Tim13p fusion proteins were expressed in *Escherichia coli* strain ER2566 (New England Biolabs), and the inclusion bodies were purified as per the manufacturer's instructions. Proteins were purified by SDS-PAGE, stained with Coomassie blue R-250, and the bands containing the fusion proteins were excised. Gel slices were frozen in liquid nitrogen, ground in a mortar and pestle, and lyophilized. Injection of antigens into rabbits and the collection of antiserum were performed by Covance, Inc. Specificity of antisera to each Tim protein was determined by immunoprecipitation of in vitro translated, ^{35}S-labeled proteins in the presence of excess cold mitochondrial proteins (see Table I).

Results

A Tim23p Translocation Intermediate Cross-links to Two Small Proteins

Tim23p is a 23-kD protein anchored in the IM by four transmembrane segments, with an ~10-kD hydrophilic, NH_2-terminal domain facing the intermembrane space. When ^{35}S-labeled Tim23p was incubated with isolated mitochondria, Tim23p was imported across the mitochondrial outer membrane and protected from externally added protease (Fig. 1 A, lane 1). When the mitochondrial OM was disrupted by osmotic shock, a ~12-kD fragment of Tim23p, diagnostic for its insertion in the IM (Davis et al., 1998; Ryan et al., 1998), was generated by protease treatment (Fig. 1 A, lane 2). When ^{35}S-labeled Tim23p was incubated with mitochondria whose membrane potential was dissipated with CCCP ($-\Delta\Psi$), Tim23p was imported across the mitochondrial OM and protected from externally added protease (Fig. 1 A, lane 3), but was not inserted into the IM. When the mitochondrial OM was disrupted by osmotic shock, no protease fragment of Tim23p was generated (Fig. 1 A, lane 4). When the membrane potential was restored by washing out the CCCP ($-\Delta\Psi$,chase), most of the Tim23p transported across the outer membrane in the absence of potential (Fig. 1 A, lane 5) was now inserted in the IM. The 12-kD Tim23p fragment was now readily formed after protease treatment of mitoplasts (Fig. 1 A, lane 6). Thus, in the absence of the membrane potential, Tim23p forms a productive translocation intermediate located inside the mitochondrial OM, before its insertion into the IM. Consistent with previous studies (Koehler et al., 1998b; Sirrenberg et al., 1998; Ryan et al., 1999), we also find that the ADP-ATP carrier protein, Aac2p, forms a translocation intermediate in a location seemingly identical to that of the Tim23p intermediate when imported into mitochondria lacking a membrane potential (Davis, A., unpublished observations).

To identify proteins in close proximity to Tim23p during its import into mitochondria, we used a chemical cross-linking approach. ^{35}S-labeled Tim23p was incubated with isolated mitochondria, either in the presence ($+\Delta\Psi$) or absence ($-\Delta\Psi$) of a membrane potential, and then treated with DSP (a lysine to lysine cross-linker) or SMPB (a lysine to cysteine cross-linker). In the presence of $\Delta\Psi$, we observed an abundant Tim23p-containing cross-linked product of ~55 kD with either DSP or SMPB that was not seen in the absence of cross-linker (Fig. 1 B, compare

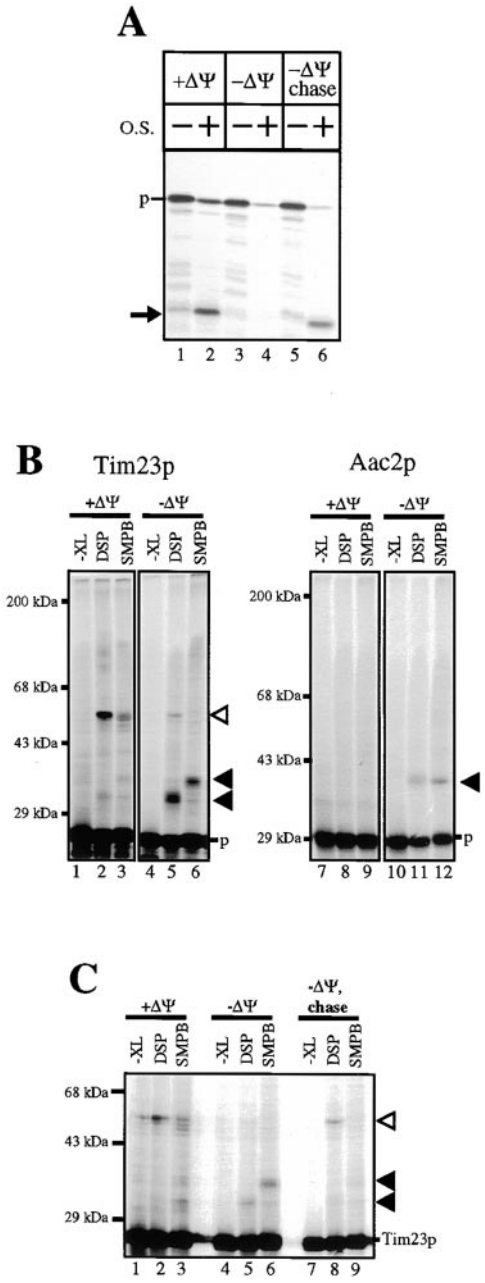

Figure 1. A Tim23p translocation intermediate cross-links to two small proteins. (A) ^{35}S-labeled Tim23p was incubated for 10 min with fully energized mitochondria (+ΔΨ) or mitochondria whose membrane potential was dissipated by pretreatment with CCCP (−ΔΨ). In half of the CCCP-treated samples, the IM potential was reestablished by washing out the CCCP, followed by an additional 10-min incubation (−ΔΨ,chase). Samples were either directly treated with proteinase K or were digested with protease after the outer membrane was disrupted by osmotic shock (O.S.). Samples were pelleted and analyzed by SDS-PAGE and fluorography. Arrow denotes the ~12-kD protease-protected fragment in mitoplasts, which is indicative of Tim23p insertion into the IM. The location of the Tim23p precursor is indicated by p. (B) ^{35}S-labeled Tim23p or Aac2p were imported into mitochondria in the presence (+ΔΨ) or absence (−ΔΨ) of an IM potential and treated with the cross-linkers DSP or SMPB, or no cross-linker (−XL). Samples were analyzed by SDS-PAGE and fluorography. Black arrowheads identify cross-links found primarily under −ΔΨ conditions; the open arrowhead identifies the primary cross-link found under +ΔΨ conditions. The location of the Tim23p or Aac2p precursor is indicated by p. The migration of the molecular mass standards that were run in adjacent lanes is shown. (C) Tim23p was imported for 10 min into mitochondria pretreated (−ΔΨ) or untreated (+ΔΨ) with CCCP. In aliquots of the −ΔΨ reactions, the IM potential was restored by washing out the CCCP, followed by another 10-min incubation (−ΔΨ, chase). Samples were treated with DSP, SMPB, or no cross-linker (−XL) and analyzed.

lanes 2 and 3 to lane 1). This cross-linked material was presumed to be a Tim23p-Tim23p dimer (Bauer et al., 1996), and was not studied further. However, in the absence of membrane potential, we found very little of the 55-kD cross-link and instead, saw a prominent Tim23p-containing cross-linked product of ~31 kD with DSP (Fig. 1 B, lane 5). With SMPB, we found a major cross-link of ~36 kD (Fig. 1 B, lane 6). In comparison to Tim23p, we observed that Aac2p forms an abundant cross-link of ~40 kD with both cross-linkers when imported into mitochondria lacking ΔΨ (Fig. 1 B, lanes 11 and 12).

The 31- and 36-kD products represent cross-links to a Tim23p translocation intermediate, because they were not detected when the Tim23p intermediate was chased into the IM. As shown in Fig. 1 C, if the membrane potential was restored before adding cross-linkers, the 31- and 36-kD Tim23p-containing cross-links seen in the absence of IM potential (Fig. 1 C, lanes 5 and 6) were absent; instead, we saw either no cross-links (Fig. 1 C, lane 9) or an ~55-kD product (Fig. 1 C, lane 8). The cross-linking pattern seen in our chase experiments looked very similar to those seen when Tim23p was imported into fully energized mitochondria (Fig. 1 C, lanes 2 and 3).

The Tim23p Translocation Intermediate Cross-links to Tim8p and Tim13p

The 31- and 36-kD cross-links suggest that two small proteins (~8 and 13 kD) are in close proximity to Tim23p when it is imported into mitochondria in the absence of the IM potential. To identify the Tim23p-interacting proteins, we immune precipitated our cross-linked samples with various antisera specific for low molecular mass TIM proteins. For example, the Tim12, Tim10, and Tim9 proteins previously have been shown to interact with Aac2p during its import into mitochondria (Koehler et al., 1998a,b; Sirrenberg et al., 1998; Adam et al., 1999; Endres et al., 1999). Consistent with these results, we found that the 40-kD cross-link formed after import of Aac2p into −ΔΨ mitochondria was precipitated with antiserum against either Tim9p or Tim10p (Fig. 2 A). In contrast, neither of the two abundant cross-links to Tim23p precipitated with Tim9p or Tim10p antiserum (Fig. 2 A). We found instead that the recently identified Tim8 and Tim13 proteins (Koehler et al., 1999) associated with and cross-linked to Tim23p. The 36-kD cross-link that formed with SMPB was efficiently precipitated with antiserum to Tim13p, while a small amount pelleted with Tim8p antiserum. The 31-kD DSP cross-link was efficiently precipitated with antiserum to Tim8p and, to a lesser degree, by Tim13p antiserum. On the other hand, the cross-linked

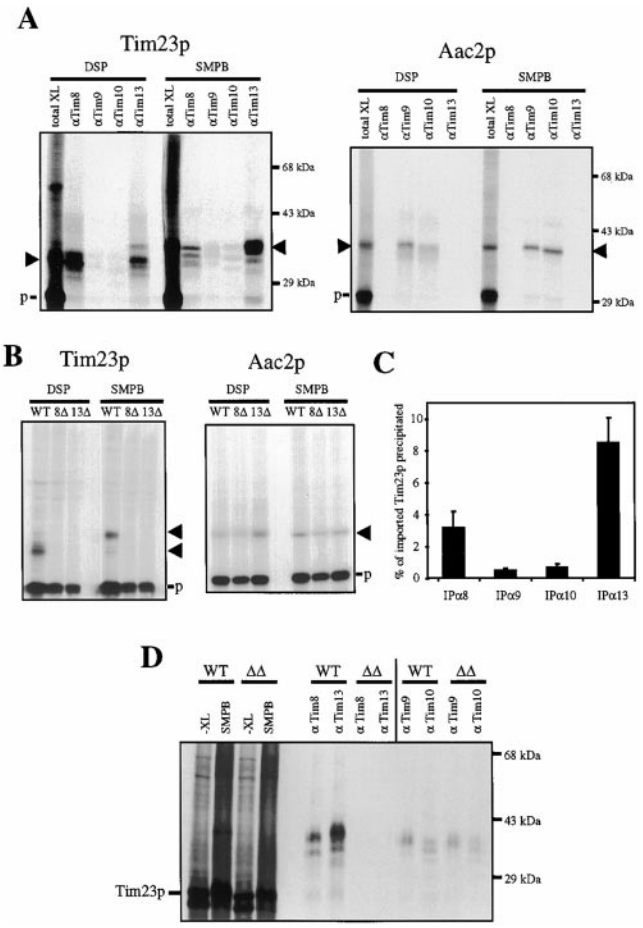

Figure 2. The Tim23p intermediate cross-links to Tim8p and Tim13p. (A) ^{35}S-labeled Tim23p or Aac2p was imported into mitochondria pretreated with CCCP and cross-linked with DSP and SMPB as described above. Mitochondria (50 μg) were subjected to SDS-PAGE (total XL). Aliquots containing 200 μg of mitochondria were solubilized, immunoprecipitated with antisera to Tim8p, Tim9p, Tim10p, or Tim13p, and then analyzed by SDS-PAGE and phosphorimaging. The location of the unmodified Tim23p or Aac2p precursor is indicated as p. Black arrowheads identify cross-links formed under $-\Delta\Psi$ conditions. (B) Mitochondria isolated from wild-type (*WT*), *tim8*Δ or *tim13*Δ cells were pretreated with CCCP and incubated with ^{35}S-labeled Tim23p or Aac2p for 10 min. Samples were treated with DSP or SMPB and analyzed. Black arrowheads identify cross-links. (C) Tim23p was imported into mitochondria, treated with SMPB, and immune precipitated as described above. Phosphorimages were quantified, and the cross-links to Tim23p (black bars) are shown. The amount of Tim23p imported and protease-protected in the absence of SMPB was set to 100%. Values shown are the average of eight separate experiments, and error bars indicate the SD from the mean. (D) Mitochondria isolated from wild-type (WT) or *tim8*Δ *tim13*Δ (ΔΔ) cells were pretreated with CCCP, incubated with Tim23p for 20 min, and cross-linked with SMPB or no cross-linker (−XL). Mitochondria (30 μg) were subjected to SDS-PAGE (−XL or SMPB). Aliquots containing 300 μg of mitochondria were solubilized, immunoprecipitated with antisera to Tim8p, Tim9p, Tim10p, and Tim13p, and analyzed by SDS-PAGE and phosphorimaging.

Table I. Antiserum Specificity for the Small Tim Proteins

	Radiolabeled protein			
Antisera	^{35}S-Tim8p	^{35}S-Tim9p	^{35}S-Tim10p	^{35}S-Tim13p
Preimmune	0.06	0.22	0.04	< 0.01
α-Tim8p	29.4	0.16	< 0.01	0.01
α-Tim9p	0.10	11.75	0.22	0.01
α-Tim10p	0.03	0.21	38.6	0.01
α-Tim12p	0.02	0.01	0.14	< 0.01
α-Tim13p	0.01	0.01	0.02	17.4

5 μl of ^{35}S-labeled Tim8p, Tim9p, Tim10p, or Tim13p was added to 100 μg of mitochondria and solubilized in 100 μl boiling buffer. Samples were incubated at 95°C for 5 min, diluted with 1 ml TNET, and immunoprecipitated as described in Materials and Methods with the indicated antiserum. Values shown are the percentage of total TIM protein added to each reaction that was precipitated with the corresponding antiserum.

material containing Aac2p, was not precipitated by either Tim8p or Tim13p antiserum (Fig. 2 A).

Supporting our observation that Tim8p and Tim13p interact with Tim23p during its import, the Tim23p-containing cross-links were dependent on the presence of the Tim8 and Tim13 proteins. We constructed disruptions in either the *TIM8* or *TIM13* genes and, consistent with previous results (Koehler et al., 1999), we found that both the Tim8 and Tim13 proteins were absent in either of the two single gene disruption strains (Davis, A., unpublished observations). We isolated mitochondria from wild-type (*WT*), *tim8*Δ, and *tim13*Δ strains. When Tim23p was imported into mitochondria lacking IM potential, cross-links were found only in *WT* mitochondria (Fig. 2 B). No major DSP or SMPB cross-links to Tim23p were observed using mitochondria lacking Tim8p and Tim13p. In contrast, cross-links to Aac2p were the same in *WT*, *tim8*Δ, and *tim13*Δ mitochondria (Fig. 2 B).

The above results suggest that Tim23p interacts with Tim8p and Tim13p, but not with Tim9p and Tim10p, whereas Aac2p interacts only with Tim9p and Tim10p. However, upon close examination of our gels, we found that a small but significant fraction of the Tim23p-containing cross-linked products formed using SMPB was precipitated by antiserum to either Tim9p or Tim10p (Fig. 2 A). We argue that the minor cross-links of Tim9 and Tim10p to Tim23p are not the result of cross-reactivity among our different antisera. Although Tim8p, Tim9p, Tim10p, and Tim13p are homologous proteins, we found that our antiserum to each protein was specific (Table I). Furthermore, quantification of phosphorimages indicated that while ~3% of Tim23p cross-linked to Tim8p and ~8.5% to Tim13p, ~0.5% of Tim23p cross-linked to Tim9p and ~0.6% to Tim10p (Fig. 2 C). In precipitations using preimmune sera or antiserum to the abundant IM protein, PiC, Tim23p cross-links to proteins in the 5–15-kD range were <0.04% (Davis, A., unpublished observations). By comparison, ~12% of Aac2p cross-linked to both Tim9 and Tim10p, and cross-links to Tim8p and Tim13p, were <0.1% and, thus, not considered to be above background (Davis, A., unpublished observations). Furthermore, we find that the cross-links to Tim9p and Tim10p are present and unchanged after imports into mitochondria isolated from *tim8*Δ *tim13*Δ strains, which lack Tim8p and Tim13p

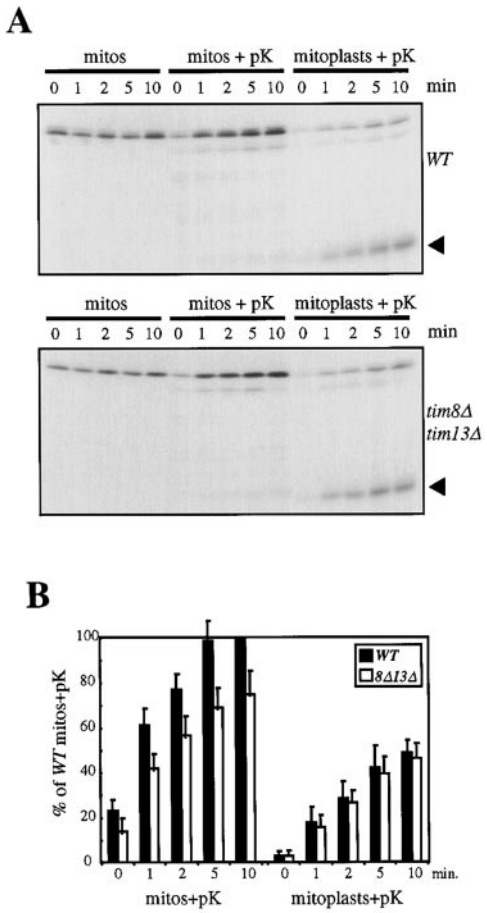

Figure 3. Tim8p and Tim13p are not essential for the import of Tim23p. (A) ^{35}S-labeled Tim23p was imported for the indicated times with mitochondria isolated from wild-type strain YRJ1206 (*WT*) or *tim8*Δ *tim13*Δ cells. Samples were treated with proteinase K either before (mitos + pK) or after the outer membrane was disrupted by osmotic shock (mitoplasts + pK), and were analyzed by SDS-PAGE and phosphorimaging. Arrowhead denotes the ~12-kD protease-protected fragment of Tim23p that has been fully integrated into the IM. (B) Quantification of Tim23p after import into *WT* or *tim8*Δ *tim13*Δ mitochondria. The percentage of Tim23p imported into a protease-protected location in mitochondria or the percentage of the ~12-kD fragment produced by protease digestion of mitoplasts is shown for *WT* (black bars) and *tim8*Δ *tim13*Δ (white bars). The amount of Tim23p imported into *WT* mitochondria at the 10-min time point was set at 100%. Values shown are the average of three separate experiments, and error bars indicate the SD from the mean.

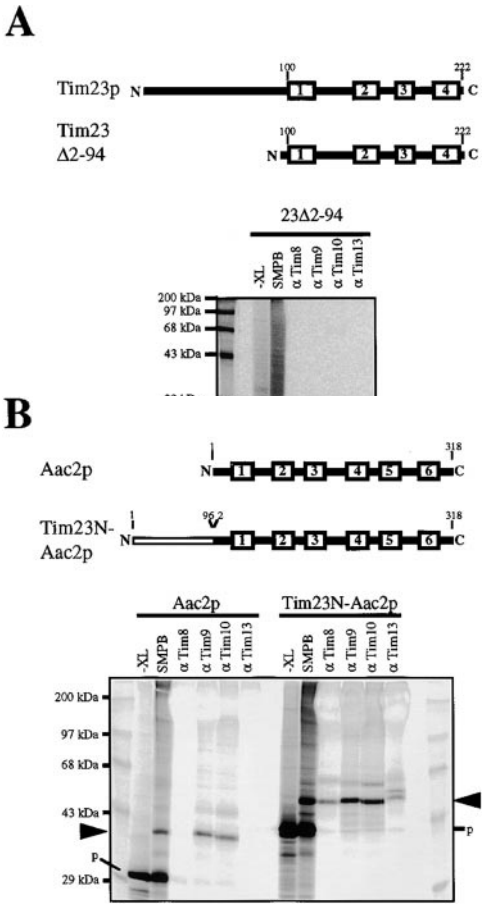

Figure 4. The Tim8p–Tim13p complex binds to the NH_2 terminus of Tim23p. (A) Tim23Δ2-94, a Tim23p construct lacking its first 94 residues, no longer cross-links to Tim8p and Tim13p. Top panel shows a schematic representation of the Tim23 protein and the Tim23Δ2-94 construct. The numbered rectangles represent the membrane-spanning segments within Tim23p. Small numbers above the figures correspond to amino acid numbers within Tim23p. In the bottom panel, ^{35}S-labeled Tim23Δ2-94 was imported into mitochondria lacking IM potential and treated with SMPB or no cross-linker (−XL). An aliquot of mitochondria (50 μg) was pelleted (SMPB or −XL). Aliquots containing 200 μg of mitochondria were solubilized, immunoprecipitated with antiserum to either Tim8p, Tim9p, Tim10p, or Tim13p, and then analyzed by SDS-PAGE and phosphorimaging. Black arrowhead identifies cross-links. (B) Fusion of the Tim23p NH_2-terminal domain to Aac2p recruits Tim8p and Tim13p binding. The top panel shows schematic representations of the Aac2 protein and Tim23N-Aac2 fusion protein. The numbered rectangles correspond to membrane-spanning segments within Aac2p. Small numbers above the black figure correspond to the amino acid number of Aac2p. The white rectangle represents residues 1–96 of the Tim23p NH_2 terminus. In the bottom panel, ^{35}S-labeled Aac2p and Tim23N-Aac2p were imported into mitochondria in the absence of a membrane potential, cross-linked with SMPB, immune precipitated, and analyzed as described above. Black arrowheads identify cross-links.

(Fig. 2 D). Additional evidence for an interaction between Tim23p and both Tim9p and Tim10p is described below.

Tim8p and Tim13p Are Not Essential for the Import of Tim23p

To further define the function of Tim8p and Tim13p, we isolated mitochondria from wild-type (*WT*) cells and a strain disrupted for both the *TIM8* and *TIM13* genes (*tim8*Δ *tim13*Δ) and examined their ability to import Tim23p. We found no significant difference in the import of Tim23p into *WT* or *tim8*Δ *tim13*Δ mitochondria (Fig. 3 A). Similar amounts of Tim23p were imported and protected from externally added protease after 1, 2, 5, or 10 min incubation with either wild-type or mutant mitochondria (Fig. 3 A, mitos + pK). Quantification of three independent time course experiments indicated that the import of Tim23p into *tim8*Δ *tim13*Δ mitochondria was at

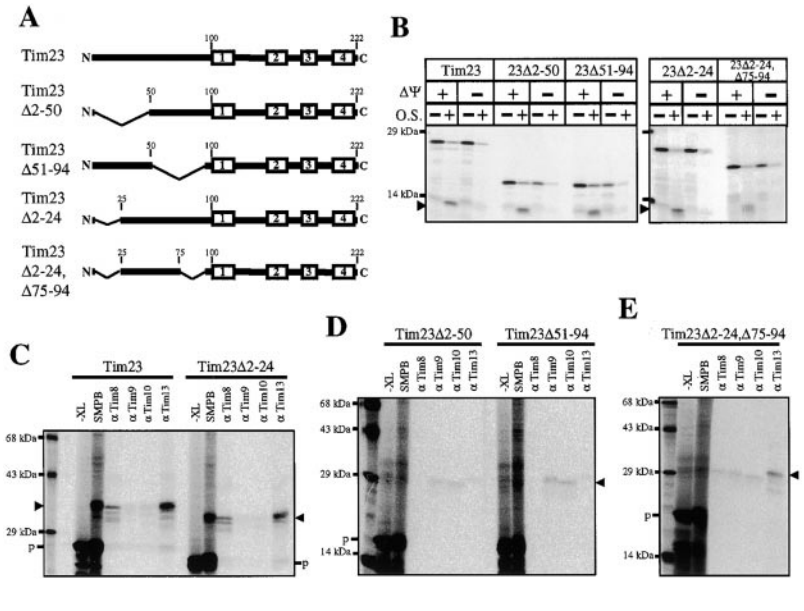

Figure 5. Tim8p and Tim13p appear to bind to a region of Tim23p between amino acids 25 and 75. (A) Schematic representation of different Tim23p deletion constructs is shown. Numbered rectangles represent the membrane-spanning segments within Tim23p. Small numbers above the figures correspond to amino acid number within Tim23p. Thin lines represent regions of the Tim23p NH_2 terminus that were deleted. (B) ^{35}S-labeled Tim23, Tim23Δ50, Tim23Δ51-94, Tim23Δ24m and Tim23Δ24,Δ75-96 proteins were imported into mitochondria in the presence (+ΔΨ) or absence (−ΔΨ) of a membrane potential. Mitochondria were either treated directly with proteinase K or the outer membrane was disrupted by osmotic shock (O.S.) before protease treatment. Samples were pelleted and analyzed by SDS-PAGE and fluorography. Arrowheads denote the ~12-kD protease-protected fragment of Tim23p, which is indicative of its insertion into the IM. The ^{35}S-labeled Tim23 and Tim23Δ2-24 proteins (C), the Tim23Δ50 and Tim23Δ51-94 proteins (D), and the Tim23Δ24, Δ75-94 protein (E) were imported into the mitochondria in the absence of IM potential and treated with SMPB or no cross-linker (−XL). Mitochondria (50 μg) were subjected to SDS-PAGE and phosphorimaging (−XL or SMPB). Aliquots containing 200 μg of mitochondria were solubilized, immunoprecipitated with antiserum to Tim8p, Tim9p, Tim10p, or Tim13p, and then analyzed. p indicates the location of the Tim23p precursor.

most 25–35% reduced compared with import into wild-type mitochondria (Fig. 3 B, mitos + pK). In addition, we saw no notable difference in the insertion of Tim23p into the IM. The rate of appearance of an ~12-kD fragment after treatment of mitoplasts with protease was virtually identical in *WT* or *tim8*Δ *tim13*Δ mitochondria (Fig. 3, A and B, mitoplasts + pK). We conclude that although Tim8 and Tim13 proteins efficiently cross-link to Tim23p, neither protein is essential for Tim23p import into isolated mitochondria.

Tim8p and Tim13p Cross-link to the Hydrophilic Tim23p NH_2 Terminus

To determine where the Tim8 and Tim13 proteins bind within Tim23p, we examined the import of different Tim23p constructs. As shown previously, all of the information required to import Tim23p into isolated mitochondria is carried in the COOH-terminal half of the protein (Davis et al., 1998). Therefore, we imported Tim23Δ2-94, which lacks the first 94 residues of Tim23p, into mitochondria in the absence of an IM potential, and added the cross-linker SMPB. Immune precipitations showed that Tim23Δ2-94 cross-linked to both Tim9p and Tim10p, but no detectable cross-links to Tim8p and Tim13p were formed (Fig. 4 A). Our results indicate that the NH_2-terminal region of the Tim23 protein is required for interactions with Tim8p and Tim13p, whereas Tim9p and Tim10p interact with the COOH terminus of Tim23p.

To determine whether the Tim23p NH_2-terminal domain is sufficient for Tim8p and Tim13p binding, we constructed a fusion protein between the NH_2-terminal region of Tim23p and Aac2p, a protein that does not normally interact with the Tim8p–Tim13p complex. As shown in Fig. 4 B, we imported the Tim23N-Aac2p fusion construct, as well as Aac2p, into mitochondria in the absence of IM potential. After cross-linking with SMPB and immune precipitation, we find cross-links between Tim23N-Aac2p and Tim8p, Tim9p, Tim10p, and Tim13p. In contrast, Aac2p only formed significant cross-links to Tim9p and Tim10p. Quantification of phosphorimages showed similar amounts of cross-linking of Tim9p and Tim10p to both Aac2p and Tim23N-Aac2p, but only Tim23N-Aac2 showed cross-links to Tim8p and Tim13p above background levels. Therefore, our results suggest that the Tim8p–Tim13p complex interacts with the NH_2-terminal domain of Tim23p.

Tim8p and Tim13p Cross-link to the Tim23p NH_2 Terminus between Amino Acids 25 and 75

To identify where in the NH_2-terminal region of Tim23p that Tim8p and Tim13p bind, we constructed additional deletions within the Tim23 protein and examined their ability to cross-link to the small TIM proteins. For example, as shown in Fig. 5 A, we deleted either the first half (Tim23Δ2-50) or the second half (Tim23Δ51-94) of the Tim23p NH_2 terminus. When ^{35}S-labeled Tim23p, Tim23Δ2-50, or Tim23Δ51-94 were incubated with isolated mitochondria, all three proteins were imported across the mitochondrial outer membrane and inserted into the IM (Fig. 5 B). When the mitochondrial outer membrane was disrupted by osmotic shock, an ~12-kD membrane-embedded fragment was generated by protease treatment with each protein. In the absence of IM potential (−ΔΨ), Tim23p, Tim23Δ2-50, or Tim23Δ51-94 were imported across the mitochondrial outer membrane and protected from externally added protease, but none were inserted into the IM. Unlike Tim23p, after import into −ΔΨ mitochondria, neither Tim23Δ2-50 nor Tim23Δ51-94 cross-

Table II. Analysis of Tim23p Deletion Constructs in tim23::URA3 Yeast Cells

	YEPD				YEPgly/eth			
	24°C	30°C	34°C	37°C	24°C	30°C	34°C	37°C
Vector	−	−	−	−	−	−	−	−
TIM23	+++	+++	+++	+++	+++	+++	+++	+++
23Δ2-24	−	−	−	−	−	−	−	−
23Δ2-50	+++	+++	+++	+++	++	++	+	−
23Δ51-94	+	+	−	−	−	−	−	−
23Δ2-24,75-94	+	+	−	−	−	−	−	−
23Δ2-94	−	−	−	−	−	−	−	−

Plasmids expressing wild-type Tim23p (pJE50) or the deletion constructs Tim23Δ2-24 (pAD109), Tim23Δ2-50 (pAD105), Tim23Δ51-94 (pAD106), Tim23Δ2-24,75-94 (pAD111), or Tim23Δ2-94 (pKR15) were transformed into *tim23::URA3 trp1 leu2 cyh2* strain KRR146, which contains the *TIM23-TRP1-CYH2* plasmid pKR1 (Ryan et al., 1998). Leu^+ transformants were patched onto SD medium lacking leucine, replica-plated onto YEPD or YEPgly/eth plates containing 10 mg/l cycloheximide to detect the loss of pKR1, and allowed to grow at 24, 30, 34, or 37°C for 3 d. Wild-type growth (+++), intermediate growth (++ or +), or no growth (−) is indicated.

linked to Tim8p and Tim13p, and only cross-links to Tim9p and Tim10p were seen (Fig. 5 D). Quantification of the phosphorimages showed that cross-linking to Tim8p and Tim13p was reduced at least 12-fold for the Tim23Δ2-50 construct as compared with wild-type Tim23p, and was undetectable for the Tim23Δ51-94 protein. Our results suggested that the Tim8p and Tim13p binding site may span the middle portion of the Tim23p NH_2-terminal.

To test this possibility, we constructed two additional Tim23p derivatives, Tim23Δ2-24, which lacks the first 24 amino acids of Tim23p, and Tim23Δ2-24,Δ75-94, which lacks residues 2–24 and residues 75–94 (Fig. 5 A). In the presence of membrane potential, both proteins were efficiently imported into mitochondria and inserted into the IM (Fig. 5 B). In the absence of a membrane potential, both Tim23Δ2-24 and Tim23Δ2-24,Δ75-94 could be cross-linked to Tim8p and Tim13p (Fig. 5, C and E). Tim23Δ2-24 formed abundant cross-links to Tim8p and Tim13p, with lower amounts of cross-linking to Tim9p and Tim10p. Tim23Δ2-24,Δ75-94 cross-links to Tim8p, Tim9p, Tim10p, and Tim13p with approximately equal efficiencies. Therefore, our data indicate that the binding site for the Tim8p and Tim13p lies within amino acids 25–75 of Tim23p. We note that the amount of Tim8p and Tim13p that cross-link to Tim23Δ2-24,Δ75-94 is less than that to wild-type Tim23p. Whether Tim23Δ2-24,Δ75-94 lacks some of the Tim8p and Tim13p binding site, or whether the NH_2-terminal region of Tim23Δ2-24,Δ75-94 is not correctly folded awaits further studies.

Tim23p is an essential protein for yeast cell viability (Emtage and Jensen, 1993), and the 9-kD NH_2-terminal domain is required for Tim23p function (Ryan et al., 1998). Therefore, we tested our different Tim23p constructs for their ability to provide Tim23p activity in yeast cells (Table II). The Tim23Δ2-24 and Tim23Δ2-94 proteins could not rescue the *tim23::URA3* disruption on either glucose-containing medium or on glycerol/ethanol medium at any temperature tested. The Tim23Δ2-28,Δ75-94 and Tim23Δ51-94 proteins partially rescued the *tim23::URA3* strain, and both strains grew very slowly on glucose-containing medium only at low temperatures. Consistent with previous studies (Bauer et al., 1996), the Tim23Δ2-50 protein provided almost wild-type Tim23p function on glucose medium, and was temperature-sensitive for activity on glycerol medium. Interestingly, we found that Tim23Δ2-50 did not cross-link to either Tim8p or Tim13p during its import (Fig. 5 D), again suggesting that Tim23p import and function is not strictly dependent upon interaction with the Tim8p–Tim13p complex.

The Positively Charged Loops in Tim23p Are Not Required for Interaction with the Small TIM Proteins

The Tim9 and Tim10 proteins bind to Aac2p during its import into mitochondria (Koehler et al., 1998a,b; Sirrenberg et al., 1998; Adam et al., 1999; Endres et al., 1999), and it has been proposed that positively charged amino acids carried within matrix-facing loops of Aac2p are important for this interaction (Sirrenberg et al., 1998; Endres et al., 1999). To examine the role of positive charges in the import of Tim23p, we examined the import of L1L3neut, a Tim23p construct in which the lysine and arginine residues in each of its matrix-facing loops, loop L1 and loop L3, were exchanged for neutral alanine residues (Davis et al., 1998). We previously showed that L1L3neut is completely translocated across the mitochondrial outer membrane, but not inserted into the IM (Davis et al., 1998). After import into mitochondria, we found that DSP and SMPB treatment generated similar cross-linking profiles with L1L3neut (Fig. 6 A) as compared with wild-type Tim23p (Fig. 1 B). However, in contrast to Tim23p, similar cross-links to L1L3neut were formed either in the presence ($+\Delta\Psi$) or absence ($-\Delta\Psi$) of IM potential (Fig. 6 A).

Like wild-type Tim23p, the L1L3neut protein cross-linked efficiently to Tim8p and Tim13p, and less efficiently to Tim9p and Tim10p using SMPB (Fig. 6 B). Although the cross-linking pattern to L1L3neut appeared similar to Tim23p, we noticed two significant differences. First, we found that the L1L3neut protein interacted more efficiently with all of the small TIM proteins. Compared with Tim23p, L1L3neut cross-linked to 7-fold more Tim8p, 3.5-fold more Tim13p, and 12-fold more Tim9p and Tim10p (Fig. 6 C). Second, we found high molecular mass cross-links to L1L3neut, which was not seen using Tim23p (Fig. 6 B, asterisk). These products appear to represent cross-links between Tim23p and multiple small TIM proteins. A further indication that Tim9p and Tim10p do not recognize positively charged amino acids in Tim23p, we find that the Tim23Δ2-94neut protein, which lacks both the NH_2-terminal segment of Tim23p, and the positive charges in the matrix-facing loops, formed efficient cross-links to Tim9p and Tim10p (Fig. 6 D). We conclude that Tim8p, Tim9p, Tim10p, and Tim13p each bind to the Tim23 protein during its import, and these interactions do not require the lysine or arginine residues in the matrix-facing loops of Tim23p.

Positively Charged Loops of Tim23p Mediate Interactions with Tim54p, Tim22p, and Tim12p

In the absence of membrane potential, the majority of imported Tim23p is arrested before its insertion into the IM. However, we noticed that a small amount of Tim23p could be cross-linked to import components in addition to Tim8p, Tim9p, Tim10p, and Tim13p. As shown in Fig. 7,

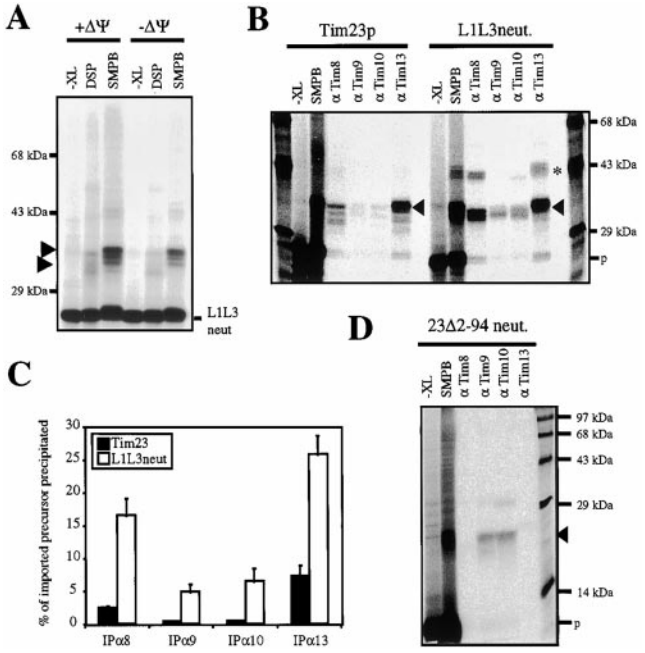

Figure 6. Cross-linking to Tim8p, Tim9p, Tim10p, and Tim13p does not require positively charged loops within Tim23p. (A) ^{35}S-labeled L1L3neut, a derivative of Tim23p in which the positively charged amino acids in the matrix-facing loops are replaced by alanine residues, was imported into mitochondria in the presence (+ΔΨ) or absence (−ΔΨ) of an IM potential. After treatment with proteinase K, mitochondria were reisolated, incubated with DSP, or SMPB, or no cross-linker (−XL), and then analyzed by SDS-PAGE and fluorography. Arrowheads indicate cross-links to L1L3neut. (B) The Tim23 and L1L3neut proteins were imported into the mitochondria in the absence of IM potential and treated with SMPB or no cross-linker (−XL). Mitochondria (50 μg) were subjected to SDS-PAGE and fluorography (−XL or SMPB). Aliquots containing 200 μg of mitochondria were solubilized, immunoprecipitated with antiserum to the Tim8p, Tim9p, Tim10p, or Tim13p, and analyzed by SDS-PAGE and phosphorimaging. Arrowheads indicate cross-links to Tim23p and L1L3neut. Asterisks indicate multiple cross-links to L1L3neut. (C) The Tim23 and L1L3neut proteins were imported into the mitochondria, treated with SMPB, and immune precipitated as described above. Phosphorimages were quantified, and the cross-links to Tim23p (black bars) and L1L3neut (white bars) are shown. The amount of Tim23p or L1L3neut imported and protease-protected in the absence of SMPB was set to 100%. Values shown are the average of three separate experiments and error bars indicate the SD from the mean. (D) Tim23Δ2-94neut, a Tim23p derivative lacking its first 94 amino acids and in which the positively charged amino acids in the matrix-facing loops are replaced by alanine residues, was imported into mitochondria in the absence of the IM potential. After treatment with proteinase K, samples were incubated with SMPB or no cross-linker (−XL). An aliquot (50 μg) of mitochondria was pelleted (SMPB or −XL). Aliquots containing 200 μg of mitochondria were solubilized, immunoprecipitated with antiserum to Tim8p, Tim9p, Tim10p, or Tim13p, and analyzed by SDS-PAGE and phosphorimaging.

when ^{35}S-labeled Tim23p was imported into mitochondria in the absence of a membrane potential, treated with SMPB, and reactions were precipitated with different antisera, we found small amounts of cross-linked products that were precipitated by antiserum to Tim54p, Tim22p, and

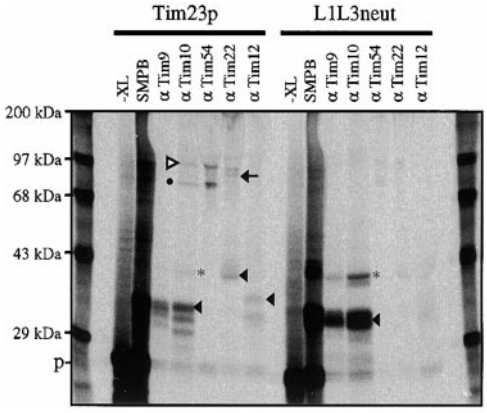

Figure 7. Positively charged loops within Tim23p are required for interaction with Tim54p, Tim22p, and Tim12p. ^{35}S-labeled Tim23 and L1L3neut proteins were imported into mitochondria in the absence of IM potential, treated with proteinase K, and incubated with SMPB or no cross-linker (−XL). An aliquot (25 μg) of the reaction was analyzed directly (SMPB or −XL). Aliquots containing 250 μg of mitochondria were solubilized, immunoprecipitated with antiserum to Tim9p, Tim10p, Tim54p, Tim22p, or Tim12p, and analyzed by SDS-PAGE and phosphorimaging. Arrowheads indicate the cross-linked products resulting from a direct interaction with Tim9p, Tim10p, Tim22p, and Tim12p. Open arrowhead, bullet, arrow and asterisks indicate products that result from multiple proteins cross-linking to Tim23p.

Tim12p. The molecular masses of some of the products were consistent with a single protein cross-linked to Tim23p (Fig. 7, arrowheads). In particular, Tim9p, Tim10p, Tim22p, and Tim12p appeared to form single cross-links to Tim23p. Other products appear to contain multiple proteins cross-linked to Tim23p. As seen in Fig. 6, cross-links containing Tim9p or Tim10p and an additional small Tim protein are apparent (Fig. 7, asterisks). A product of ~110 kD appears to contain Tim10p, Tim54p, and Tim22p since a similar sized cross-link comigrates in precipitations with all three antisera (Fig. 7, open arrowhead). Another cross-link of ~90 kD appears to contain both Tim54p and Tim10p (Fig. 7, see bullet). In addition, we see a product of ~105 kD that contains at least Tim22p (Fig. 7, arrow). We also found that Aac2p imported into −ΔΨ mitochondria forms high molecular mass cross-links that precipitate with Tim54p, Tim22p, Tim10p, and Tim9p antisera, very similar to those seen with Tim23p (Davis, A., unpublished observations).

Cross-linking to Tim54p, Tim22p, and Tim12p is dependent upon the positively charged loops within Tim23p. In particular, when the L1L3neut protein was imported into −ΔΨ mitochondria, treated with SMPB, and immune precipitated, cross-links to Tim54p, Tim22p, and Tim12p were not observed, and only the cross-links to Tim9p and Tim10p remained. Thus, the positively charged loops of Tim23p, which are necessary for the insertion of Tim23p into the IM (Davis et al., 1998), are required for interaction with components of the IM insertion machinery.

Discussion

During its import into mitochondria, Tim23p interacts

with two different intermembrane space TIM complexes, Tim8p–Tim13p and Tim9p–Tim10p. In the absence of membrane potential, imported Tim23p was arrested at a point after complete translocation across the outer membrane, but before insertion into the IM. Using chemical cross-linking we found that Tim8p, Tim9p, Tim10p, and Tim13p all interacted with Tim23p at this stage of the import pathway. However, the small TIM complexes recognized different domains of Tim23p. Tim8p and Tim13p cross-linked to the Tim23p hydrophilic NH_2-terminal region, whereas the Tim9–Tim10p complex cross-linked to the COOH-terminal domain.

We previously showed that Tim23p contains separate and distinct signals for its import into the mitochondria (Davis et al., 1998). Information carried within the first and the fourth transmembrane segments of Tim23p is required for translocation across the mitochondrial outer membrane, and positively charged residues located in the two matrix-facing loops of Tim23p are required for insertion into the IM. We find that Tim9p and Tim10p interact with the COOH-terminal hydrophobic domain of Tim23p, raising the possibility that the Tim9p–Tim10p complex plays a critical role in the translocation of Tim23p across the outer membrane and its subsequent insertion into the IM. We speculate that Tim9p and Tim10p recognize the targeting information carried within transmembrane segments one and four. Supporting this idea, we find that the positively charged loops of Tim23p are not required for Tim9–Tim10p interaction. Furthermore, derivatives of Tim23p devoid of all lysine residues form cross-links to Tim9p and Tim10p using SMPB, a lysine–cysteine cross-linker, but not with DSP, a lysine–lysine cross-linker (Davis, A., unpublished observations). We argue that SMPB is reacting with one of the three cysteine residues in Tim23p, one located in the first transmembrane segment, and the other two located in transmembrane segment four. Experiments to directly test this prediction are in progress.

It has been proposed that during the import of Aac2p and other members of the mitochondrial carrier family, Tim9p and Tim10p recognize a conserved motif in one or more of the matrix-facing loops of the imported IM protein (Sirrenberg et al., 1998; Endres et al., 1999). These so-called carrier motifs include a number of positively charged amino acids. Tim23p, Tim22p, and Tim17p lack the carrier motif but, nonetheless, each protein interacts with the Tim9p–Tim10p complex during their import (Fig. 2 and Figs. 4–6; Leuenberger et al., 1999; Davis, A., unpublished observations). We now find that L1L3neut, a derivative of Tim23p which lacks the positively charged residues in loops L1 and L3, was efficiently translocated across the outer membrane and cross-linked to Tim9p and Tim10p. Thus, Tim9p and Tim10p must recognize structural features or properties of Tim23p other than the positive charges in its matrix-facing loops. It remains to be seen whether there is a common site on IM proteins for recognition by Tim9p and Tim10p.

Although the basic residues within the loops of Tim23p are not required for interaction with Tim9p and Tim10p, these residues appear to be important for interaction with Tim54p, Tim22p, and Tim12p. In the absence of a membrane potential, Tim23p is arrested in its import before insertion into the IM, but appears to interact with components of the inner membrane insertion machinery, because Tim23p can be cross-linked to Tim54p, Tim22p, and Tim12p with low efficiency. In contrast, Tim23p lacking lysines and arginines within loops L1 and L3 does not detectably cross-link to Tim54p, Tim22p, or Tim12p. Instead, these constructs cross-link more efficiently to Tim9p and Tim10p, suggesting that in the absence of the positive charges, more Tim23p molecules are trapped at the Tim9p-Tim10p–dependent step. Although we previously showed that the positively charged loops of Tim23p are required for insertion into the IM (Davis et al., 1998), it is not clear how the lysine and arginine residues are recognized by Tim54p, Tim22p, and/or Tim12p to facilitate insertion.

If Tim9p and Tim10p are required for the import of both Tim23p and Aac2p, then why does the Tim9p–Tim10p complex appear to form more abundant cross-links to Aac2p than to Tim23p. The observed differences between Tim23p and Aac2p cross-linking could reflect the extent of complex formation, but could also result from differences in the relative positions of the residues involved in forming the cross-link (e.g., even a subtle conformational change could separate two lysines, or a lysine and a cysteine, sufficiently to eliminate their cross-linking). Alternatively, it is possible that the Tim23p and Aac2p translocation intermediates are at slightly different positions in the import pathway. Furthermore, although Tim23p and Aac2p both utilize the TIM22 translocon for their insertion into the IM, they may rely on subsets of the translocation machinery. Supporting this possibility, *tim9*, *tim10*, *tim12*, and *tim22* mutants show reduced steady state levels of Aac2p, but normal levels of Tim23p (Sirrenberg et al., 1996, 1998; Koehler et al., 1998b, 1999, 2000).

Consistent with previous studies (Leuenberger et al., 1999), we find that Tim23p interacts with Tim8p and Tim13p during its import. The Tim8p–Tim13p complex is proposed to be required for the transport of one set of membrane proteins, such as Tim23p, whereas Tim9p–Tim10p mediates the transport of other IM proteins, such as Aac2p (Leuenberger et al., 1999). Supporting this idea, we found that Tim23p forms abundant cross-links to Tim8p and Tim13p, whereas Aac2p cross-links primarily to Tim9p and Tim10p. However, several observations indicate that it is unlikely that the Tim8p–Tim13p complex is solely responsible for the import of Tim23p. First, Tim23p is essential for yeast cell viability, yet neither Tim8p nor Tim13p are essential proteins. Second, mitochondria isolated from yeast strains disrupted for both Tim8p and Tim13p import Tim23p at nearly wild-type rates. Third, the Tim8p–Tim13p complex appears to interact with the NH_2-terminal region of Tim23p, which does not carry essential targeting information (Davis et al., 1998; Ryan et al., 1998). Fourth, we have observed significant cross-linking between Tim23p and both Tim9p and Tim10p. We also find that Tim8p and Tim13p are not required for a later step in the insertion of Tim23p into the IM. Tim23p molecules, which are accumulated in the IMS in the absence of an IM potential, are efficiently chased to the IM in both wild-type mitochondria and mitochondria isolated from *tim8*Δ *tim13*Δ strains (Davis, A., unpublished observations).

However, it is possible that Tim8p and Tim13p do play

some role in Tim23p import. For example, Tim23p, in contrast to Aac2p, Tim22p, and Tim17p, contains a large hydrophilic domain. Tim8p and Tim13p may facilitate the transport of this hydrophilic segment of Tim23p. We do observe a small (~25–35%) decrease in the rate of Tim23p import into *tim8*Δ *tim13*Δ mitochondria, and *tim8*Δ *tim13*Δ strains contain ~50% reduced levels of Tim23p as compared with wild-type (Sepuri, N., unpublished observations). Also supporting a role for Tim8p and Tim13p in import, *tim8*Δ and *tim13*Δ strains exhibit synthetic lethality with *tim10-1* mutants (Koehler et al., 1999). In addition, *tim8*Δ *tim13*Δ mitochondria show an altered submitochondrial distribution of Tim9p and Tim10p (Koehler et al., 1999; Sepuri, N., unpublished observations).

Alternatively, it is possible that Tim8p and Tim13p do not play a direct role in import, but rather play a different role in the biogenesis of Tim23p. For example, the NH_2-terminal domain of Tim23p has been proposed to function as a presequence receptor, recognizing precursor proteins after their translocation across the outer membrane (Emtage and Jensen, 1993; Bauer et al., 1996). In addition, the NH_2 terminus of Tim23p mediates the formation of Tim23p-Tim23p dimers, which apparently blocks the opening of the Tim23p-Tim17p translocation pore (Bauer et al., 1996). Perhaps binding of Tim8p and Tim13p to the NH_2 terminus of Tim23p prevents either premature Tim23p dimerization or inappropriate precursor association before the assembly of Tim23p into a functional translocon. Further studies are underway to clarify the role of the Tim8 and Tim13 proteins during the import of Tim23p and other IM proteins.

We thank Klaus Pfanner for the pGEM4z-AAC2 plasmid. We also thank Carolyn Machamer, Kathy Wilson, Hiromi Sesaki, Kara Cerveny, Alyson Aiken Hobbs, Oliver Kerscher (all from Johns Hopkins University, Baltimore, MD), and Joe Kelleher (University of Minnesota, Minneapolis, MN) for comments on the manuscript.

This work was supported United States Public Health Service grants RO1-GM46803 (to R.E. Jensen) and RO1-GM26494 (to A.E. Johnson), a National Institutes of Health Predoctoral Training Grant 5T32GN07445 (to A.J. Davis), and The Robert A. Welch Foundation (to A.E. Johnson).

Submitted: 13 April 2000
Revised: 2 August 2000
Accepted: 4 August 2000

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