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Molecular Chaperones Hsp90 and Hsp70 Deliver Preproteins to the Mitochondrial Import Receptor Tom70

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Summary

The role of cytosolic factors in protein targeting to mitochondria is poorly understood. Here, we show that in mammals, the cytosolic chaperones Hsp90 and Hsp70 dock onto a specialized TPR domain in the import receptor Tom70 at the outer mitochondrial membrane. This interaction serves to deliver a set of preproteins to the receptor for subsequent membrane translocation dependent on the Hsp90 ATPase. Disruption of the chaperone/Tom70 recognition inhibits the import of these preproteins into mitochondria. In yeast, Hsp70 rather than Hsp90 is used in import, and Hsp70 docking is required for the formation of a productive preprotein/Tom70 complex. We outline a novel mechanism in which chaperones are recruited for a specific targeting event by a membrane-bound receptor.

Introduction

Most mitochondrial proteins are nuclear encoded, synthesized as preproteins in the cytosol, and imported into mitochondria via translocation complexes in the outer and inner mitochondrial membranes (Neupert, 1997; Pfanner and Geissler, 2001; Hoogenraad et al., 2002). While the import process itself is understood in considerable molecular detail, particularly in *Saccharomyces cerevisiae* and *Neurospora crassa*, the mechanism by which newly synthesized preproteins are transferred from the cytosol to their receptors in the outer mitochondrial membrane is still largely unresolved.

The protein translocation machinery in the outer mitochondrial membrane (TOM) includes two surface receptors, Tom20 and Tom70 (Söllner et al., 1989, 1990). Tom20 recognizes classical N-terminal mitochondrial targeting sequences, which form positively charged amphipathic helices (Söllner et al., 1989; Brix et al., 1997). In contrast, the Tom70 receptor interacts with internal targeting sequences such as those in the multitransmembrane carrier proteins of the inner mitochondrial membrane (Söllner et al., 1990; Brix et al., 1997). Preproteins are transferred from the receptors to the common Tom22/Tom40 translocation pore and further sorted by the inner membrane translocation machinery (TIM) (Pfanner and Geissler, 2001).

While most secretory proteins are targeted cotranslationally to the endoplasmic reticulum (ER) by signal recognition particle and its membrane-bound receptor (Rapoport et al., 1996), many mitochondrial preproteins are targeted posttranslationally. In particular, Tom70-dependent preproteins may contain multiple targeting signals throughout the polypeptide sequence (Pfanner and Neupert, 1987; Endres et al., 1999; Wiedemann et al., 2001). The ATP-dependent Hsc70/Hsp70 chaperones (referred to as Hsp70 for simplicity) and a 14-3-3 protein termed MSF (mitochondrial import stimulating factor) in the cytosol have been implicated in the import process (Deshaies et al., 1988; Murakami et al., 1988; Hachiya et al., 1994), but mitochondrial receptors for these factors have not been established. Cytosolic Hsp70 is generally involved in the folding of newly synthesized proteins (Bukau et al., 2000; Hartl and Hayer-Hartl, 2002), and its role in mitochondrial import may be an extension of this activity.

Tom70 contains in its cytosolic segment seven tetratricopeptide repeat (TPR) motifs (Steger et al., 1990), which are degenerate 34 amino acid helix-turn-helix sequences. The four C-terminal TPR motifs lie within a core region of Tom70, which recognizes the internal targeting sequences of preproteins (Brix et al., 2000). Interestingly, the three N-terminal TPR motifs have some similarity to a class of proteins that act as cofactors of the Hsp90 and Hsp70 chaperones in the folding of a subset of proteins, including many signal transduction proteins (Young et al., 2001a). Some of these cochaperones, typified by Hop, recognize the C-terminal sequences of Hsp90 and/or Hsp70 through specialized TPR domains in which three TPR motifs are organized into a superhelical structure (Scheufler et al., 2000). A dicarboxylate clamp in the TPR domain coordinates the C-terminal aspartate residue conserved in both chaperones, and the specificity for Hsp70 or Hsp90 is determined by hydrophobic contacts with neighboring residues (Scheufler et al., 2000; Brinker et al., 2002). Hop contains an Hsp70- and an Hsp90-specific TPR clamp domain. Other TPR cochaperones either recognize only Hsp90 or both Hsp90 and Hsp70 in the same TPR domain (Young et al., 2001a).

We now demonstrate the function of a chaperone binding dicarboxylate clamp domain in Tom70. Through this domain, Hsp90 in cooperation with Hsp70 mediates the targeting of a subset of mitochondrial preproteins to the Tom70 receptor in mammals, a novel cellular function of Hsp90 that expands the range of client proteins for this chaperone. In yeast, Hsp70 recruitment is specifically required for preprotein recognition by Tom70 to result in productive import. Thus, the Tom70 receptor is a membrane-localized cochaperone that integrates the Hsp70/Hsp90 chaperones with mitochondrial preprotein targeting and translocation.

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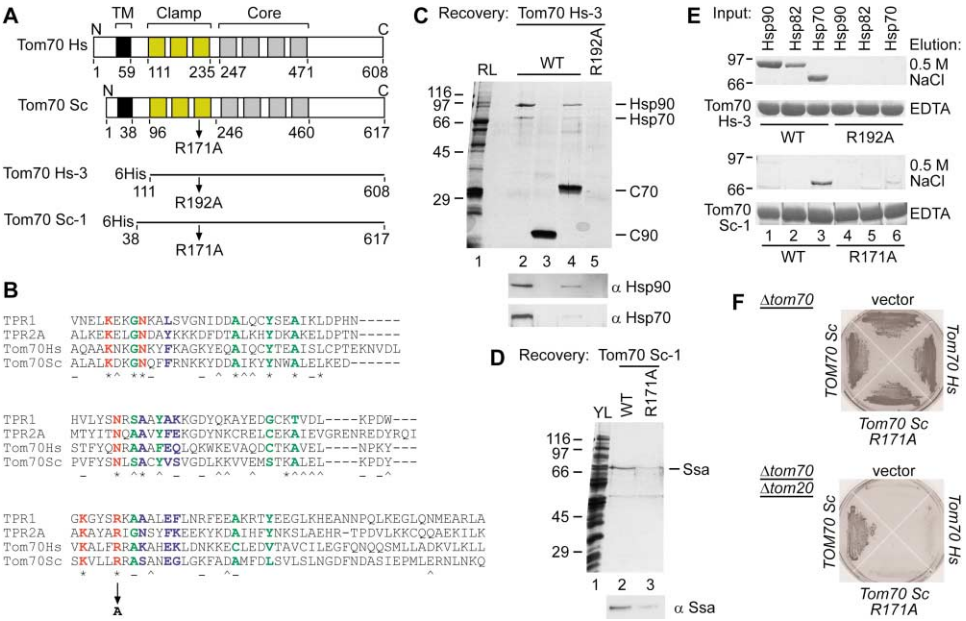

Figure 1. Interaction of Tom70 with Hsp70 and Hsp90

(A) Schematic representation of human (Hs) and *S. cerevisiae* (Sc) Tom70 (top), showing the transmembrane domain (TM, black), and the TPR motifs organized into a dicarboxylate clamp domain (yellow) and preprotein binding core domain (gray). The His-tagged soluble cytosolic segments of human (Hs-3) and *S. cerevisiae* (Sc-1) Tom70 containing the TPR clamp mutations R192A and R171A, respectively, are represented below.

(B) Sequence alignment of the conserved region of the Hsp70 binding TPR1 domain (residues 4–121) and Hsp90 binding TPR2A domain (residues 225–348) of human Hop, human Tom70 (Hs, residues 114–235), and *S. cerevisiae* Tom70 (Sc, residues 99–214). Red, residues of the dicarboxylate clamp recognizing the C-terminal aspartate in Hsp70 and Hsp90; blue, residues responsible for chaperone specificity; green, residues forming intramolecular contacts; the symbols (*), (^), and (−) represent absolute, strong, and weak conservation, respectively. Arrow indicates location of R to A mutation.

(C) His-tagged Tom70 Hs-3 was incubated with reticulocyte lysate (RL, lane 1) and recovered with NiNTA-agarose. Bound proteins were eluted with 0.5 M NaCl (lanes 2–5). Binding was performed with either wild-type (WT, lanes 2–4) or clamp mutant Tom70 (R192A, lane 5); binding reactions were also supplemented with 50 μM C90 (lane 3) or C70 (lane 4) without His-tags. Top, Coomassie-stained gel; bottom, immunoblot with antibodies against Hsp90 and Hsp70. Molecular weight standards in kDa and the positions of identified proteins are marked.

(D) His-tagged Tom70 Sc-1 was incubated with yeast lysate (YL, lane 1) and recovered with NiNTA-agarose. Bound proteins were eluted with 0.5 M NaCl (lanes 2 and 3). Binding was performed with either wild-type (WT, lane 2) or clamp mutant Tom70 (R171A, lane 3). Top, Coomassie-stained gel; bottom, immunoblot with antibodies against Ssa.

(E) His-tagged Tom70 Hs-3 (top) and Tom70 Sc-1 (bottom), either wild-type (lanes 1–3) or TPR clamp mutants (R192A and R171A, lanes 4–6), were incubated with 2 μM purified bovine Hsp90 (lanes 1 and 4), yeast Hsp82 (lanes 2 and 5) or bovine Hsp70 (lanes 3 and 6) and recovered with NiNTA-agarose. Bound chaperones were eluted with 0.5 M NaCl, and Tom70 proteins eluted with 25 mM EDTA. Coomassie-stained gels are shown.

(F) The Tom70 TPR clamp is essential for Tom70 function in vivo. Top, Δtom70 yeast harboring the plasmid *pRS315* with either no open reading frame (vector) or encoding wild-type (*TOM70 Sc*), clamp mutant (*Tom70 Sc-R171A*), or human Tom70 (*Tom70 Hs*) were grown on synthetic media. Bottom, Δtom70 Δtom20 yeast harboring the plasmids *p416ADH/TOM20*, and the same *pRS315* plasmids as above were grown on synthetic media containing 5-fluoroorotic acid to remove the *TOM20* plasmid. Growth at 30°C was observed after 3–5 days.

Results

Tom70 Specifically Recognizes Hsp70 and Hsp90

The three N-terminal TPR motifs of Tom70 were predicted to form a dicarboxylate clamp domain based on their similarity to the TPR1 and TPR2A domains of Hop, which interact with the C termini of Hsp70 and Hsp90, respectively (Figures 1A and 1B) (Scheufler et al., 2000). Several residues in the Hop TPR domains critical for chaperone recognition are conserved in both human and yeast Tom70 (Figure 1B). One of these residues, the arginine at position 192 in human Tom70 (position 171 in *S. cerevisiae*), was mutated to an alanine to provide Tom70 clamp mutants (Figures 1A and 1B). The corresponding mutations in the TPR domains of Hop disrupt binding to Hsp90 and Hsp70 (Brinker et al., 2002). The residues of Hop that determine the specificity for Hsp90 or Hsp70 are not absolutely conserved in Tom70, so it was possible that Tom70 would recognize either Hsp90 or Hsp70, or both. A similar TPR clamp domain was not identified in the Tom20 receptor.

To test the prediction of the Tom70 TPR clamp, the cytosolic segments of human and yeast Tom70 were purified as His-tagged proteins and used in capture experiments with cell lysates. Both wild-type and clamp mutant proteins fractionated upon gel filtration as homodimers (data not shown). Human Tom70 (Hs-3) recognized predominantly Hsp90 and less efficiently Hsp70 in rabbit reticulocyte lysate (RL). The chaperones were readily eluted from Tom70 with 0.5 M salt (Figure 1C, lane 2), similar to the interaction of Hop with Hsp70 and Hsp90 (Brinker et al., 2002). Chaperone binding to Tom70 was competed effectively by an excess of the Hsp90 and Hsp70 C-terminal domains (C90 and C70),

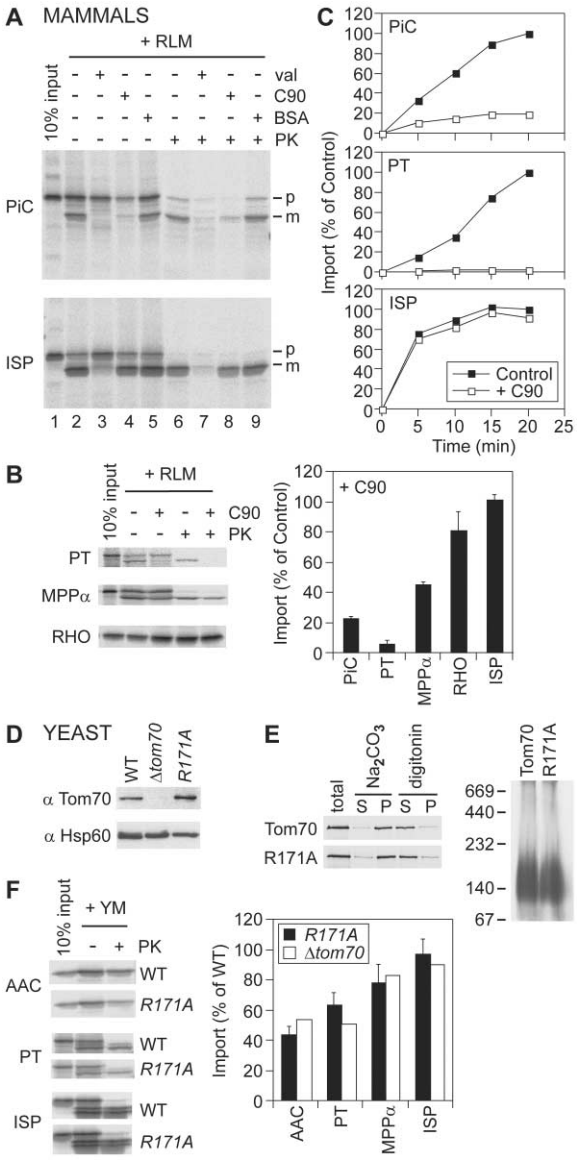

Figure 2. Chaperone/Tom70 Interactions Are Required for Preprotein Import

(A) Cell-free translated ^{35}S-labeled PiC and ISP (lane 1) were incubated with isolated rat liver mitochondria (RLM). Mitochondria were reisolated and either left untreated (lanes 2–5) or treated with proteinase K (PK, lanes 6–9), followed by SDS-PAGE and phosphorimager scanning. Where indicated, reactions contained 1 μM valinomycin (val) to disrupt the membrane potential, 20 μM C90, or an equivalent amount of control protein (BSA). Precursor (p) and mature (m) forms of the imported proteins are marked.

(B) Left, the import of PT, MPPα, and RHO in the presence or absence of excess C90 was assayed as in (A). Right, quantitation of import in the presence of C90 relative to control reactions. In all figures, only PK-resistant, mature proteins were quantified. Error bars are shown for at least three replicates.

(C) Time course of import of PiC, PT, and ISP into RLM in the absence (closed squares) or presence of excess C90 (open squares).

(D) The clamp mutant Tom70 was introduced into Δtom70 yeast. Mitochondria were isolated from wild-type or Δtom70 yeast, or Δtom70 yeast expressing the clamp mutant Tom70 (*R171A*) and characterized by immunoblotting with antibodies against yeast Tom70 and Hsp60.

(E) Cell-free translated ^{35}S-labeled wild-type yeast Tom70 and the clamp mutant (R171A) were incubated with equal amounts of mitochondria from Δtom70 yeast. Left, mitochondria were reisolated (total) and extracted with 0.1 M Na_2CO_3 (pH 10.5) or 1% digitonin and, following centrifugation, the supernatant (S) and pellet fractions (P) were analyzed by SDS-PAGE and autoradiography. Right, the digitonin-solubilized samples were analyzed by blue native PAGE and autoradiography.

(F) Left, cell-free translated ^{35}S-labeled AAC, PT, and ISP were incubated with equal amounts of mitochondria (YM) from yeast bearing wild-type (WT) and mutant Tom70 (*R171A*). Mitochondria were reisolated and left either untreated or treated with PK as indicated. Right, quantitation of import into mitochondria bearing clamp mutant Tom70 (R171A, black bars) or no Tom70 (Δtom70, open bars) relative to mitochondria from wild-type (WT) yeast.

suggesting that like Hop, Tom70 recognizes the C-terminal sequences of the chaperones (Figure 1C, lanes 3 and 4). C90 competed more strongly, consistent with the preferential recognition of Hsp90 by Tom70. The R192A clamp mutant of human Tom70 did not recognize either Hsp90 or Hsp70 (Figure 1C, lane 5), indicating that both chaperones bind to a single site on Tom70. Interestingly, yeast Tom70 (Sc-1) recognized only Hsp70 (Ssa), but not Hsp90 (Hsp82) from yeast cell lysate, and the R171A clamp mutant showed only background binding of Hsp70 (Figure 1D). These results were confirmed in similar experiments using purified bovine Hsp90 and Hsp70 as well as yeast Hsp82. Mammalian Tom70 Hs-3 efficiently recovered both Hsp90 and Hsp82 as well as Hsp70 in a salt-elutable manner, whereas the R192A mutation abolished specific binding of all the chaperones (Figure 1E, top). In contrast, yeast Tom70 Sc-1 recovered only Hsp70 in the salt-eluted fraction, and this binding was blocked by the R171A mutation (Figure 1E, bottom). Thus, the difference in chaperone specificity between mammalian and yeast Tom70 is determined by the receptor and not the chaperones.

The importance of the chaperone/Tom70 interactions was next examined in vivo. In *S. cerevisiae*, deletion of Tom70 produces no phenotype under standard growth conditions due to partial functional overlap between the yeast Tom70 and Tom20 receptors (Figure 1F, top) (Steger et al., 1990). However, the Δtom70 Δtom20 double-deletion mutant is not viable (Ramage et al., 1993) but can be complemented by expression of yeast Tom70. Importantly, the Tom70 R171A mutant failed to rescue growth (Figure 1F, bottom). This result suggests that the chaperone/Tom70 interaction is essential under conditions where Tom70 function is stringently required for growth. Human Tom70 could not substitute for yeast Tom70 (Figure 1F, bottom), most likely due to the relatively low overall homology between the two proteins (20% sequence identity).

Chaperone/Tom70 Interactions Are Required for Preprotein Import

To examine the function of the chaperone/Tom70 interactions, the in vitro import of cell-free translated preproteins into isolated rat liver mitochondria was tested in the presence of C90 as a potent competitor of chaperone docking. Import of the Tom70-dependent mitochondrial phosphate carrier (PiC) (Söllner et al., 1990) and Tom20-dependent Rieske iron-sulfur protein (ISP) (Söllner et al.,

1989) was observed by the appearance of the processed, proteinase K (PK)-resistant mature forms of the proteins (Figure 2A, lanes 2 and 6). Import was inhibited by disrupting the inner membrane potential ($\Delta\Psi$) with valinomycin (Figure 2A, lanes 3 and 7). The proteolytic processing of imported PiC is a maturation step of this inner membrane protein that leaves its internal targeting signals intact (Zara et al., 1992). Excess C90 strongly inhibited the import of PiC, while identical amounts of a control protein (bovine serum albumin) had no effect (Figure 2A, lanes 4 and 5, 8 and 9). The import of ISP to the intermembrane space was not affected by C90 (Figure 2A). As described below (Figure 5B), inhibition of import by C90 was not caused by direct interactions between C90 and the preproteins.

The inhibitory effect of C90 varied between preproteins. The import of PiC and of the mitochondrial peptide transporter (PT) (Young et al., 2001b) into their protease-protected location in the inner membrane was strongly inhibited by C90 (Figures 2B and 2C). C90 had no effect on the import of ISP or the matrix protein rhodanese (RHO), but it partially impaired import of the matrix-processing peptidase α subunit (MPPα) (Figures 2B and 2C). Thus, in mammals, inner membrane proteins such as PiC and PT appear to depend the most strongly on the chaperone/Tom70 interaction.

To test the importance of the chaperone/Tom70 interaction in yeast, mitochondria were isolated from both wild-type and Δtom70 strains and a Δtom70 strain expressing the Tom70 R171A mutant. Similar levels of wild-type and mutant Tom70 as well as matrix-localized Hsp60 were observed per mass of isolated mitochondria (Figure 2D, and data not shown). To rule out effects of the R171A mutation on the targeting or assembly of Tom70, cell-free translated wild-type and mutant yeast Tom70 were incubated with Δtom70 mitochondria. Both forms of Tom70 were efficiently targeted to the mitochondria, where they became resistant to extraction at pH 10.5 but were solubilized by 1% digitonin, characteristic of correctly inserted outer membrane proteins (Figure 2E, left). In addition, blue native PAGE (BNP) analysis showed that wild-type and R171A mutant Tom70 migrated identically between 140 and 200 kDa (Figure 2E, right), forming the expected subcomplexes with the TOM machinery (Dekker et al., 1998). Therefore, the chaperone/Tom70 interaction is not required for assembly of the Tom70 receptor itself.

Preprotein import into mitochondria bearing wild-type and mutant Tom70 was next tested in vitro. Because bovine PiC was recognized poorly by yeast mitochondria, Tom70-mediated import of the homologous fungal ADP/ATP carrier (AAC) was tested instead. AAC is not proteolytically processed, and its import into the inner membrane can only be assessed after proteinase K treatment of the mitochondria. The import of both AAC and PT was significantly reduced in Tom70 R171A mitochondria, to the same level observed with mitochondria from Δtom70 yeast (Figure 2F). The residual import observed with Δtom70 mitochondria is consistent with earlier studies and is attributed to the Tom20 receptor (Steger et al., 1990). The import of ISP and MPPα, known to use the Tom20 receptor in yeast, was largely unaffected by Tom70 mutation or deletion (Figure 2F). Thus, the chaperone/Tom70 interactions are essential for the preprotein import function of the Tom70 receptor in both yeast and mammals.

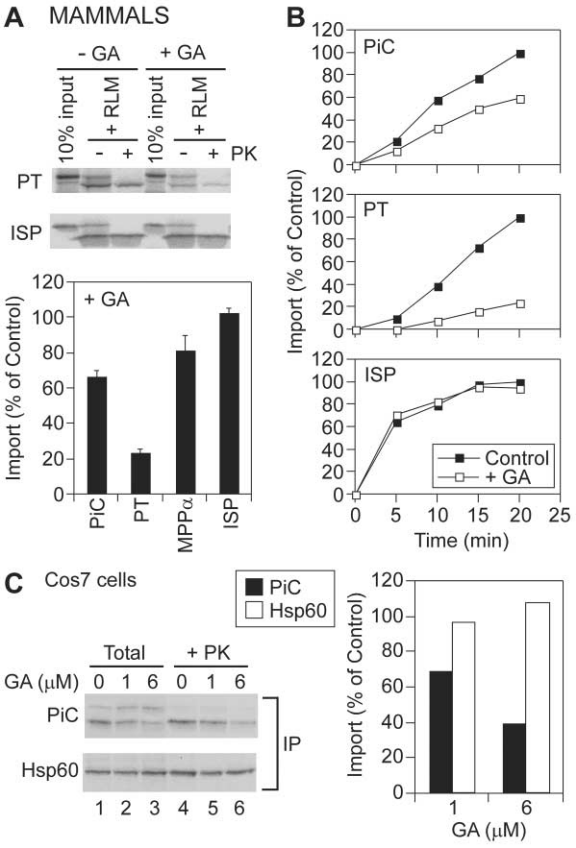

Figure 3. Involvement of Hsp90 in Preprotein Import

(A) Cell-free translations of ^{35}S-labeled preproteins were treated with 18 μM GA or mock-treated, and free GA was removed by rapid gel filtration. Import into RLM was then assayed as in Figure 2. Top, import into RLM of PT and ISP without or with GA treatment. Bottom, quantitation of import after GA treatment relative to untreated control, normalized to input translation product.

(B) Time course of import of PiC, PT, and ISP into RLM without (closed squares) or with GA treatment (open squares).

(C) Cos7 cells were transfected with vectors encoding HA-tagged PiC or Hsp60. Cells were radiolabeled with ^{35}S-Met/Cys and treated with 0, 1, or 6 μM GA for 2 hr. After labeling, cells were either solubilized completely, or the mitochondria were isolated, PK-treated, and solubilized. PiC-HA and Hsp60 were immunoprecipitated (IP) from total cell lysate (Total, lanes 1–3) and PK-treated mitochondria (+PK, lanes 4–6). Right, quantitation of PK-resistant PiC (black bars) and Hsp60 (white bars) relative to control without GA treatment.

Hsp90 Functions in Tom70-Dependent Import in Mammals

To analyze the individual contribution of Hsp90 to the targeting process, we used the Hsp90-specific inhibitor geldanamycin (GA), which blocks with high affinity the ATP-driven chaperone cycle of Hsp90 (Young and Hartl, 2000). GA, added during translation and then removed, significantly decreased the import of PT and PiC compared to the untreated control (Figures 3A and 3B). In contrast, GA treatment had little effect on the import of MPPα and no effect on ISP (Figures 3A and 3B). Thus,

it appears that Hsp90 is specifically involved in the import of the Tom70-dependent inner membrane proteins, consistent with the preprotein-specific effect of C90 competition in Figure 2. The inhibitory effect of GA was in all cases less pronounced than that of C90, which disrupts the contribution of both Hsp90 and Hsp70 to import.

The contribution of Hsp90 to mitochondrial protein import in vivo was tested in Cos7 cells transiently transfected with PiC or Hsp60. The latter protein is imported to the mitochondrial matrix by an N-terminal targeting sequence for Tom20. Newly synthesized proteins were radiolabeled in the presence of levels of GA that did not impair cell growth. Although total protein synthesis was not affected, immunoprecipitation of PiC from total cell lysates revealed a marked decrease in the mature form of the protein and a corresponding accumulation of the unprocessed precursor in the presence of GA (Figure 3C, lanes 1–3, top). A reduced amount of the mature form of PiC was immunoprecipitated from a proteinase K-treated mitochondrial fraction (Figure 3C, lanes 4–6, top). Transfected Hsp60 immunoprecipitated from total cell lysates or PK-treated mitochondria was unaffected by GA treatment (Figure 3C, bottom), ruling out any effect of GA on protein expression or mitochondrial import competence. These results unambiguously establish a critical role of Hsp90 in mammalian mitochondrial import, a novel cellular function for this chaperone.

Hsp70 Functions in Tom70-Dependent Import in Both Mammals and Yeast

The individual contribution of Hsp70 to import was also examined. The Hsp70 ATPase cycle is regulated by co-chaperone proteins, and we used this feature to specifically disable Hsp70. Bag-1 binds to the ATPase domain of Hsp70 through its C-terminal Bag domain (CBag) to promote the exchange of ADP for ATP (Takayama et al., 1997; Höhfeld and Jentsch, 1997; Brehmer et al., 2001), thereby dissociating Hsp70 from polypeptide substrates without affecting Hsp90/substrate interactions (Young and Hartl, 2000; Sondermann et al., 2001). Cell-free translations of preproteins were incubated with or without CBag before addition of mitochondria to initiate import.

With mammalian mitochondria, CBag strongly inhibited the import of PiC and PT but did not affect import of ISP (Figures 4A and 4B). As with GA (Figure 3A), CBag had only a small effect on the import of MPPα (Figure 4A). Furthermore, treatment of the translation reactions with both GA and CBag practically abolished import of PiC and PT relative to the untreated control and significantly inhibited MPPα import without affecting that of ISP (Figure 4C). These effects were similar to the inhibition of import by C90 (Figures 2A and 2B), which would also interfere with the targeting of both Hsp90 and Hsp70. Thus, the inner membrane proteins PiC and PT are dependent on both Hsp90 and Hsp70 for import into mammalian mitochondria.

The import of AAC and PT into yeast mitochondria was also significantly inhibited by excess CBag, whereas the import of MPPα or ISP was only marginally affected (Figure 4D). This inhibition by CBag parallels the import defects observed with mitochondria bearing the Tom70

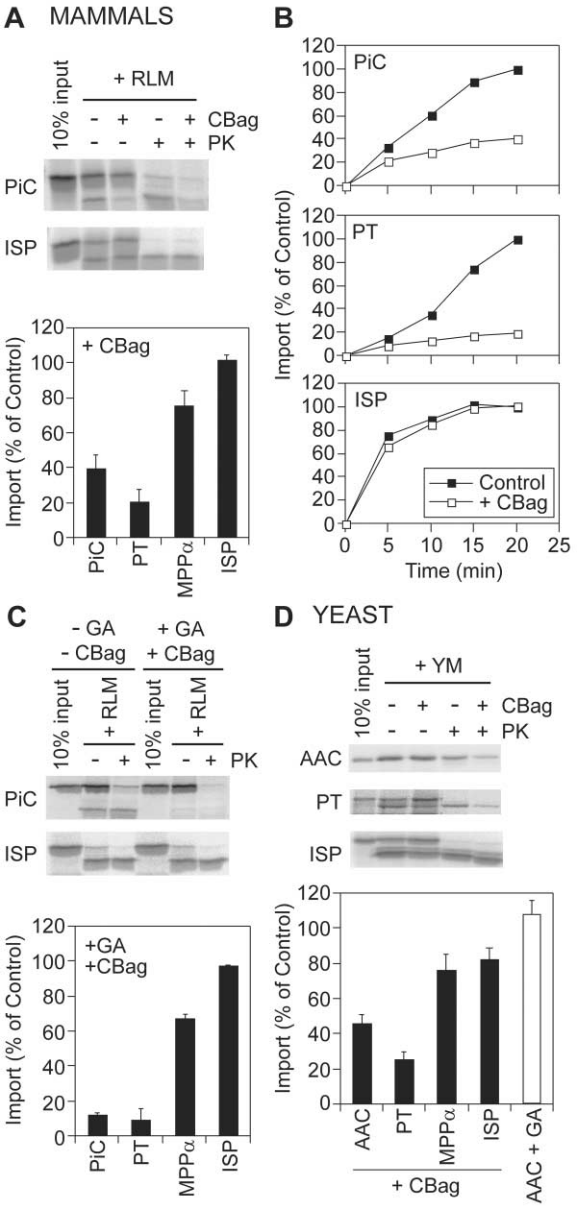

Figure 4. Involvement of Hsp70 in Preprotein Import

(A) Cell-free translations of ^{35}S-labeled preproteins were incubated with 5 μM CBag and 2 mM ATP, or 2 mM ATP alone for 5 min at 30°C before addition of mitochondria. Import into RLM was then assayed as in Figure 2. Top, import into RLM of PiC and ISP without or with CBag. Bottom, quantitation of import with CBag relative to untreated control.

(B) Time course of import of PiC, PT, and ISP into RLM without (closed squares) or with CBag (open squares).

(C) ^{35}S-labeled preproteins were mock- or GA-treated as in Figure 3, then mock- or CBag-treated as in (A) before the gel filtration step. Import into RLM was then assayed as in Figure 2. Top, import into RLM of PiC and ISP without or with GA and CBag treatment. Bottom, quantitation of import with GA and CBag relative to untreated control, normalized to input translation product.

(D) Top, import of AAC, PT, and ISP into YM without or with CBag. Translations were treated with or without CBag as in (A), and import was assayed as in Figure 2. Bottom, quantitation of import with excess CBag relative to untreated control (black bars). The import of AAC into YM after GA treatment was tested as in Figure 3, and the quantitation of import relative to untreated control is shown in the bottom (white bar).

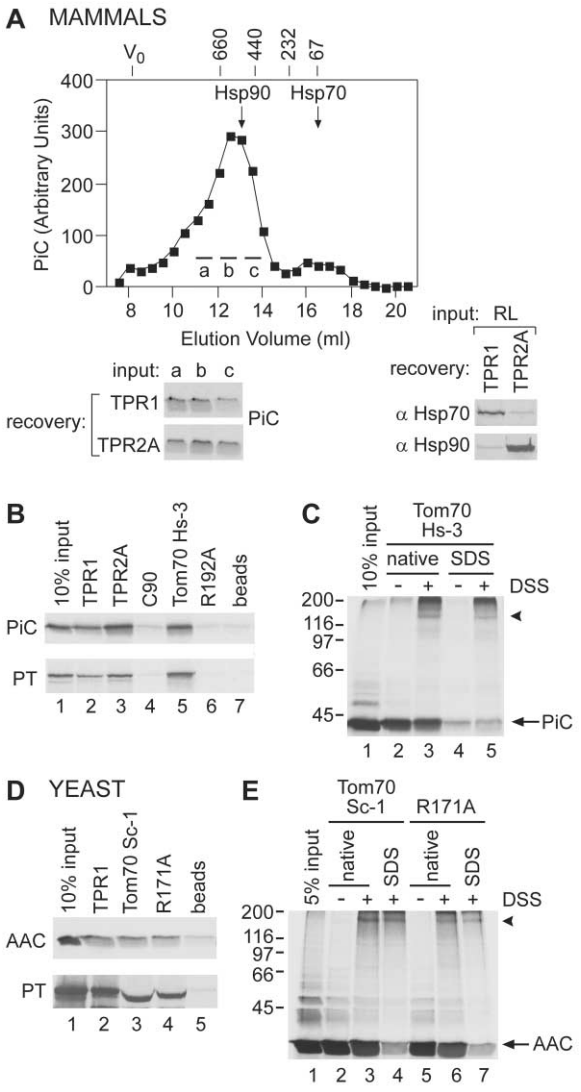

Figure 5. Binding of Preprotein by a Mammalian Multichaperone Complex and Targeting to Tom70

(A) Top, RL-translated ^{35}S-labeled PiC was resolved on a Superose 6 gel filtration column, and the amount of PiC in each fraction determined by SDS-PAGE and phosphorimager analysis. The void volume (V_0), elution of molecular size standards (in kDa), and of purified Hsp90 and Hsp70 are marked above and pooled fractions (a–c) indicated. Bottom left, pooled gel filtration fractions (a–c) were incubated with the His-tagged Hsp70 binding (TPR1) and Hsp90 binding (TPR2A) domains of Hop and recovered with NiNTA-agarose. Bound PiC was detected by SDS-PAGE and autoradiography. Bottom right, His-tagged TPR1 and TPR2A domains of Hop were incubated with unlabeled RL, recovered with NiNTA-agarose, and bound chaperones identified by immunoblotting.

(B) ^{35}S-labeled PiC and PT in RL (lane 1) were incubated with various purified His-tagged proteins, bound proteins recovered with NiNTA-agarose and analyzed by SDS-PAGE, and autoradiography. The His-tagged proteins used were the TPR1 (lane 2) and TPR2A (lane 3) domains of Hop, C90 (lane 4), the cytosolic segment of either wild-type (Hs-3, lane 5), or clamp mutant human Tom70 (R192A, lane 6), and a control with no added protein (beads, lane 7).

(C) ^{35}S-labeled PiC (lane 1) was bound to human Tom70 cytosolic segment (Hs-3) and recovered with NiNTA-agarose as in (A). Reactions were incubated either with 200 μM DSS to crosslink proteins (lanes 3 and 5) or mock-treated (lanes 2 and 4). Samples were washed with a nondenaturing native buffer (lanes 2 and 3) or buffer containing 1% SDS (lanes 4 and 5) and eluted with buffer containing 25 mM EDTA. The position of noncrosslinked PiC is marked on the right; a major crosslinked species is marked with an arrowhead.

(D) ^{35}S-labeled AAC and PT (lane 1) were recovered from RL with various purified His-tagged proteins as in (B). The His-tagged proteins used were the TPR1 domain of Hop (lane 2), the cytosolic segment of either wild-type (Tom70 Sc-1, lane 3), or the TPR clamp mutant (R171A, lane 4) yeast Tom70, and a control with no added protein (beads, lane 5).

(E) ^{35}S-labeled AAC (lane 1) was crosslinked to either wild-type (Tom70 Sc-1, lanes 2–4) or TPR clamp mutant yeast Tom70 (R171A, lanes 5–7) as in (C). Samples were washed with native buffer (lanes 2 and 3 and lanes 5 and 6) or buffer containing 1% SDS (lanes 4 and 7). The position of noncrosslinked AAC is marked on the right and a major crosslinked species is marked with an arrowhead.

TPR clamp mutant (Figure 2F). Thus, the import activity of Hsp70 is most important for those preproteins following the Tom70 receptor pathway. GA did not affect the import of AAC into isolated yeast mitochondria (Figure 4D), consistent with the lack of interaction between yeast Tom70 and Hsp90.

A Multichaperone Complex Targets Preproteins to Tom70 in Mammals

The chaperone association of Tom70-dependent preproteins was tested by gel filtration chromatography. Purified, monomeric Hsp70 eluted at about 70 kDa, but purified Hsp90 homodimer eluted with an apparent molecular size of ~500 kDa due to its unusual shape (Young et al., 1998). Intriguingly, cell-free translated PiC (42 kDa) in RL eluted in a complex with an apparent molecular size of around 600 kDa (Figure 5A, top). Radiolabeled PiC from the peak fractions was recovered with both the His-tagged Hsp70 binding (TPR1) and Hsp90 binding (TPR2A) domains of Hop bound to NiNTA-agarose (Figure 5A, bottom left), indicating that the preprotein was complexed with both chaperones. The TPR1 and TPR2A domains were chaperone specific, as shown by capture experiments from complete RL not containing mitochondrial preprotein (Figure 5A, bottom right). Hop itself does not interact as a chaperone with substrate polypeptides (Bose et al., 1996), and no recovery of PiC was observed without addition of the His-tagged TPR domains (Figure 5B). Thus, both Hsp90 and Hsp70 bind the preprotein in a multichaperone complex for mitochondrial targeting.

The core domain of yeast Tom70 as a purified protein fragment is known to bind preprotein in vitro (Brix et al., 2000). To investigate the role of chaperone docking onto Tom70 in mitochondrial targeting, we examined the binding of chaperone-dependent preproteins to the complete cytosolic segments of wild-type and TPR clamp mutant Tom70 from both mammals and yeast (Figure 1A). Cell-free translation reactions containing radiolabeled PiC and PT were incubated with various His-tagged proteins, and bound preprotein was recovered with NiNTA-agarose. As positive controls, both PiC and PT were recovered with TPR1 and TPR2A domains of Hop, confirming their association with Hsp70 and Hsp90 (Figure 5B). As a negative control, C90 was shown not to bind preprotein (Figure 5B). Importantly, the preproteins were recovered with the wild-type mammalian Tom70, but not with the R192A clamp mutant (Figure 5B), suggesting that chaperone docking onto Tom70 is critical for preprotein targeting in mammals.

To determine whether preprotein in the presence of chaperones directly bound to mammalian Tom70, radiolabeled PiC recovered with wild-type Tom70 Hs-3 on NiNTA-agarose was crosslinked with DSS. A single crosslinked species of about 130 kDa was observed, which was absent in the input translation and in control reactions without crosslinker (Figure 5C, arrowhead). This species remained associated with the beads under both native and denaturing conditions (1% SDS), although noncrosslinked PiC was almost entirely removed by the denaturing wash (Figure 5C). Furthermore, the crosslinked species could not be coprecipitated with antibodies against Hsp90 or Hsp70 after the denaturing wash (data not shown). Thus, the crosslinked species represents preprotein directly bound by Tom70. We therefore conclude that chaperone-bound preprotein is first delivered to mammalian Tom70 via the chaperone/Tom70 interaction, then directly bound by the Tom70 core domain.

The binding of preproteins to yeast Tom70 was similarly tested. AAC as well as PT interacted with Hsp70 and were recovered with the Hsp70 binding TPR1 domain of Hop (Figure 5D). In contrast to the mammalian system, the preproteins were recovered equally well with both the wild-type and R171A clamp mutant Tom70 (Figure 5D). Crosslinking of radiolabeled AAC to both wild-type yeast Tom70 and TPR clamp mutant Tom70 produced a high-molecular weight species slightly larger than that found with mammalian Tom70 (Figure 5E), in agreement with the ~10 kDa larger size of the purified yeast Tom70 fragment used (Figure 1). Again, the crosslinked species was not removed by denaturing conditions (Figure 5E), indicating that AAC is covalently linked to Tom70. Thus, although Tom70 defective in Hsp70 binding is not functional in vivo (Figure 1F), chaperone docking onto yeast Tom70 is not essential for preprotein binding per se.

Chaperones Are Required for Productive Tom70 Binding

The pathway of Tom70-dependent import has been defined for AAC as consisting of five stages (Figure 6, top) (Pfanner and Neupert, 1987): stage I is the newly-synthesized preprotein in the cytosol; stage II, preprotein bound to the Tom70 receptor; stage III, translocation through the Tom40 import pore; stage IV, binding to the inner membrane TIM complex; and stage V, the native homodimeric protein inserted into the inner membrane. Stages II, III, and V can be resolved in yeast mitochondria by blue native PAGE (BNP) (Ryan et al., 1999), and we applied this technique to mitochondria with mutated Tom70. The stage II intermediate behaves as a broadly migrating high molecular weight complex that is thought to result from the recruitment of up to three Tom70 dimers to the internal targeting sequences of AAC (Wiedemann et al., 2001).

When in vitro import of AAC into yeast mitochondria was initiated under normal conditions, with ATP and the membrane potential ($\Delta\Psi$), AAC accumulated as previously established in stages III and V (Figure 6, lane 1). Disruption of $\Delta\Psi$ prevented progression beyond stage III (Figure 6, lane 2) and removal of ATP and $\Delta\Psi$ trapped AAC in the Tom70-bound stage II (Figure 6, lane 3) (Ryan et al., 1999). In stages II and III, but not in stage V, the AAC was accessible to externally added protease

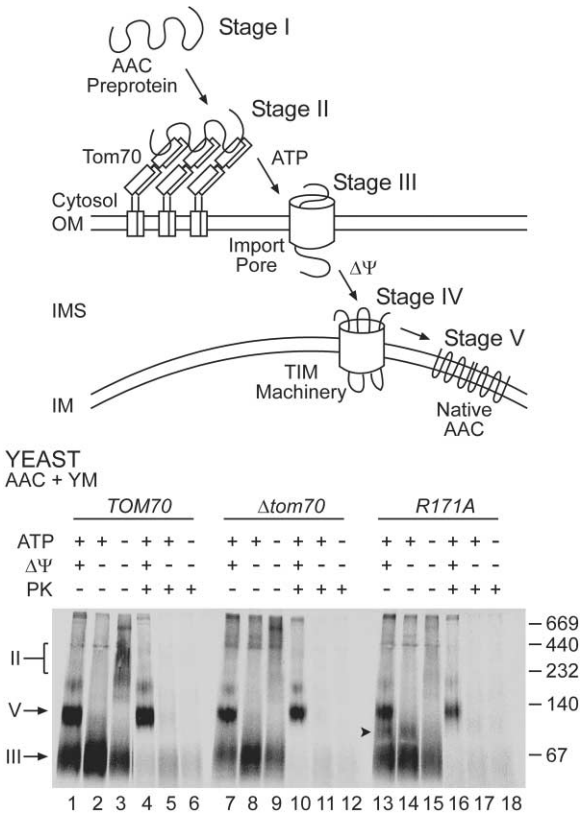

Figure 6. Specific Involvement of Hsp70 in Preprotein Recognition by Tom70

^{35}S-labeled AAC was imported into mitochondria (YM) isolated from wild-type or Δtom70 yeast, or Δtom70 yeast expressing the clamp mutant Tom70 (*R171A*). Where indicated, the inner membrane potential ($\Delta\Psi$) was disrupted, and external ATP was depleted. Mitochondria were reisolated and treated with PK as in Figure 2 where indicated. Samples were solubilized in 1% digitonin and analyzed by blue native PAGE and autoradiography. Conceptual stages of AAC import into mitochondria are shown above (abbreviations: OM, outer membrane; IMS, intermembrane space; IM, inner membrane) (Pfanner and Neupert, 1987). Stages resolved by PAGE are marked. An arrowhead identifies a different species appearing on mitochondria bearing clamp mutant Tom70. Native molecular weight markers (in kDa) are shown.

(Figure 6, lanes 4–6). Mitochondria from Δtom70 yeast, as expected, did not accumulate AAC in the Tom70-bound stage II but still supported a ~50% reduced import of AAC to stages III and V, mediated by Tom20 (Figure 6, lane 9) (Steger et al., 1990). Intriguingly, the stage II intermediate was also not observed on Δtom70 mitochondria bearing the TPR clamp mutant Tom70 (Figure 6, lane 15). Most of the AAC associated with these mitochondria after ATP depletion was not solubilized by the mild detergent conditions (1% digitonin) used for BNP analysis but was irretrievably aggregated instead (data not shown). Also, in the presence of ATP, mitochondria with the mutant Tom70 formed a new, PK-sensitive AAC species of about 100 kDa (Figure 6, lanes 13–18, arrowhead) that was not observed in Δtom70 mitochondria (lanes 7–12). Thus, preprotein binding to yeast Tom70 independently of Hsp70 (Figure 5D) is insufficient to form the productive, high-molecular weight Tom70/preprotein complex. Rather, the presentation of

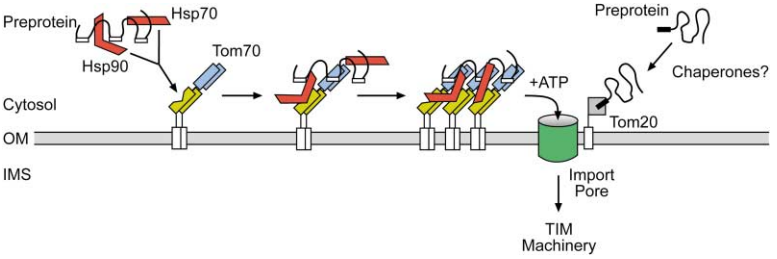

Figure 7. The Chaperone/Tom70 Pathway for Mitochondrial Protein Targeting

Tom70-dependent preprotein in the cytosol is associated with a multichaperone complex including Hsp90 and Hsp70 in mammals or with Hsp70 in yeast. Docking of chaperones onto the Tom70 clamp domain (yellow) is required for productive recognition of internal targeting sequences (white boxes) in the preprotein by the Tom70 core domain (blue). Multiple Tom70 dimers are recruited to form a high molecular weight complex. ATPase cycling, most likely by the chaperones, transfers preprotein from the Tom70 complex through the import pore to the TIM machinery. Tom20-mediated preprotein targeting via an N-terminal presequence (black box) appears to be less dependent on chaperones. Abbreviations: OM, outer membrane; IMS, intermembrane space.

preprotein to Tom70 through specific Hsp70 docking is a prerequisite for this stage. The disruption of Hsp70 docking apparently allows nonproductive targeting of AAC to the outer membrane.

Discussion

Our results outline a specific role of the cytosolic chaperones Hsp90 and Hsp70 in preprotein targeting to mitochondria (Figure 7). As shown in yeast, Hsp70 docking onto the import receptor Tom70 at the outer mitochondrial membrane is required for productive preprotein recognition. In mammals, preprotein in the cytosol is associated with both Hsp90 and Hsp70 in a multichaperone complex, and docking of Hsp90 and/or Hsp70 onto Tom70 is essential for preprotein targeting. After chaperone docking, preprotein is transferred to the Tom70 core domain, and multiple Tom70 dimers are recruited to form a high molecular weight complex. ATPase cycling of the chaperones may then drive the transfer of preprotein from the Tom70 complex to the import pore. The requirement for cytosolic chaperones in the Tom20-mediated import route seems to be less pronounced.

The action of Hsp90 in mitochondrial targeting in mammals is a novel cellular function of this chaperone. This discovery considerably broadens the range of client proteins handled by Hsp90, which has been thought to be largely restricted to signal transducing proteins (Young et al., 2001a). The mitochondrial inner membrane proteins that follow the Tom70 pathway can have multiple internal targeting sequences requiring posttranslational import and, because of their overall hydrophobicity, would be highly aggregation prone in the cytosol. These preproteins are most likely bound by Hsp90 similarly to other Hsp90 client proteins.

Why is Hsp90, in addition to Hsp70, used for mitochondrial targeting in mammals but apparently not in yeast? Hsp70 is universally involved in both systems and may represent an evolutionarily older mechanism. Also, Hsp90 is more abundant compared to Hsp70 in the mammalian cytosol than in yeast, whereas TOM complexes are more abundant in yeast than in mammals (J.C.Y. and N.J.H., unpublished data). Thus, there may be a greater accumulation of mitochondrial preproteins in the mammalian cytosol, requiring the involvement of Hsp90 in addition to Hsp70. Another possibility is that mitochondrial protein import in mammals may be more tightly regulated, perhaps in part by the action of Hsp90.

The cytosolic preprotein complex containing Hsp90 and Hsp70 intriguingly resembles the multichaperone machinery involved in the folding of other Hsp90 client proteins. Hsp90 binding TPR clamp cochaperones such as Hop may participate in the initial formation of the complex but most likely will be displaced by the docking of Hsp90 onto Tom70. The mammalian mitochondrial targeting factor MSF is unlikely to be a part of the chaperone/preprotein complexes analyzed here. MSF recognizes Tom20-dependent presequences (Hachiya et al., 1994), and its function is independent of Tom70 (Suzuki et al., 2002). In any case, the targeting functions of Hsp90 and Hsp70 cannot be replaced by other components of the preprotein complex, as demonstrated by the strong defect in import upon disruption of chaperone/Tom70 docking. Sorting of mitochondrial preproteins from other Hsp90 and Hsp70 client proteins may ultimately be performed by the preprotein binding core domain of Tom70.

Preprotein complexes on the outer membrane of yeast mitochondria can contain up to three Tom70 homodimers (Wiedemann et al., 2001) but may also include Hsp70. While it is possible that Hsp70 interacts only transiently with Tom70 during preprotein transfer, it seems more likely that Hsp70 remains bound and helps Tom70 maintain the preprotein in a nonaggregated, translocation-competent conformation. Hsp70 in this complex could then be involved in the subsequent ATP-dependent transfer of preprotein to the import pore (Pfanner and Neupert, 1987). In the mammalian system, the impairment of import by geldanamycin, which specifically inhibits the ATP-driven Hsp90 cycle (Young and Hartl, 2000), suggests that the ATPase of Hsp90 contributes to Tom70-dependent protein import.

The chaperone/Tom70 targeting pathway for mitochondrial inner membrane proteins may be contrasted with the posttranslational translocation of some soluble secretory proteins across the ER membrane (Rapoport et al., 1996). In the latter system, chaperones including Hsp70 keep the preproteins unfolded before translocation, but apparently no specific docking sites for chaperones are required for targeting. Instead, preproteins are recognized by components on the cytosolic face of the ER membrane, followed by translocation involving lumenal Hsp70 (Rapoport et al., 1996). In the Tom70 pathway, the functions of chaperone docking and preprotein recognition are combined within the Tom70 receptor, perhaps reflecting a more active role of the cytosolic chaperones in preprotein import.

Experimental Procedures

Plasmids

Sequences encoding human Tom70 (Kazusa DNA Research Institute, Japan) or yeast Tom70 were inserted into *pGEM11Zf(-)* (Pro-

mega), *p415ADH (LEU2)*, and *pRS315 (LEU2)*, and the yeast Tom70 promoter (476 base pairs preceding the start codon) inserted upstream of the coding sequences in *pRS315*. Sequences encoding amino acids 111–608 of human Tom70 (Hs-3), 38–617 of *S. cerevisiae* Tom70 (Sc-1), full-length yeast Hsp82, 566–732 of human Hsp90α (C90), and 383–646 of human Hsc70 (C70) were inserted into *pPROEXHTa* or *pRSETA* (Invitrogen). The R192A mutation in human Tom70 and the R171A mutation in yeast Tom70 were introduced by PCR. The sequence encoding yeast Tom20 was inserted into *p416ADH*. Expression vectors for CBag (residues 151–263 of human Bag-1), the TPR1 and TPR2A domains of human Hop (residues 1–118 and 223–352, respectively), and TEV protease were as published (Scheufler et al., 2000; Sondermann et al., 2001). The bovine PiC and human Hsp60 coding sequences were inserted into *pCAGGS* (Zhao et al., 2002) and a triple HA-tag inserted in frame with PiC immediately before the stop codon.

Yeast Methods

Yeast strains JY002 (*tom70::HIS3*) and JY009 (*tom70::HIS3 tom20::TRP1 p416ADH/TOM20 [URA3]*) were created by directed gene replacements of *TOM70* and *TOM20* in YPH499 (*Mata ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) (Stratagene). Yeast cell lysates were made from YPH499 by glass bead lysis (Sondermann et al., 2002). Mitochondria were isolated from yeast strain D273-10B (Daum et al., 1982). For in vitro experiments comparing Tom70 proteins, mitochondria were isolated from YPH499 or JY002 containing either *pRS315* or *p415ADH/Tom70 Sc R171A* grown in synthetic medium. Complementation of *TOM70* was tested by transforming JY009 with either *pRS315*, *p315/TOM70 Sc*, *p315/Tom70 Sc R171A*, or *p315/Tom70 Hs* and growing on synthetic medium -leu, -ura with 2% glucose, followed by restreaking on synthetic complete medium containing 0.1% 5-fluoroorotic acid with 2% glucose.

Chaperone Binding

Proteins were expressed in BL21(DE3)pLysS *Escherichia coli* cells (Stratagene), and purified by chromatography on NiNTA-agarose (Qiagen) and Mono Q and Superdex 200 columns (Amersham Biosciences) when required. Hsp90 and Hsp70 were purified from bovine brain (Young et al., 1998; Sondermann et al., 2001). The His-tag was removed from Hsp82, C70, and C90 where indicated by digestion with TEV protease (Scheufler et al., 2000). Binding of mammalian chaperones was performed at 4°C using 5 μM purified wild-type or R192A mutant Tom70 Hs-3 in either 50% reticulocyte lysate (Green Hectares, WI) or 2 μM purified chaperones in buffer CG (100 mM KOAc, 20 mM HEPES-KOH [pH 7.5], 5 mM $MgOAc_2$), NiNTA-agarose and, where indicated, 50 μM C90 and C70. Recovered beads were eluted with buffer containing 500 mM NaCl and, where indicated, with 25 mM EDTA. Binding of yeast chaperones (Sondermann et al., 2002) was performed using 5 μM wild-type or R171A mutant Tom70 Sc-1. Chaperones were identified by immunoblotting with antibodies against Hsp90, Hsc70/Hsp70 (Stressgen), or Ssa.

Mitochondrial Import Assays

Rat liver mitochondria were isolated, and import into rat liver and yeast mitochondria was assayed (Daum et al., 1982; Lingelbach et al., 1986) in buffer MC (250 mM sucrose, 80 mM KOAc, 20 mM HEPES-KOH [pH 7.5], and 5 mM $MgOAc_2$). Cell-free translations were performed with the TNT SP6 or T7 coupled RL translation systems (Promega) supplemented with ^{35}S-methionine (Amersham Biosciences) at 30°C. Reactions were adjusted to 250 mM sucrose and incubated with rat liver mitochondria or yeast mitochondria at 30°C or 25°C, respectively, for 20 min. RL typically constituted 25% of the final import reaction, and control reactions were inhibited with 1 μM valinomycin. Half of each sample was treated with 50 μg/ml PK for 10 min at 4°C, digestion was stopped with 1 mM PMSF, and the mitochondria were reisolated. For GA treatment, 18 μM GA or the equivalent volume of DMSO (1%) was added to the cell-free translations. GA was removed using MicroBioSpin 6 columns (Bio-Rad) preequilibrated in buffer MC containing 0.4 mM EGTA, centrifuged at 16°C with 2 mM ATP added to the collection tube. To analyze targeted Tom70, mitochondria were reisolated after the targeting reaction and extracted with 0.1 M Na_2CO_3 (pH 10.5) for 10 min at 4°C and centrifuged at 100,000 × g for 30 min or solubilized with 1% digitonin as for blue native PAGE analysis.

Cell Culture

Cos7 cells were grown in DMEM with 3.7 g/l $NaHCO_3$ and 4.5 g/l glucose (Biochrom), 10% fetal calf serum, and 2 mM glutamine (Gibco). At ~50% confluence, cells were transfected with 6 μg of *pCAGGS/PiC-HA* or *pCAGGS/Hsp60* with 1 μg β-galactosidase control plasmid per 10 cm plate, using Lipofectamine PLUS (Invitrogen). Cells were split evenly into 6-well plates, and 36 hr after transfection, labeled for 2 hr in starvation medium containing 80 μCi ^{35}S-ProMix (Amersham Biosciences) per well, and 1 μM or 6 μM GA or the equivalent volume of DMSO (0.1%). Cells were harvested and divided into equal samples, and one sample lysed with PBS containing 1% NP40. Mitochondria were isolated from the other sample (Zhao et al., 2002) and similarly lysed. The lysates were corrected for transfection efficiency with the β-galactosidase Enzyme Assay system (Promega), and the total cell and mitochondrial lysates were immunoprecipitated with antibodies against the HA epitope (Babco) or Hsp60.

Preprotein Binding

For gel filtration analysis, cell-free translations of preproteins were terminated with 0.1 unit/μl apyrase and resolved at 4°C on a Superose 6 10/30 column (Amersham Biosciences) equilibrated in buffer CG. Peak fractions were pooled and adjusted to 2 mg/ml ovalbumin, 0.1% NP40, and either 4 μM TPR1 or TPR2A domains of Hop or with no addition, and bound to NiNTA-agarose at 4°C. Beads were washed with buffer CG containing 0.1% NP40. For direct analysis of preprotein binding, cell-free translations of preproteins were diluted with buffer CG containing 2 mg/ml ovalbumin, 0.1% NP40, and 10 μM His-tagged TPR1, TPR2A, or C90, or 2 μM of the wild-type or mutant Tom70 cytosolic segments, or with no addition. Reactions were stopped with 0.1 unit/μl apyrase and bound to NiNTA-agarose as above. For crosslinking, 200 μM disuccinimidyl suberimidate (DSS) or the equivalent volume of DMSO (0.4%) was added for 15 min at 4°C. Crosslinking was terminated with 20 mM ethanolamine (pH 8.0), and beads were washed with either buffer CG containing 0.1% NP40 or buffer containing 1% SDS and 500 mM NaCl.

Blue Native PAGE

BNP analysis was performed as published (Ryan et al., 1999). Where indicated, $\Delta\Psi$ was disrupted with 1 μM valinomycin, or 1 μM valinomycin, 6 μM oligomycin, and 10 μM antimycin A with 0.1 unit/μl apyrase to remove ATP. Mitochondria were solubilized at 4°C in 10% glycerol, 1% digitonin, 50 mM KOAc, and 20 mM HEPES-KOH (pH 7.5), insoluble material separated by centrifugation at 20,000 × g for 10 min, and samples resolved on 6%–16.5% gradient gels.

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