A Multisubunit Complex of Outer and Inner Mitochondrial Membrane Protein Translocases Stabilized in Vivo by Translocation Intermediates*

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Mitochondria are organelles of eukaryotes that perform essential functions involved in energy generation and many metabolic pathways. Most mitochondrial proteins are nuclear encoded, synthesized on cytoplasmic ribosomes, and then imported into mitochondria. Thus, the mitochondrial import machinery is essential for viability of the eukaryotic cell. Numerous components of this machinery have been identified through genetic and biochemical methods; only the core elements have been shown to be essential, and thus, deletion of the corresponding genes in yeast leads to non-viability. Here we describe the isolation of a complex containing these essential constituents, an important step for obtaining a complete molecular and functional picture of the mitochondrial import apparatus.

Mitochondrial preproteins synthesized in the cytoplasm are targeted to various compartments of the mitochondrion. Proteins that are destined for the matrix must cross both the outer and inner mitochondrial membranes. This import process is mediated by two independent but cooperating translocases: one (Tom (translocase of outer mitochondrial membrane)) is located in the outer membrane, and the other (Tim (translocase of inner mitochondrial membrane)) in the inner membrane (for review, see Ref. 1). The Tom machinery contains at least eight proteins: four receptor subunits (Tom70 (2), Tom37 (3), Tom22 (4), and Tom20 (5)), three small proteins (Tom7 (6), Tom6 (7), and Tom5 (8)), and a structural component of the outer membrane channel (Tom40 (9, 10)). The Tom22-Tom20 subcomplex recognizes preproteins with cleavable signal sequences, whereas the Tom70-Tom37 subcomplex recognizes mainly preproteins that have internal signal sequences (1). Tom22 is the only receptor essential for cell viability and protein import, presumably because this is also a component of the outer membrane translocation channel (11, 12). The genes for Tom70, Tom37, or Tom20 can be deleted from haploid yeast strains without severe functional consequences; the cells remain viable, and protein import into mitochondria is only slightly affected (13, 14). Tom5 presumably provides a link between import receptors and the outer membrane import channel. Tom6 and Tom7 appear to modulate the stability of the Tom machinery, but do not directly interact with preproteins. In contrast to these small Tom proteins, Tom40 is an essential protein. Purified Tom40 forms a hydrophilic channel that specifically binds to and transports mitochondrial signal peptides (15).

The Tim complex consists of Tim17 (16, 17), Tim23 (18, 19), and Tim44 (20, 21), which are essential elements for translocation of preproteins across the inner membrane into the matrix. Tim17 and Tim23 are integral membrane proteins and likely represent the structural elements of the inner membrane channel. Tim44, on the other hand, behaves as a peripheral protein and recruits mt-Hsp70 (matrixlocalized heat shock protein of 70 kDa) to the site where the preprotein emerges from the Tim channel (22–24).

Isolation of a complex containing the essential constituents of both the outer and inner membrane translocation systems is a prerequisite for obtaining a complete molecular and functional picture of the mitochondrial import apparatus. Solubili-
zation of mitochondrial membranes with nonionic detergents, however, yields various subcomplexes of either Tom or Tim proteins (3, 7, 10, 14, 25–27). This is perhaps due to the fact that the outer and inner membrane channels are not permanently linked together. Instead, they are likely to interact with each other only transiently during import. When preproteins spanning both outer and inner mitochondrial membranes were trapped during in vitro import into the matrix and subsequently detergent-solubilized, some of the Tom and Tim proteins were found to be associated with the translocation intermediates (26–28), but the composition of these complexes was variable and inconsistent. Furthermore, these observations were made in vitro and remain to be confirmed by in vivo experiments.

We have recently constructed a chimeric protein (pPGPrA (pPut-GST-protein A)) comprising an authentic N-terminal mitochondrial precursor (Δ1-pyrroline-5-carboxylyl-dehydrogenase, pPut) linked, through glutathione-S-transferase (GST),1 to IgG-binding domains derived from staphylococcal protein A (29). This construct becomes trapped en route to the matrix, spanning both outer and inner membranes in such a way that the entire Put moiety reaches the matrix while only the folded protein A domain remains outside. During in vivo import of pPGPrA, the outer and inner membranes of mitochondria become progressively “zippered” together. Under the electron microscope, these appear as long stretches of close membrane contact. Based on these results, we proposed that the outer and inner mitochondrial membrane channels, which normally interact only transiently, can be tightly joined by means of arrested PGPrA translocation intermediates (29). We now provide biochemical evidence for this hypothesis. We demonstrate that pPGPrA follows the same import pathway as authentic pPut both in vitro and in intact yeast cells. Blocking of import sites with PGPrA intermediates inhibits protein import and cell growth. These findings are extended by the isolation of a multisubunit complex containing PGPrA intermediates generated in vivo and essential components of both outer and inner membrane channels.

**EXPERIMENTAL PROCEDURES**

**Constructs**

pET21b/pPGPrA—The pPGPrA open reading frame was excised with XbaI and NcoI from the plasmid pET3a/pPGPrA (29) and subcloned into the same sites of pET21b/pPGPrA with a C-terminal His6 tag.

pET21b/ΔN-PGPrA—The NdeI/NsiI fragment of the plasmid pET21b/pPGPrA was replaced by a short linker using the oligonucleotides 5′-CTGAGTGCTGACAGTGCAGGATCT-3′ (sense) and 5′-TACTGGCA-CATACA-3′ (antisense). The resulting plasmid, pET21b/ΔN-PGPrA, encodes a truncated protein in which the first 124 amino acids of pPGPrA-His6 are replaced by Met-Tyr-Ala-

pYES/m*PGPrA—The HindIII/XbaI fragment of pYES2/pPGPrA was replaced by a short linker using oligonucleotides 5′-AAGCCTCA GGATCTATGGCACTAGGCTATCC-3′ (sense) and 5′-CAGAATGAGGCATCATGAAATCGTAA-3′ (antisense). The resulting plasmid, pET21b/ΔN-PGPrA, encodes a truncated protein in which the first 124 amino acids are replaced by Met-Tyr-Ala-

pYES/pPut—The plasmid pYES/pPGPrA was digested with KpnI and BamHI. The fragment containing the vector and most of pPut was re ligated in the presence of oligonucleotides 5′-CAGAATGAGGCATCATGAAATCGTAA-3′ (sense) and 5′-GATCCTCA GGATCTATGGCACTAGGCTATCC-3′ (antisense) to generate pYES/pPut.

pRS145GAL1/mGFP—A chimeric protein (mGFP) in which the first 90 amino acids of mGFP were linked to the green fluorescent protein (GFP) was constructed as follows. The HindIII/SacI fragment of pYES2/pPGPrA was cloned into the HindIII/SmaI sites of pBluescript II SK+ (pBS, Stratagene), resulting in pNS161. The GFP open reading frame was excised from pGFPuv (CLONTECH) with Xbal and EagI and ligated into the same sites of pNS161 to yield pBS/mGFP. The HindIII/EagI fragment of GFP from pBS/mGFP was subsequently subcloned into the same sites of pRS145GAL1 to yield pRS145GAL1/mGFP (pNS168), which encodes the chimeric protein (mGFP) under the control of the GAL1 promoter for expression in yeast.

**Expression of Proteins in Bacteria and Their Purification**

Proteins were expressed in bacteria as described previously (30). pPGPrA, sequestered in insoluble inclusion bodies, was solubilized in 20 mM Tris-HCl, pH 6.0, containing 8 M urea and 1 mM β-mercaptoethanol, applied to a Mono M 5/20 column (Amersham Pharmacia Biotech), and subsequently eluted with a NaCl gradient (0–100 mM). Peak fractions containing pPGPrA were concentrated using a N2+-NTA column (QIAGEN Inc.). The final eluate was in 20 mM Hepes/KOH, pH 7.5, 0.4 M imidazole, 8 M urea, and 1 mM dithiothreitol. Inclusion bodies containing ΔN-PGPrA were solubilized in 8 M urea and directly purified on N2+-NTA.

**Expression of Proteins in Yeast**

A protease-deficient yeast strain, ABYS 86 (MATa prl-1 prb-1-1 prc-1-1 urs3-1 ura3 Δ5 leu2-3,112 his 1), was cotransformed with pNS168 and pYES/pPGPrA, pYES/m*PGPrA, or pYES/pPut, and Ura'Leu+ transformants were selected. As a control for nuclear import, strain ABYS 86 was cotransformed with pYES/pPGPrA and a CEN-HIS3 plasmid containing the GAL1 promoter-driven gene fusion of histone H2B1 to GFP (31), and Ura' His+ transformants were selected. Various transformants were grown at 30°C to mid-log phase in selective medium containing 2% dextrose and subsequently shifted to medium containing 2% galactose and 1% raffinose. The subcellular distribution of GFP reporter proteins was analyzed by direct fluorescence microscopy as described (31).

**Affinity Chromatography**

Mitochondria were purified from yeast cells that carried the plasmid pYES/pPGPrA after 4 h of induction with 1% galactose (29). Mitochondria were resuspended by sonication in 20 mM Tris-HCl, pH 7.5, 72% sucrose, 0.15 M NaCl, 1 mM EDTA, and a protease inhibitor mixture. Samples (4 ml) were transferred to a SW 40 tube and layered with 6 and 2.5 ml of 68% and 5% sucrose in the same buffer, respectively. Membranes were floated by centrifugation at 202,000 × g for 40 h. Purified membranes (68%/55% interface) were solubilized in 20 mM Tris-HCl, pH 7.5, 0.7% digitonin, 0.25 M NaCl, 1 mM EDTA, 10% glycerol, protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 45% phospholipid, 10% phosphatidylethanolamine, 10% phosphatidylinositol, and 10% cardiolipin). Samples were centrifuged (TLA-100.3, 153,000 × g, 30 min), and the supernatant thus obtained was loaded onto a column containing 0.1 ml of rabbit IgG-Sepharose (Cappel). The column was sealed and rotated end-over-end at 4 °C for 2 h. Following washing, the column was eluted with 0.1 M glycine HCl, pH 2.8, 0.15 M NaCl, and 0.02% digitonin. The eluate was immediately neutralized with 2 M Tris base. PGPrA interferes with Western blot analysis since protein A has a high affinity for rabbit IgG. Therefore, PGPrA was specifically removed from the affinity eluate as follows. SDS (0.2% final concentration) was added to the affinity eluate and incubated at room temperature for 5 min. Samples were then adjusted to 1% Triton X-100 and again passed through a fraction of IgG-Sepharose column. The unbound fraction was analyzed by all immunoblot analysis. Under these conditions, only PGPrA was retained by the IgG-Sepharose column; all other proteins were quantitatively recovered in the unbound fraction.

**Antibodies**

All antibodies were raised in rabbits. Antibodies against mt-Hsp70, Tom70, Tom40, Tom20, Tim44, Tim11, and ponin were generated against bacterially expressed and purified full-length proteins. The first 97 amino acids of Tom22 were expressed in bacteria as a GST fusion protein using the vector pGEX-4T (Amersham Pharmacia Biotech). Anti-Tom22 antibodies were against the purified fusion protein. Antibodies against Tim23 (residues 1–14) and Tim17 (residues 142–156) were raised against chemically synthesized peptides as described (32). Antibodies against p32 have been described earlier (32, 33). Antibodies against the ADP/ATP carrier and cytochrome c peroxidase were from H. Murakami and J. Kaput, respectively.

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1 The abbreviations used are: GST, glutathione S-transferase; GFP, green fluorescent protein; BioTis, 2-[bis[2-hydroxyethyl]amino]-2-(hydroxymethyl)-propane-1,3-diol; NTA, nitroliatroic acid; PAGE, polyacrylamide gel electrophoresis; GIP, general insertion pore.

2 N. Schulke and D. Pain, unpublished data.
In vitro import reactions were performed using mitochondria isolated from *Saccharomyces cerevisiae* strain D273-10B (ATCC 24657) in the presence of 1 mM ATP and 1 mM GTP (30, 34). Briefly, urea-denatured precursors were diluted 50-fold in the import reactions; the final urea concentration was 0.16 M. A final urea concentration as high as 0.6 M does not inhibit import of native precursors (35). Following import, reaction mixtures were left untreated or were treated with trypsin (0.1 mg/ml) for 30 min at 0 °C. Trypsin was inactivated by a mixture of inhibitors, and mitochondria were reisolated. Samples were analyzed by SDS-PAGE and autoradiography.

### RESULTS

**pPGPrA Fusion Protein Used to Generate Translocation Intermediates**—pPGPrA synthesized in reticulocyte lysate becomes trapped as a translocation intermediate en route to the matrix; hence, it could be of potential use for blocking mitochondrial import sites (29). However, the quantity of proteins that can be synthesized in cell-free translation systems is very low. Therefore, to maximize our ability to saturate import sites with the translocation intermediates, we decided to use pPGPrA with a C-terminal His$_6$ tag overexpressed in bacteria. The overexpressed protein was sequestered in insoluble inclusion bodies (Fig. 1A, lane 2, Load) and solubilized in 8 M urea. When this fraction was directly chromatographed on Ni$^{2+}$-NTA resin, three other smaller molecular mass proteins copurified with pPGPrA (data not shown). These contaminants are probably N-terminally truncated forms of pPGPrA resulting from internal initiations. On SDS-PAGE, one of these contaminants migrated very closely to the mature chimeric protein obtained after removal of the signal sequence (mPGPrA) and allows visualization of the ladder of intermediates. The migration position of mPut-GST (mPG; molecular mass of ~84 kDa) is indicated in order to provide a relative topology of various protected fragments. Lane 1 represents 2% of the precursor used per import assay. Schematic representations of pPGPrA, mPGPrA, and mPut-GST are shown.

We next sought to determine the location of mPGPrA molecules. The susceptibility of the precursor protein to proteolytic cleavage both on the matrix side (by signal peptidase) and on the cytosolic side (by externally added protease) is evident that the precursor has become lodged or stuck in the membrane.
as a translocation intermediate, spanning both the outer and inner membranes. Only ~50% of the mPGPrA molecules remained completely protected from externally added trypsin. The remaining 50% were digested to varying degrees by external trypsin (Fig. 1B, lane 4; equivalent to 20 times the lane 2 load), generating a ladder of bands. The ladder likely resulted from the partial cleavage of imported molecules arrested at various stages of translocation. Although the signal sequence of these molecules was cleaved by the matrix-localized signal peptidase, different lengths of the C-terminal domain remained exposed outside. A more slowly migrating band in the ladder corresponds to an intermediate with a longer protease-protected N-terminal segment, i.e., an intermediate in which more of the N terminus of pPGPrA has been translocated across the outer membrane. The band representing mPut-GST (mPG) was the major band in the ladder (Fig. 1B, lane 4), indicating that a significant portion of the intermediates were trapped in such a way that only the protein A domain remained exposed outside the organelle. The ladder was not the result of incomplete digestion of non-imported molecules by trypsin since (i) pPGPrA was completely digested by trypsin when import was inhibited in the presence of valinomycin (Fig. 1B, lane 5), and (ii) both precursor and mature forms of PGPrA were completely digested by trypsin in the presence of Triton X-100 (data not shown).

The import of urea-denatured proteins at 30 °C is very rapid (34). As a result, the protein A domain of mPGPrA may be unable to fold sufficiently to serve as an efficient C-terminal block during the import reaction. We reasoned that if the import of urea-denatured PGPrA could be slowed by lowering the incubation temperature, the protein A moiety of the chimeric precursor might fold sufficiently, thereby yielding a larger number of translocation intermediates trapped within the import channels and fewer completely imported mature molecules in the matrix. Indeed, this was the case. When the import was carried out at 0 °C, intermediate formation was greatly increased (compare mPG/mPGPrA ratio in Fig. 1B, lane 4, with that in Fig. 1C, lane 4). We therefore used these conditions for import inhibition studies.

Protease Protection of PGPrA Intermediates at Tom/Tim Contact Sites—To investigate how stably the outer and inner membrane channels were held together by PGPrA intermediates, import reactions were subjected to trypsin treatment in isotonic (Fig. 2A, lane 3) or hypotonic (the latter disrupts the outer membrane and exposes the intermembrane space; lane 4) buffer. The pattern and the intensity of each band in the ladder were almost identical. This suggests that no further degradation of intermediates by trypsin occurred when the intermembrane space was exposed by hypotonic shock. As a control, we monitored the release of endogenous cytochrome c peroxidase (an intermembrane space protein) as an indicator of outer membrane disruption (Fig. 2B). More than 90% of cytochrome c peroxidase was released under hypotonic conditions. Taken together, these data suggest that PGPrA translocation intermediates were able to hold the outer and inner membrane channels together, preventing trypsin access to the intermediates through the intermembrane space.

Blocking of Translocation Sites with PGPrA Intermediates—To test whether pPGPrA and pPut compete with each other for import, mitochondria were incubated with [35S]Met-labeled native pPut (synthesized in reticulocyte lysate) in the presence of different concentrations of unlabeled urea-denatured pPGPrA. About 50% of pPut import was inhibited by pPGPrA at 2 µg/ml (Fig. 3A). The inhibition indeed occurred in a dose-dependent manner (Fig. 3B). However, a relatively high concentration of pPGPrA was required for complete inhibition of pPut import. This was perhaps due to the rapid import of urea-denatured pPGPrA at 30 °C, making it a weak competitor. Nevertheless, to rule out any nonspecific effect of a relatively high concentration of pPGPrA, we tested ΔN-PGPrA (with the first 172 amino acids, including the signal sequence of PGPrA, deleted) as a control. Even at the highest concentration, urea-denatured ΔN-PGPrA had no significant inhibitory effect on the import of native pPut (Fig. 3B, lane 7).

To test whether preformed PGPrA intermediates would inhibit subsequent pPut import, intermediates were first generated by incubating unlabeled urea-denatured pPGPrA with mitochondria at 0 °C as in Fig. 1C (lane 4). Mitochondria were then resolated to remove unbound pPGPrA and tested for their ability to import [35S]Met-labeled native pPut at 30 °C. PGPrA intermediates (Mito + TI) were able to inhibit pPut import by ~60% compared with the mock control (Fig. 3C, compare lanes 2 and 5). Complete inhibition was not achieved because some of

**Fig. 2.** Trypsin sensitivity of translocation intermediates in isotonic (intact mitochondria) and hypotonic (mitoplasts) buffers. A, import of urea-denatured pPGPrA was carried out at 0 °C for 30 min, followed by a shift to 30 °C for 5 min. Reaction mixtures were immediately chilled on ice and divided into two sets. Samples were diluted 6-fold so that the initial isotonic sorbitol concentration (0.6 M) in one set was maintained (Iso) while that of the other set was lowered to 0.1 M (Hypo). Following incubation on ice for 10 min, samples were directly analyzed or treated with trypsin (0.1 mg/ml). Untreated (lane 2) and trypsin-treated (lanes 3 and 4) samples represent 5 and 100% of reaction mixtures, respectively. Lane 1 represents 2% of the precursor used per import assay. B, mitochondria (2 mg/ml) in isotonic buffer were diluted 6-fold either with the same buffer (0.6 M sorbitol) or buffer containing no sorbitol, as in A. Following incubation on ice for 10 min, mitochondria or mitoplasts were sedimented. Equivalent aliquots of the pellet (P) and supernatant (S) fractions were analyzed by Western blotting using anti-cytochrome c peroxidase antibodies (αCCP). mPG, mPut-GST.
samples were treated with trypsin and analyzed. Affinity-purified rabbit IgG. Following incubation for 15 min at 30 °C, were performed in the absence or presence of different concentrations of pPut used per import assay. A pPut used per import assay. Analyzed by SDS-PAGE and autoradiography. The urea-denatured precursor (mtGFP) with a mitochondrial targeting sequence. Mitochondria were reisolated and tested for import of 35S-labeled native pPut. These import reactions were carried out for 30 min at 0 °C in the presence of unlabeled urea-denatured pPGPrA or ΔN-PGPrA. The final urea concentration in all import reactions was 0.16 M. Lane 1 represents 15% of pPut used per import assay. C, import reactions were performed in the absence or presence of different concentrations of affinity-purified rabbit IgG. Following incubation for 15 min at 30 °C, samples were treated with trypsin and analyzed. Lane 1 represents 15% of pPut used per import assay.

To obtain a more quantitative inhibition of pPut import, we took advantage of the high affinity of protein A for rabbit IgG. As expected, intermediates with bound IgG remained unchased, occupying the import sites (data not shown), and the inhibition of pPut import was enhanced and dependent on the dose of IgG (Fig. 3C, lanes 6 and 7). As a control, import of pPut into mitochondria containing no intermediate (Mock Mito) was not affected by IgG (Fig. 3C, compare lanes 2–4).

**Inhibition of Protein Import and Cell Growth by in Vivo Overexpression of pPGPrA**—To directly visualize mitochondrial protein import in intact yeast cells, we constructed a fluorescent precursor (mtGFP) with a mitochondrial targeting sequence. mtGFP was created by fusing the first 90 amino acids (including the signal sequence) of pPut to the N terminus of GFP, and the construct was placed under the control of the GAL1 promoter. Plasmid-borne mtGFP was expressed when yeast cells were grown in medium containing galactose, but not glucose. The GFP fluorescence showed a vesicular and reticular pattern characteristic of mitochondrial localization and co-localized with the fluorescence pattern seen with anti-porin antibodies (data not shown).

To validate our *in vitro* findings in intact cells, mtGFP and pPGPrA were expressed simultaneously from separate plasmids in transformed yeast. As a control, mtGFP was coexpressed with pPut or m*PGPrA* (the latter is an N-terminally truncated form of pPGPrA and lacks a signal sequence). Galactose-induced expression was comparable for pPGPrA, pPut, and m*PGPrA*, as monitored by Western blotting using anti-pPut antibodies (data not shown). Following induction with galactose, cells were examined at various time points by fluorescence microscopy (Fig. 4A). In control cells coexpressing pPut or m*PGPrA*, GFP fluorescence associated with mitochondria was clearly visible within 3–6 h, and levels increased with time. By contrast, cells coexpressing mtGFP and pPGPrA showed a much reduced mitochondrial GFP fluorescence. These results suggest that when import channels were occupied by trapped PGPrA intermediates, import of mtGFP was strongly inhibited. The inhibition of mitochondrial import by pPGPrA was specific and not a general transport defect since, under identical conditions, the nuclear import of histone H2B1 fused to GFP was not affected (Fig. 4B). Furthermore, direct measurement of the membrane potential showed no change in the PGPrA-blocked mitochondria (data not shown). Thus, the inhibition of mtGFP import was likely due to a blockade of mitochondrial import sites and was not a nonspecific effect due to uncoupling of mitochondria resulting from the accumulation of PGPrA intermediates. This agrees well with earlier reports that saturation of import sites with other translocation intermediates (that span both outer and inner membranes) did not dissipate the membrane potential (36–38).

Accumulation of PGPrA intermediates in import sites would likely interfere with the import of other proteins in addition to mtGFP. If so, growth of yeast cells overexpressing pPGPrA might be impaired. We therefore investigated the effect of progressive expression of pPGPrA on cell growth. When cells were grown in glucose-based medium, the growth rates of all transformants were similar (data not shown). However, following a shift to galactose, cells expressing pPGPrA grew significantly slower than the control cells expressing pPut or m*PGPrA* (Fig. 4C). These results suggest that when import channels were occupied by trapped PGPrA intermediates, import of mtGFP was strongly inhibited. The inhibition of mitochondrial import by pPGPrA was specific and not a general transport defect since, under identical conditions, the nuclear import of histone H2B1 fused to GFP was not affected (Fig. 4B). Furthermore, direct measurement of the membrane potential showed no change in the PGPrA-blocked mitochondria (data not shown). Thus, the inhibition of mtGFP import was likely due to a blockade of mitochondrial import sites and was not a nonspecific effect due to uncoupling of mitochondria resulting from the accumulation of PGPrA intermediates. This agrees well with earlier reports that saturation of import sites with other translocation intermediates (that span both outer and inner membranes) did not dissipate the membrane potential (36–38).

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Tim23 are structural elements of the inner membrane channel. Tim44 (a peripheral membrane protein) and mt-Hsp70 (a soluble matrix protein), while clearly present, were not as enriched as the other four integral membrane proteins. Tim44 interacts only weakly with the Tim machinery and only transiently with a small fraction of the total mt-Hsp70 (22–24, 26). The interaction of Tim44 with preproteins is also fleeting (39). It is therefore not surprising that Tim44 and mt-Hsp70 were only moderately enriched in the eluate.

Neither of the redundant receptors (Tom70 and Tom20) was detected in the affinity eluate. These proteins may not be core components of the outer membrane translocation channel (14), or they may have been dissociated from other channel subunits during solubilization and affinity chromatography. We also tested the affinity eluate with antibodies against two other membrane proteins, p32 (32, 40) and Tim11 (41), which have been suggested to be components of the import machinery. Neither of these proteins was present. Both p32 and Tim11 are not essential for cell viability, and their functions remain controversial. The protein p32 has been identified as a phosphate carrier (42). Likewise, Tim11 has been recently shown to be identical to ATPase subunit e (43). The data presented here make it less likely that these proteins directly participate in mitochondrial protein import, although the other possibility, that they might have been dissociated during chromatography, cannot be completely ruled out.

To evaluate the specificity of the association of the essential Tom and Tim proteins with the in vivo intermediates, we performed additional controls. First, we examined two abundant proteins that are unrelated to import: porin (a major outer membrane protein) and the ADP/ATP carrier (a major inner membrane protein). Both were absent from the PGPrA affinity eluate (Fig. 5). Second, mitochondria were isolated from cells expressing the signal-less fusion protein m*PGPrA, and affinity chromatography was performed. None of the 12 proteins

![Fig. 4. Inhibition of protein import and cell growth by in vivo PGPrA translocation intermediates.](image)

A, yeast cells carrying the plasmid pRS415GAL1/mtGFP were transformed with pYES/pPGPrA, pYES/m*PGPrA, or pYES/pPut. Transformants were grown overnight in dextrose and shifted to galactose for simultaneous coexpression of proteins: mt-GFP together with pPGPrA, m*PGPrA, or pPut. At the indicated time points, cells were examined by phase-contrast and fluorescence microscopy. B, yeast cells carrying a CEN-HIS3 plasmid containing the GAL1 promoter-driven gene fusion of histone H2B1 to GFP were transformed with the pYES2 vector or pYES/pPGPrA. Following expression of proteins with galactose for 12 h, cells were analyzed exactly as described for A. C, yeast cells carrying the plasmid pYES/pPGPrA, pYES/m*PGPrA, or pYES/pPut were grown overnight in dextrose and then shifted to galactose at zero time. The growth rates of cells were determined by measurement of the absorbance of the cultures at 600 nm.

![Fig. 5. Identification of a multisubunit complex stabilized by PGPrA intermediates in intact cells.](image)

Mitochondria were isolated from yeast cells after induction of plasmid-borne pPGPrA with galactose for 4 h. Purified membranes with trapped PGPrA intermediates were solubilized with digitonin and affinity-chromatographed on IgG-Sepharose. Proteins were analyzed by immunoblotting using various antibodies. The lane marked Load represents 0.2% of the total digitonin-solubilized material loaded onto IgG-Sepharose. The lane marked Eluate represents 7.5% of the total eluate fraction. αAAC, anti-ADP/ATP carrier antibodies.
that we tested was detected on immunoblots of this control eluate (data not shown).

**DISCUSSION**

Protein import into the mitochondrial matrix is mediated by two translocases: one (Tom) in the outer and the other (Tim) in the inner membrane of mitochondria. We have isolated a multisubunit complex from yeast cells containing essential elements of both Tom and Tim. These translocases normally interact only transiently during protein import. However, they can be tightly joined when a novel chimeric precursor (pPGPrA) becomes physically trapped en route to the matrix as it spans both the outer and inner membranes. Under these conditions, mitochondrial protein import is inhibited, and this, in turn, leads to reduced cell growth.

The key to successfully isolating Tom and Tim complexes as a single entity from intact cells resides mainly in the choice of pPGPrA as the import substrate. Trapped intermediates spanning both mitochondrial membranes have previously been achieved in several ways. Two popular methods are based on (i) the use of ligands (e.g. methotrexate) to stabilize the tertiary structure of the preprotein (e.g. dihydrofolate reductase), preventing it from unfolding, thereby blocking its import (36, 44), and (ii) covalent attachment of a highly cross-linked protein moiety (e.g. bovine pancreatic trypsin inhibitor) to the precursor’s C-terminus in order to “plug up” the translocation machinery (37). Although these translocation intermediates have been successfully used to saturate mitochondrial import sites in vitro, their application in vivo as a “fishing hook” for isolating Tom and Tim complexes is restricted because of their inherent limitations. For example, bovine pancreatic trypsin inhibitor is unlikely to form the required intramolecular disulfide bridges to serve as a highly cross-linked moiety in the reducing environment of the cytosol. Likewise, a ligand-induced block requires the presence of excess ligand (36, 38), which precludes subsequent affinity chromatography.

How do our data relate to other studies on Tom and Tim complexes? Previous in vitro studies used COXIV-DHFR-BPTI (the signal sequence of cytochrome c oxidase subunit IV linked, through dihydrofolate reductase, to bovine pancreatic trypsin inhibitor) as an import substrate, generating an intermediate that spanned both outer and inner membranes. Horst et al. (28) showed that antibodies against Tom40 coprecipitated mt-Hsp70/Tim44 (and vice versa) only in the presence of the intermediate. However, none of the other Tom and Tim components was coprecipitated with this intermediate. Among several possibilities, two explanations are the most likely. First, these experiments were done with samples solubilized with the detergent MEGA-8, whereas many other studies, including ours, used digitonin. It is possible that other Tom and Tim proteins are easily dissociated by MEGA-8, but not by digitonin. Second, the intermediate became entirely accessible to proteases when the outer membrane was ruptured by hypotonic shock, being cleaved evidently from the intermembrane space in mitoplasts (45). Taken together, these experiments suggest that COXIV-DHFR-BPTI fusion protein “tethers” the inner and outer import channels. By contrast, in the presence of trapped PGPrA intermediates, the outer and inner membrane translocases are so closely associated that they perhaps form a continuous channel with no “slack” in the intervening segment. Consequently, a protease has no additional access to the intermediates through the intermembrane space in mitoplasts.

Tom22-Tom20 and Tom70-Tom37 subcomplexes have been suggested to function as mitochondrial receptors. How do we then explain the absence of Tom20 and Tom70 in the complex that we have isolated? We have not tested for Tom37, but it is unlikely to be present since it has not been shown to be part of the Tom complex.) Our data are in good agreement with the reports by Pfanner and co-workers (14). They have extensively characterized the molecular architecture of the Tom complex, particularly by blue native gel electrophoresis. They demonstrated that Tom40 and Tom22 were stably associated in a complex with a molecular mass of ~400 kDa referred to as the GIP complex. Very little, if any, Tom70 or Tom20 was detected with the GIP complex. In fact, a yeast mutant lacking both Tom70 and Tom20 was still able to form the GIP complex when sufficient amounts of Tom22 were synthesized. More importantly, mitochondria isolated from this mutant strain were still able to import preproteins, although at a reduced level. Tom70 and Tom20 likely facilitate import by recruiting preproteins dispersed over the entire outer membrane and delivering them to the GIP complex. These two proteins are not, however, an intrinsic part of the outer membrane translocation channel. In addition to Tom22 and Tom40, the GIP complex was found to contain three small subunits: Tom5, Tom6, and Tom7. It remains to be determined whether these small proteins are also present in our purified fraction. Finally, accumulation of an in vivo translocation intermediate spanning both outer and inner mitochondrial membranes has been recently shown to link a portion of the GIP complex to the Tim complex, generating an ~600-kDa supercomplex. Whereas Tim17 and Tim23 were almost quantitatively present in this supercomplex, neither Tim44 nor mt-Hsp70 was present in stoichiometric equivalents (27). This is consistent with our in vivo data.

A more complete molecular picture of the mitochondrial import machinery is now emerging. Neupert and co-workers (46) have recently reconstituted purified Tom complex from Neurospora crassa into liposomes. Analysis of negatively stained Tom complexes shows stain-filled openings with an apparent diameter of 20 Å, which may represent import pores. In planar lipid membranes, the Tom complex forms a cation-selective high conductance channel. A similar channel activity has also been demonstrated by Pfanner and co-workers (15) using purified yeast Tom40. The methods described here will certainly help in the visualization of not only the outer, but also the inner membrane pores of mitochondria. Furthermore, our techniques should help elucidate the topology and the three-dimensional structure of the entire mitochondrial import machinery.

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**REFERENCES**

1. Neupert, W. (1997) *Annu. Rev. Biochem.* 66, 863–917
2. Sollner, T., Pfaller, R., Griffiths, G., Pfanner, N., and Neupert, W. (1990) *Cell* 62, 107–115
3. Graber, S., Lithgow, T., Bauer, R. E., Lamping, E., Paltauf, F., Kohlewein, S. D., Haecke, V., Junne, T., Schatz, G., and Horst, M. (1995) *J. Cell Biol.* 129, 23–34
4. Kiebler, M., Keil, P., Schneider, H., van der Klei, I. J., Pfanner, N., and Neupert, W. (1993) *Cell* 74, 483–492
5. Sollner, T., Griffiths, G., Pfaller, R., Pfanner, N., and Neupert, W. (1989) *Cell* 59, 1061–1070
6. Hönlinger, A., Bömer, U., Alconada, A., Eckerskorn, C., Lottspeich, F., Dietermeier, K., and Pfanner, N. (1996) *EMBO J.* 15, 2125–2137
7. Kassnenbrock, C. K., Cao, W., and Douglas, M. G. (1993) *EMBO J.* 12, 3023–3034
8. Dietermeier, D., Hönlinger, A., Bömer, U., Dekker, P. J. T., Eckerskorn, C., Lottspeich, F., Kührich, M., and Pfanner, N. (1997) *Nature* 388, 195–200
9. Vestweber, D., Brunner, J., Bacher, A., and Schatz, G. (1989) *Nature* 341, 205–209
10. Kiebler, M., Pfaller, R., Sollner, T., Griffiths, G., Horstmann, H., Pfanner, N., and Neupert, W. (1990) *Nature* 348, 619–620
11. Lüthi-Aujogue, J., Junne, T., Suda, K., Gräter, S., and Schatz, G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 11973–11977
12. Hönlinger, A., Kührich, M., Moczkö, M., Gartner, F., Mallet, L., Bussereau, F., Eckerskorn, C., Lottspeich, F., Dietermeier, K., Jacquet, M., and Pfanner, N.
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(1995) Mol. Cell. Biol. 15, 3382–3389
13. Lithgow, T., Junne, T., Wachter, C., and Schatz, G. (1994) J. Biol. Chem. 269, 15325–15330
14. Dekker, P. J. T., Ryan, M. T., Brix, J., Muller, H., Hinlenger, A., and Pfanner, N. (1998) Mol. Cell. Biol. 18, 6515–6524
15. Hill, K., Model, K., Ryan, M. T., Dietmeier, K., Martin, F., Wagner, R., and Pfanner, N. (1998) Nature 395, 516–521
16. Maarse, A. C., Blom, J., Keil, P., Pfanner, N., and Meijer, M. (1994) FEBS Lett. 349, 215–221
17. Ryan, K. R., Menold, M. M., Garrett, S., and Jensen, R. E. (1994) Mol. Biol. Cell 5, 529–538
18. Dekker, P. J., Keil, P., Rassow, J., Maarse, A. C., Pfanner, N., and Meijer, M. (1993) FEBS Lett. 330, 66–70
19. Emtage, J. L. T., and Jensen, R. E. (1993) J. Cell Biol. 122, 1003–1012
20. Maarse, A. C., Blom, J., Krainer, E., Ku¨ brich, M., Muller, H., Meijer, M., Craig, E. A., and Pfanner, N. (1994) J. Cell Biol. 127, 1547–1556
21. Schneider, H.-C., Berthold, J., Bauer, M. F., Dietmeier, K., Guiard, B., Brunner, M., and Neupert, W. (1994) Nature 371, 768–774
22. Mosko, M., Dietmeier, K., Sollner, T., Segui, B., Steger, H. P., Neupert, W., and Pfanner, N. (1992) EMBO Lett. 310, 265–268
23. Berthold, J., Bauer, M. F., Schneider, H.-C., Klaus, C., Dietmeier, K., Neupert, W., and Brunner, M. (1995) Cell 81, 1085–1093
24. Dekker, P. J. T., Martin, F., Maarse, A. C., Bomer, U., Muller, H., Guiard, B., Meijer, M., Rassow, J., and Pfanner, N. (1997) EMBO J. 16, 5408–5419
25. Horst, M., Hilfiker-Rothenuhr, S., Oppliger, W., and Schatz, G. (1995) EMBO J. 14, 2293–2297
26. Schulke, N., Sepuri, N. B. V., and Pain, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7314–7319
27. Sepuri, N. B. V., Gordon, D. M., and Pain, D. (1998) J. Biol. Chem. 273, 20941–20950
28. Enenkel, C., Schulke, N., and Blobel, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12986–12991
29. Pain, D., Murakami, H., and Blobel, G. (1999) Nature 347, 444–449
30. Murakami, H., Blobel, G., and Pain, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3358–3362
31. Sepuri, N. B. V., Schulke, N., and Pain, D. (1998) J. Biol. Chem. 273, 1420–1424
32. Eilers, M., Hwang, S., and Schatz, G. (1988) EMBO J. 7, 1139–1145
33. Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F.-U., and Neupert, W. (1989) J. Cell Biol. 109, 1421–1428
34. Vestweber, D., and Schatz, G. (1988) J. Cell Biol. 107, 2037–2043
35. Wienhues, U., Becker, K., Schleyer, M., Guiard, B., Tropschug, M., Horwich, A. L., Pfanner, N., and Neupert, W. (1991) J. Cell Biol. 115, 1601–1609
36. Blom, J., Kubrich, M., Rassow, J., Voss, W., Dekker, P. J., Maarse, A. C., Meijer, M., and Pfanner, N. (1993) Mol. Cell. Biol. 13, 7364–7371
37. Tokatlidis, K., Junne, T., Moes, S., Schatz, G., Glick, B. S., and Kronidou, N. (1996) Nature 384, 585–588
38. Phelps, A., Schobert, C. T., and Wohlrab, H. (1991) Biochemistry 30, 248–252
39. Arnold, I., Bauer, M. F., Brunner, M., Neupert, W., and Stuart, R. A. (1997) FEBS Lett. 411, 195–200
40. Eilers, M., and Schatz, G. (1986) Nature 323, 228–232
41. Jascur, T., Goldenberg, D. P., Vestweber, D., and Schatz, G. (1992) J. Biol. Chem. 267, 13636–13641
42. Künkele, K.-P., Heins, S., Dombrowski, M., Nargang, F. E., Benz, R., Thieffry, M., Walz, J., Lilli, R., Nussberger, S., and Neupert, W. (1998) Cell 93, 1009–1019