An Unusual TOM20/TOM22 Bypass Mechanism for the Mitochondrial Targeting of Cytochrome P450 Proteins Containing N-terminal Chimeric Signals

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Previously we showed that xenobiotic-inducible cytochrome P450 (CYP) proteins are bimodally targeted to the endoplasmic reticulum and mitochondria. In the present study, we investigated the mechanism of delivery of chimeric signal-containing CYP proteins to the peripheral and channel-forming mitochondrial outer membrane translocases (TOMs). CYP+33/1A1 and CYP2B1 did not require peripheral TOM70, TOM20, or TOM22 for translocation through the channel-forming TOM40 protein. In contrast, CYP+5/1A1 and CYP2E1 were able to bypass TOM20 and TOM22 but required TOM70. CYP27, which contains a canonical cleavable mitochondrial signal, required all of the peripheral TOMs for its mitochondrial translocation. We investigated the underlying mechanisms of bypass of peripheral TOMs by CYP proteins. In contrast to the prevailing dogma, studies from our and other laboratories have shown that a number of proteins are bimodally targeted to different subcellular compartments (14–20). Our results showed that different CYPs, glutathione S-transferase (GST) isoforms, and the amyloid precursor protein (APP) are targeted to mitochondria in addition to their well established subcellular locations in the ER, cytosol, and plasma membranes, respectively (21, 22). Bimodal targeting of CYPs and APP was facilitated by the N-terminal chimeric signal, which consists of a cryptic mitochondria-targeting signal immediately flanking the N-terminal ER-targeting signal. By contrast, a C-terminal cryptic mitochondria-targeting signal was critical for the mitochondrial translocation of cytosolic GSTs (21). We also showed that mitochondrial targeting of these proteins required the activation of a cryptic mitochondrial signal either by sequence-specific processing by a cytosolic endoprotease as in the case of CYP1A1 (14) or by PKA-mediated phosphorylation of Ser residues at positions 128 or 129 as in the case of CYP2B1 and CYP2E1 (16, 18, 23). In contrast, phosphorylation of a tandem PKA/PKC (protein kinase C) target

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get site close to the C terminus was involved in the activation of the mitochondria-targeting signal of GSTA4-4 (21).

Proteins with canonical mitochondria-targeting signals specifically bind to the outer membrane protein import complex, TOM, as the critical initial step in mitochondrial protein targeting (13, 24–27). The TOM complex consists of nine subunits, of which TOM70, TOM40, TOM22, and TOM20 are the major components. The C termini of TOM20 and TOM70 and the N terminus of TOM22 are exposed to the cytosol and provide receptor sites for binding to client proteins. The small TOMs (TOM7, TOM6, and TOM5), on the other hand, are associated with the channel-forming TOM40 protein, which is embedded in the outer membrane (13, 24, 26). Current models suggest that the N-terminal signal regions of pre-proteins bind to the cytosol-exposed domains of TOM20 and TOM22 through both hydrophobic and ionic interactions. TOM20 and TOM70 exhibit overlapping substrate specificity. TOM22 is believed to regulate the gating activity of the TOM40 channel (28–31). A recent study suggests the involvement of Hsp70 in the targeting of phosphorylated CYP2E1 to TOM40 (18). However, the precise mode of transport of chimeric signal-containing proteins to mitochondria and the interaction of chimeric signals with members of the TOM complex remain unclear. A number of studies have observed low levels of mitochondrial protein import by mitochondria pretreated with proteases or those depleted of peripheral TOMs (28, 32–35), suggesting a distin

phorylated CYP2B1 and 2E1, respectively) that contain N-terminal chimeric signals. We found that the activated chimeric signals remains unclear. In the mitochondrial import of proteins containing chimeric signals, the targeting of phosphorylated CYP2E1 to TOM40 (18). How-

differentiation. The peripheral TOMs (TOM7, TOM6, and TOM5), on the other hand, are associated with the channel-forming TOM40 protein, which is embedded in the outer membrane (13, 24, 26). Current models suggest that the N-terminal signal regions of pre-proteins bind to the cytosol-exposed domains of TOM20 and TOM22 through both hydrophobic and ionic interactions. TOM20 and TOM70 exhibit overlapping substrate specificity. TOM22 is believed to regulate the gating activity of the TOM40 channel (28–31). A recent study suggests the involvement of Hsp70 in the targeting of phosphorylated CYP2E1 to TOM40 (18). However, the precise mode of transport of chimeric signal-containing proteins to mitochondria and the interaction of chimeric signals with members of the TOM complex remain unclear. A number of studies have observed low levels of mitochondrial protein import by mitochondria pretreated with proteases or those depleted of peripheral TOMs (28, 32–35), suggesting a distinct role for these peripheral TOMs in the mitochondrial import of proteins containing chimeric signal. We found that the activated chimeric signals remains unclear.

Experimental Procedures

Yeast Strains—Strains BY4741 (MATa and MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), tom70Δ (MATa tom70::KAN his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), and tom20Δ (MATa tom70::KAN his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) were obtained from Research Genetics Inc. Strain tom22Δ (his3ΔΔ200 leu2Δ1 ura3Δ52 trp1Δ63 TOM22::HIS3 rho) and the control haploid strain OL223 (his3ΔΔ200 leu2Δ1 ura3Δ52 trp1Δ63 rho+) were a kind gift from N. Pfanner (Freiburg, Germany). Standard yeast genetic techniques and media were used (36).

Construction of Expression Plasmids—Rat CYP2E1, 2B1, +5/1A1, and +33/1A1 cDNAs were generated by reverse transcription-PCR and cloned in pGEM7zf (Promega Biotech, Madison, WI) plasmid vectors. Full-length rat liver cDNA to TOM40 was purchased from ATCC (IMAGE clone). N-terminal truncated TOM40 cDNA (Δ143TOM40) was generated by PCR amplification using full-length TOM40 DNA as a template. Both full-length and truncated TOM40 constructs were cloned in pGEM7zf vector and pET vectors to carry out expression in vitro and bacterial cells, respectively.

Wild-type and tom70Δ, tom20Δ, and tom22Δ yeast strains were used for expressing rat CYP cDNAs driven by the elongation factor promoter on either 2-μm or centromeric URA3 plasmids. The cDNAs for full-length CYP2E1, +5/1A1, +33/1A1, and su9-DHFR were cloned into the 2-μ vector, pTEF-URA3, or the centromeric pTEF-URA3 plasmid (37).

Disruption of Outer Membrane Receptors by Protease Treatment—Rat liver mitochondria were isolated by differential centrifugation in H medium (5 mM HEPES (pH 7.4), 210 mM mannitol, 70 mM sucrose, 1 mM EDTA), as described previously (38) and washed three times with H medium. Mitochondria were subjected to limited Pronase treatment (30 μg/mg protein) at 4 °C for 30 min. Following the addition of a 10-fold excess of protease inhibitor, the mitochondrial pellet was reisolated by passing through 1.2 m sucrose and used for protein import as described below.

In Vitro Import of Labeled Proteins into Mitochondria—In vitro translation products were generated in the transcription-linked translation RRL system (Promega, Madison, WI) in the presence of added 14C Met (40 Ci/50-μl reaction, 1175 Ci/mmol, PerkinElmer Life Sciences) according to the manufacturer's instructions. For immunoinactivation experiments, rat liver mitochondria at 1 mg/ml were solubilized in 2× Laemmli sample buffer (42) for 10 min at 75 °C and analyzed by SDS-PAGE and fluorography.

Effects of Immunoinhibition of Membrane Translocases on Mitochondrial Import—Rat liver mitochondria in H medium containing 1 mM phenylmethylsulfonyl fluoride were incubated with antibodies to TOM20, TOM22, TOM70, TOM40, porin, or preimmune serum at 30 °C for 30 min. The reaction mixtures were centrifuged at 5,000 × g for 5 min to remove excess antibody, and the resultant mitochondria were washed twice with H medium and used in import assays.

Immunodepletion of Hsp70 and Hsp90 from the Rabbit Reticulocyte Lysate—For immunodepletion, RRL (200 μl each) was incubated with 20 μg of Hsp70 and/or Hsp90 antibodies overnight (12–16 h) at 4 °C. Immune complexes were pulled down by incubation with protein A-agarose (1 h at 4 °C) followed by centrifugation at 5000 × g for 5 min. In parallel experiments, RRL was incubated with preimmune IgG (20 μg/200 μl RRL) as antibody controls. The depleted RRL were used for in vitro translation as described above.

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Interaction of CYPs with Cytosolic Hsp70 and Hsp90—Interaction of CYP proteins with cytosolic Hsp70 under in vitro and in vivo conditions was tested by co-immunoprecipitation. For studying in vitro interactions, nascent proteins were synthesized in the presence (for CYP2E1 and CYP2B1) and absence (for +51A1, +33/1A1, and CYP27) of PKA in the RRL system. Interaction of CYP proteins with cytosolic Hsp70 or Hsp90 present in the lysate under these conditions was estimated by co-immunoprecipitation. Reaction mixtures were immunoprecipitated with polyclonal antibodies against the respective CYP proteins. One portion of the immunoprecipitate was resolved by SDS-PAGE on a 10% gel and subjected to immunoblot analysis with anti-Hsp70 and -Hsp90 antibodies. The other portion was subjected to SDS-PAGE, and gels were subjected to autoradiography.

Bacterial Expression of TOM40 and Binding of CYPs to Reconstituted TOM40—Full-length and truncated Δ143TOM40 expressed in Escherichia coli BL21 cells were purified from inclusion bodies to near homogeneity and used for reconstitution in liposomes. Liposomes were prepared from azolectin (Sigma, type IVS) in MOPS-Tris (pH 6.9) buffer using a Branson sonifier as described previously (39). Liposomes were freeze-thawed three times and solubilized by the addition of n-octyl glucopyranoside (6% v/v) on ice for 30 min. Solubilized liposomes (10 mg) were mixed with 9–300 μg of TOM40 and diluted 1:2 with 10 mM MOPS-Tris (pH 6.9) containing 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM MgCl₂—Proteins were imported and rendered resistant to Pronase treatment degraded most of the cytosolic Hsp70 and Hsp90 proteins as described (40). As a negative control, purified P4501A1, which does not form a channel, was used following reconstitution in lipid vesicles. Reconstituted vesicles were suspended in 2 mM HEPES buffer (pH 7.4) containing 20 mM NaCl, 2 mM MgCl₂, 0.3 mM NaN₃, and 2 mM sucrose. Reaction mixtures were incubated with equal amounts (~100,000 cpm) of [³H]dextran (7.5 μCi/mmol, American Radiolabeled Chemicals Corp.) or [¹⁴C]sucrose (7.5 μCi/mmol, American Radiolabeled Chemicals Corp.) or [¹⁴C]sucrose (7.5 μCi/mmol; American Radiolabeled Chemicals Corp.) at room temperature for 30 min. Following vortexing, tubes were placed in a bath-temperature sonic oscillator for 10 s. The tubes were incubated at 45 °C for 30 min and allowed to cool down to room temperature. The reaction mixture was passed through a Sepharose 4B column; the vesicles were eluted with 10 mM HEPES-KOH buffer (pH 7.4) containing 100 mM NaCl, 0.1 mM MgCl₂, and 0.3 mM NaN₃ and assayed for the amount of radioactivity retained by using a Beckman 5000 liquid scintillation counter.

Preparation of Mitochondria from tom-deleted Yeast Strains—Mitochondria were isolated from both the control and tom-deleted yeast strains essentially as described previously (41). Steady-state levels of mitochondrial proteins were analyzed by separation via SDS-PAGE and immunoblotting.

SDS-PAGE and Immunoblot Analysis—Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblot analysis as described previously (42). Polyclonal antibodies against CYP2E1, CYP2B1, CYP1A1, and monoclonal antibody to CYP27, as well as monoclonal antibodies against cytosolic Hsp70 and Hsp90 (Sigma), were used. Immunoblots were developed using a chemiluminescence SuperSignal ULTRA kit (Pierce), and the blots were imaged and quantitated using a Fluor-S imaging system (Bio-Rad).

RESULTS

Distinctive TOM Protein Requirements for the Import of Chimeric Signal-containing CYPs—Fig. 1A shows the N-terminal chimeric signals of CYP 1A1, CYP 2B1, and CYP 2E1 that encompass the N-terminal transmembrane domain and the cryptic mitochondria-targeting signal sequences. We have shown previously that mitochondrial import of these CYPs is (14, the mitochondrial inner membrane protein, cytochrome c oxidase I (CcO1), and matrix protein Hsp70 were also fully protected. Using these protease-treated mitochondria, we tested the import efficiency of CYP proteins. CYP27 (43), which contains a canonical (N-terminal cleavable) mitochondria-targeting signal sequence, was used as a positive control. The results of in vitro import showed that nearly intact 2B1 protein was imported and rendered resistant to Pronase treatment, whereas 2E1 and +5/1A1 were not imported into Pronase-treated mitochondria (Fig. 1C). Fig. 1D shows that +33/1A1 was imported efficiently into Pronase-treated mitochondria, whereas CYP27 was not. These results suggest that different chimeric signal-containing proteins have different requirements for peripheral TOM proteins.

The results of import with protease-treated mitochondria were further ascertained using two parallel approaches. In the first approach, we used immunoinhibition of individual TOM proteins with saturating levels of specific antibodies. As shown in vitro reaction...
Import of CYP 2E1 and +5/1A1 was blocked by TOM70 antibody (Fig. 2, D and E) but not by antibodies to either TOM20 or TOM22. Also, the import of +33/1A1 and 2E1 in the presence of TOM22 antibodies was incomplete as seen by relatively shorter protease-protected products (Fig. 2, B and D). The import of CYP27 was affected by all antibodies tested (Fig. 2C). Antibody to porin was used as a negative control and had no effect on the import of any of the proteins tested (Fig. 2, A–E).

The specificity of the TOM40 antibody effect observed above was ascertained using Bcl-XL protein, which is targeted to the mitochondrial outer membrane in the absence of functional TOM40 protein (44). Fig. 2F shows that, as expected, TOM40 antibody and also porin antibody had no significant effect on the membrane association of Bcl-XL. The transmembrane orientation of the protein is apparent from its relative insolubility in Na2CO3. Furthermore, trypsin treatment caused substantial reduction in protein level as expected of a protein which is mostly exposed outside of the outer membrane (Fig. 2F).

In the second approach, we used mitochondria from yeast strains that contained selectively deleted tom genes. As seen from Fig. 3A, +33/1A1 was imported into yeast mitochondria that were devoid of TOM70, TOM20, and TOM22 proteins at the same level as in WT mitochondria. However, the protease-protected fragment in TOM22-deficient mitochondria was shorter by almost 3–4 kDa. Similarly, CYP2B1 was imported into mitochondria devoid of all three TOM proteins at a level similar to that in WT mitochondria (Fig. 3B). On the other hand, +5/1A1 and CYP2E1 were not imported efficiently by mitochondria deficient in TOM70, suggesting a critical requirement for TOM70 in the import of these two proteins (Fig. 3, C and D). Additionally, the protease-protected fragment of CYP2E1 was shorter (~1 kDa) in mitochondria deficient in TOM22 (Fig. 3D). Finally, CYP27 was not imported significantly in mitochondria deficient in any of the three TOMs (Fig. 3E). The topology of incompletely translocated CYP2E1 and +33/1A1 in tom22Δ mitochondria were tested by treatment with digitonin prior to treatment with trypsin. The results shown in Fig. 3F reveal that digitonin treatment rendered both proteins completely sensitive to trypsin digestion, suggesting that imported CYP2E1 and +33/1A1 imported in The long of g. 3G, ein, is tment inner

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In vitro translated CYPs show that in the absence of Hsp90, all of the CYPs tested showed the levels of Hsp70 and Hsp90 in control and immunodepleted RRL used in this experiment. Fig. 5E shows that in the absence of Hsp90, all of the CYPs tested were able to interact with Hsp70. Surprisingly, these CYPs failed to co-immunoprecipitate Hsp90 in Hsp70-depleted lysates (Fig. 5F). However, supplementation with purified Hsp70 restored CYP binding to Hsp90. The only exception was CYP27, which did not show any significant interactions with Hsp90 either in complete or Hsp70-depleted extracts. As seen in Fig. 5G, the level of translation of all CYP proteins was comparable in Hsp70-depleted reticulocyte lysates. Although not shown, Hsp90 depletion also did not adversely affect the translation of CYP proteins. These results show that the interaction between chimeric signal-containing proteins and Hsp90 is dependent on the presence of Hsp70, suggesting a degree of cooperativity between the two factors.

We next investigated whether the binding of various CYPs to Hsp70 and Hsp90 are interdependent or independent of each other. Various CYPs were translated in RRLs depleted of either Hsp90 or Hsp70 and subjected to immunoprecipitation with CYP-specific antibodies (Fig. 5, E and F). The immunoprecipitates were then probed with Hsp-specific antibodies by immunoblot analysis. Fig. 5D shows the levels of Hsp70 and Hsp90 in control and immunodepleted RRL used in this experiment. Fig. 5E shows that in the absence of Hsp90, all of the CYPs tested were able to interact with Hsp70. Surprisingly, these CYPs failed to co-immunoprecipitate Hsp90 in Hsp70-depleted lysates (Fig. 5F). However, supplementation with purified Hsp70 restored CYP binding to Hsp90. The only exception was CYP27, which did not show any significant interactions with Hsp90 either in complete or Hsp70-depleted extracts. As seen in Fig. 5G, the level of translation of all CYP proteins was comparable in Hsp70-depleted reticulocyte lysates. Although not shown, Hsp90 depletion also did not adversely affect the translation of CYP proteins. These results show that the interaction between chimeric signal-containing proteins and Hsp90 is dependent on the presence of Hsp70, suggesting a degree of cooperativity between the two factors.
First, Hsp90 depletion did not have a significant effect on the import of CYP27 into WT mitochondria. Second, deletion of \( \text{tom70} \), \( \text{tom20} \), or \( \text{tom22} \) almost completely inhibited the import of CYP27 (Fig. 6C). These results taken together suggest that Hsp70 and Hsp90 are important factors that facilitate the presentation of different chimeric signals to the TOM complex. They also play a major role in the ability of chimeric signals to bypass peripheral TOMs. The results also suggest different requirements for peripheral TOMs in terms of the import of the four chimeric signal-containing proteins tested.

**Targeting of CYP Proteins to Mitochondria in Intact Cells Involves TOM Bypass Mechanism**—The occurrence of a TOM bypass mechanism for the mitochondrial targeting of chimeric signal-containing CYPs was confirmed by the expression of various CYPs in control (parent strains of \( \text{tom70} \) and \( \text{tom20} \) null mutant) and \( \text{tom} \) null mutant yeast cells. Immunoblot analysis of mitochondrial proteins in Fig. 7 shows that mutant strains express intact peripheral TOM20 and TOM22 proteins at levels comparable with the wild-type yeast. Thus, the deficiency in these strains was restricted to the specified gene product.

The \( \text{tom22} \) null mutation is known to induce \( \rho^o \) status. To assess the effects of the \( \rho^o \) mutation on mitochondrial-protein targeting, we expressed different CYP cDNAs in the respective parent strain made \( \rho^o \) by ethidium bromide treatment (Rho\(^o\) control). As shown in Fig. 7B, CYP +33/1A1 was targeted efficiently to mitochondria in both controls and \( \text{tom} \) null mutant cells. The protein was inefficiently targeted to the ER, as expected of a protein that lacks the SRP (signal recognition particle)-binding signal domain. CYP +5/1A1, on the other hand, showed a distinct requirement for TOM70, as no detectable protein was found in the mitochondrial compartment of \( \text{tom70} \) cells.

These results essentially confirm the in vitro data (Fig. 6), which suggest the distinctive nature of chimeric signals of CYP2E1 and +5/1A1 requiring TOM70 for mitochondrial translocation. Although not shown, CYP2B1
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We used pSU9/DHFR as a control for matrix-targeted protein, because Stan et al. (45) have shown that the protein is efficiently transported in *tom70Δ* cells. In contrast, results from other groups (46, 47) show that deletion or depletion of TOM70, TOM20, and TOM22 affects mitochondrial import of pSU9/DHFR. However, we did not observe significant inhibition of mitochondrial import of this protein in *tom70Δ* and *tom22Δ* cells under either *in vivo* (Fig. 7C) or *in vitro* (not shown) conditions.

Association of Hsp90 with the delivery of the client protein to the translocase complex (48, 49). The *in vitro* and *in vivo* experiments physically associate with TOM70 for the delivery of the client protein to the translocase complex (48, 49).

## FIGURE 5. Interaction of chimeric and canonical import signal-containing CYPs with Hsp70 and Hsp90

**A** Interaction of chimeric and canonical import signal-containing CYPs with Hsp70 and Hsp90. **B** Western blot analysis of 35S-labeled wild-type (*WT*) CYP27 and CYP27 matrix- and microsome-associated proteins. **C** Autoradiogram representing the 35S-labeled products of CYPs translated in reticulocyte lysates depleted of Hsp70. **D** Autoradiogram showing the Coomassie Blue-stained gel of purified WT and *33/1A1* proteins. **E** Autoradiogram showing the Coomassie Blue-stained gel of purified WT and *33/1A1* proteins. **F** Autoradiogram showing the Coomassie Blue-stained gel of purified WT and *33/1A1* proteins. **G** Autoradiogram showing the Coomassie Blue-stained gel of purified WT and *33/1A1* proteins.

Behavioral studies and results from other groups (46, 47) show that deletion or depletion of TOM70, TOM20, and TOM22 affects mitochondrial import of pSU9/DHFR. However, we did not observe significant inhibition of mitochondrial import of this protein in *tom70Δ* and *tom22Δ* cells under either *in vivo* (Fig. 7C) or *in vitro* (not shown) conditions.

The topology of mitochondria- and microsome-associated CYPs in *tom* null mutants was tested by limited digestion with trypsin followed by immunoblot analysis. As shown in Fig. 7C, mitochondria-associated CYPs were resistant to trypsin digestion, whereas microsome-associated CYPs were sensitive to trypsin treatment (Fig. 7D). In this respect, mitochondria-associated CYPs exhibited resistance to trypsin similar to the matrix-associated protein pSU9-DHFR used as a control. Confirming the results in Figs. 3, 4, and 6, CYP2E1 and +5/1A1 were not targeted to mitochondria significantly in *tom70Δ* cells. Also, the trypsin-protected fragments of CYP2E1 and +331A1 were shorter in *tom22Δ* cells compared with *ρ^0* control cells. These results confirm that mitochondria-associated proteins are probably localized inside the inner membrane compartment, whereas the microsome-associated proteins are exposed externally to the ER in a protease-accessible orientation. These results together confirm the occurrence of the TOM20/TOM22 bypass protein-targeting mechanism *in vivo*.

in this study (Figs. 2–7) suggested that in the presence of Hsp70 and Hsp90, +33/1A1 and CYP2B1 proteins are translocated to mitochondria in the absence of TOM20, TOM22, and TOM70. To understand the molecular basis of this bypass mechanism, we tested the ability of Hsp90 to bind to purified and reconstituted TOM40 under *in vitro* conditions. Our preliminary results with antibody pulldown assays showed that 35S-labeled +33/1A1 efficiently bound to WT TOM40 but not to Δ143 TOM40 lacking the N-terminal cytosol exposed Pro-rich domain. We have therefore used purified and reconstituted WT and Δ143 TOM40 proteins in lipid vesicles for binding in these studies. Fig. 8A (left panel) shows the Coomassie Blue-stained gel profiles of purified WT and Δ143 TOM40 proteins. The reconstituted lipid vesicles were recovered by sedimentation at 125,000 *×* g for 1 h, and both the vesicular fraction (Fig. 8A, middle panel) and the supernatant representing “free” proteins (right panel) were analyzed by polyacrylamide gel electrophoresis. The middle and right panels (Fig. 8A) show that a large fraction of proteins in both cases is associated with lipid vesicles and a relatively small fraction is recovered in the supernatant fraction. Another control experiment, in Fig. 8B, shows that...
only vesicles containing TOM40 protein binds efficiently to +33/1A1 in complete RRL, whereas vesicles devoid of TOM40 fail to bind to the protein indicating the specificity.

The proper orientation of reconstituted TOM40 and Δ143 TOM40 proteins and the presence of TOM40 pore was tested using [13C]sucrose and [3H]dextran (~70kDa) as permeable and impermeable substrates, respectively. As shown in Table 1, reconstituted vesicles with CYP1A1 showed nearly complete retention of both 13C and 3H radioactivity, suggesting no pore activity. Vesicles containing increasing amounts of intact TOM40 as well as Δ143 TOM40 proteins showed increasing release of [13C]sucrose but complete retention of [3H]dextran, suggesting efficient pore activity.

In Fig. 8C, we tested the efficiencies of two types of chimeric signal-containing proteins, +5/1A1 and +33/1A1, for binding to reconstituted vesicles containing WT TOM40 protein. 35S-Labeled client proteins translated in intact RRL were incubated with lipid vesicles at 30 °C for 20 min; the vesicles were recovered by centrifugation at 125,000 × g for 1 h, washed twice with buffer containing 10 mM NaCl, and used for analysis. The autoradiogram in Fig. 8C, left panel, shows that DHFR, a cytosolic protein and +5/1A1 did not bind significantly to TOM40 vesicles. The inability of +5/1A1 protein to bind to TOM40 may be due to a lack of TOM70 in the vesicle. The CCoVb subunit (Fig. 8C, left panel) and also +33/1A1 (right panel) bound efficiently to TOM40 in the absence of other TOMs. These results are consistent with our in vitro and in vivo results that only +33/1A1, but not +5/1A1, can bypass all of the peripheral TOMs. In Figs. 8D and 8E, we used WT and Hsp90-depleted RRL for the vesicles. Hsp70 or Hsp90. Also, both Hsp90 and Hsp70 induced the binding in a concentration-dependent manner. These results further confirm the need for both Hsp70 and Hsp90 for support of the bypass pathway.

In Fig. 8E we tested whether Hsp90 could physically associate with the reconstituted TOM40 vesicles for delivery of the +33/1A1 protein and whether the binding requires the cytosol-exposed region of TOM40. In this experiment, HSP90-depleted RRL was used for translating +33/1A1 and Hsp90 proteins together in the presence of [35S]Met and used for binding to vesicles containing TOM40 and Δ143 TOM40 proteins. The vesicles were recovered and washed sequentially with buffers containing 10, 40, and 80 mM NaCl, and both the eluted proteins and that remaining with the vesicles (resid-
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Mitochondrial targeting signals are heterogeneous, ranging from α-helical, β-sheet, or unstructured (13). The most common type of mitochondrial signals are pre-sequences, which consist of 15–40 N-terminal amino acids that form amphiphilic helices with positively charged residues lining one side of each helix. These signals are commonly observed in mitochondrial matrix- and inner membrane-targeted proteins and cleaved by matrix metalloprotease(s) after the protein is translocated into the mitochondrial compartment (13), typically occurring in transmembrane domains. This type of targeting signal is known to occur in a number of membrane-anchored proteins such as Bcs1, Tim14, Mdj2, D-AKAP1, and others (13, 51–56). We have described a unal) were analyzed for assessment of the type of association of Hsp90 and +33/1A1 with TOM40. Fig. 8E shows that both the +33/1A1 and Hsp90 bound to WT TOM40, and most of the bound Hsp90 protein was eluted with 40 mM NaCl (Fig. 8, E and F), whereas elution of +33/1A1 required 80 mM NaCl, suggesting a more stable interaction of the latter with TOM40 (Fig. 8, E and G). Additionally, Hsp90 and +33/1A1 proteins bound at markedly reduced levels with Δ143 TOM40 (Fig. 8, E–G), suggesting that the binding requires the intact cytosol exposed Pro-rich domain of TOM40. These results provide insight into the mechanism of the bypass pathway, which involves direct binding of Hsp90 for delivery of the client protein to TOM40.

**FIGURE 8.**

A. Vesicles containing WT and Δ143 TOM40 proteins were incubated with liposomes containing WT and Hsp90-depleted RRL, and 50,000 cpm of each were incubated with liposomes containing WT TOM40 protein for 20 min at 30 °C. Liposomes were recovered by layering on 0.4 M sucrose followed by centrifugation at 125,000 × g for 1 h and then washed twice with 20 mM Tris-HCl (pH 7.4) and 10 mM NaCl; equal portions were analyzed on two companion polyacrylamide gels. One gel was subjected to SDS-PAGE and fluorography (upper panels), and the second gel was subjected to immunoblot analysis using TOM40 antibody (lower panels). B, effect of Hsp90 and Hsp70 on binding of +33/1A1 to TOM40 vesicles. +33/1A1 protein was translated in Hsp90- and Hsp70-depleted RRL, and 50,000 cpm of each were incubated with liposomes containing WT TOM40 in the presence or absence of various amounts of purified Hsp90 and Hsp70 as indicated. In E, Hsp90 and +33/1A1 proteins were co-translated in Hsp90-depleted RRL, and 50,000 cpm of each were incubated with liposomes containing WT and Δ143 TOM40 proteins (2 μg protein each) as described in C. Liposomes were recovered and sequentially washed with Tris-HCl buffer containing 10, 40, and 80 mM NaCl. The washes and also residual proteins (RES) in the vesicle were analyzed by SDS-PAGE and fluorography. F and G, quantification of Hsp 90 (F) and +33/1A1 (G) protein bands from autoradiogram in E. The Input lanes represent 20% of total counts used for binding. NS, non-specific.

**TABLE 1**

Pore function of reconstituted TOM40 and Δ143 TOM40 proteins

Reconstitution of lipid vesicles with different proteins, introduction of radioactive sucrose and dextran into vesicles, and purification of vesicles were carried out as described under “Experimental Procedures.” S/D, sucrose/dextran.

| Vesicle type* | Input radioactivity | Retained in vesicles | S/D ratiob |
|---------------|---------------------|----------------------|------------|
|               | [3H]Dextran [14C]Sucrose | [3H]Dextran [14C]Sucrose |            |
|               | cpm                  | cpm                  |            |
| CYP1A1 (30 μg) | 102,370              | 109,050              | 33,630     |
| TOM40 (4 μg)  | 100,750              | 98,000               | 26,320     |
| TOM40 (30 μg) | 105,120              | 106,280              | 25,930     |
| ΔTOM40 (4 μg) | 109,010              | 102,870              | 28,731     |
| ΔTOM40 (30 μg) | 97,146               | 98,315               | 29,562     |

* Amounts of protein reconstituted in 1 mg of lipid vesicles are shown in parentheses.

b Values represent averages of assays run in duplicate.
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fourth type of mitochondria-targeting signal, designated as a chimeric signal, based on its ability to direct the same primary translation product to two different subcellular compartments, namely, microsomes and mitochondria or cytosol and mitochondria (16, 18, 21, 22). The chimeric signals we defined are positionally similar to the Bcs1-type internal signal and follow immediately downstream of an N-terminal transmembrane domain. However, in contrast to the transmembrane localization of proteins containing internal signals (category 3), proteins containing chimeric signals are targeted to the mitochondrial matrix (15). The reason for this difference is probably because the stop transfer signals of CYPs, which enable the proteins to associate with the ER membranes through a single transmembrane anchor (54, 55), are incapable of eliciting a similar stop transfer event in the mitochondrial outer or inner membranes. Similar to the Bcs1-type signals, chimeric N-terminal or C-terminal signals are not cleaved by matrix proteases (17, 52).

Our studies with xenobiotic-inducible CYPs, GST, and APP led to the discovery of a new family of mitochondria-targeting signals that are referred to as chimeric signals (14–18, 21, 22). In the case of CYPs and APP, chimeric signals are located at the N terminus, whereas in GSTs these signals are located at the C terminus (14–18, 21, 22). The characteristic feature of these signals is that the first half (20–30 amino acids) of the protein is hydrophobic and the second half (10–12 amino acids) of the proteolytic cleavage in the cytosol, as in the case of CYP1A1 (14–18, 21, 22). The characteristic feature of these proteins is that the first half (20–30 amino acids) of the protein is hydrophobic and the second half (10–12 amino acids) of the protein consists of sequences rich in positively charged amino acids followed by a Pro-rich domain. Studies have also shown that chimeric signals are not substrates for mitochondrial proteases (14). Unlike the canonical signals that target proteins exclusively to mitochondria, the chimeric signals are thus mimicking the characteristic feature of canonical pre-sequences. In the presence of Hsp70 and Hsp90, CYP2B1 and +33/1A1 are delivered directly to TOM40, thus bypassing all peripheral TOMs. The signals of CYP2E1 and +5/1A1, on the other hand, mimic the features of internal targeting signals by first interacting with TOM70 (Fig. 9B). Here again, in the presence of Hsp70 alone, these proteins require all three peripheral TOMs, whereas in the presence of Hsp70 and Hsp90, they can bypass TOM20 and TOM22 but require TOM70. These results clearly show the divergent nature of chimeric signals in addition to the functional evolution of the TOM complex, which is capable of interacting with diverse types of mitochondria-targeting signals.

Hsp family cytoplasmic chaperones are known to facilitate the mitochondrial import of client proteins partly by preventing misfolding or protein aggregation (21, 58–61). More recent studies by Hartl and co-workers provided evidence for the direct physical and functional interactions between cytoplasmic chaperones, Hsp70 and Hsp90 and mitochondrial import machinery (48, 60). Hsp70 and Hsp90 dock onto the TOM complex through the specialized tetratricopeptide (TRP) domain of TOM70 and deliver client proteins to the TOM complex for import (60). In yeast, Hsp70 alone appears to be sufficient for pre-protein import, whereas in mammalian cells, both Hsp70 and Hsp90 are involved in pre-protein delivery and thus mimicking the characteristic feature of canonical pre-sequences.

In this study, we tested four different chimeric signals, including two N-terminally truncated forms to mimic endo-proteolytic cleavage in the cytosol, as in the case of CYP1A1 (+5/1A1 and +33/1A1), and two that are targeted to mitochondria as intact uncleaved signals, namely CYP2B1 and CYP2E1 (15, 16, 18, 21, 23), for their ability to bind different TOM receptor proteins under both in vitro and in vivo conditions. Mitochondrial targeting of both CYP2B1 and 2E1 was enhanced markedly by PKA-mediated phosphorylation at the unique target sites, Ser-128 and Ser-129, respectively (16, 18). The results presented here show that the client protein binding to Hsp70 and Hsp90 is enhanced by internal phosphorylation, which may be the reason for increased mitochondrial import. Our results also suggest that the chimeric signals fall into at least two distinct classes: one class, i.e. +33/1A1 and CYP2B1, which bind to TOM40 directly in the absence of other TOM proteins, and the second class, i.e. +5/1A1 and CYP2E1, which require other peripheral TOMs for binding to reconstituted TOM40 (Fig. 8). In this respect, CcOVb, which also binds to TOM40 in the absence of some other peripheral TOM receptors (TOM70, TOM20, and TOM22), can bypass TOM20 and TOM22 for association with TOM40. In the presence of TOM70 and Hsp70, these proteins require only TOM70 and thus mimicking the characteristic feature of canonical pre-sequences. In the presence of Hsp70 alone, +33/1A1 and CYP2B1 proteins associate with TOM20 and TOM22 on their way to TOM40 without requiring TOM70. In the presence of Hsp70 and Hsp90, CYP2B1 and +3331A1 bypass all peripheral TOMs and associate directly with TOM40. In the presence of Hsp70 only, +51A1 and 2E1 proteins associate with TOM70, TOM20, and TOM22 and are delivered to TOM40. Thus, the mitochon-

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ATP-dependent translocation (62, 63). Using a combinatorial approach, we show that all four CYP proteins with chimeric signals interact with both Hsp70 and Hsp90 and, more importantly, that client protein binding to Hsp90 is dependent on the presence of Hsp70. Notably, chimeric signal-containing client proteins also facilitate the binding of Hsp90 to form the ternary complex. Furthermore, our results show that Hsp90 physically binds to the cytosol exposed Pro-rich domain of TOM40 for client protein delivery through the bypass pathway. These results suggest a degree of cooperativity between the two chaperones. The only exception was CYP27, which did not bind to Hsp90 either in the presence or absence of Hsp70. Similarly, mitochondrial import of CYP27 did not require Hsp90.

Our observations on distinctly different requirements for TOM receptors and Hsp family chaperones by client proteins containing canonical and chimeric signals likely point to different phases of evolution of mitochondrial import machinery. Based on the fact that Hsp70 protein alone can support yeast mitochondrial protein import, it was suggested that the pathway requiring Hsp90 in mammalian cells might represent a later step in the evolution of the import pathway as it exists in the mammalian cells (64–66). It is likely that the pathway for the mitochondrial import of chimeric signal-containing proteins represents a more recent evolutionary event, consistent with the increased metabolic and physiologic roles of mitochondria in mammalian cell function. Proteomic studies suggest that the imported proteins also facilitate the binding of Hsp90 to form the ternary complex containing canonical and chimeric signals likely point to different phases of evolution of mitochondrial import machinery.

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