Role of Protein Kinase C-mediated Protein Phosphorylation in Mitochondrial Translocation of Mouse CYP1A1, Which Contains a Non-canonical Targeting Signal*§

Venkata Ramesh Dasari1, Hindupur K. Anandatheerthavarada1, Marie-Anne Robin2, Ettickan Boopathi‡, Gopa Biswas6, Ji-Kang Fang1, Daniel W. Nebert3, and Narayan G. Avadhani†,†

From the †Department of Animal Biology and the Marie Lowe Center for Comparative Oncology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the ‡Department of Environmental Health and Center for Environmental Genetics, University of Cincinnati Medical Center, P. O. Box 670056, Cincinnati, Ohio 45267-0056

A large number of mitochondrial proteins lack canonical mitochondrial-targeting signals. The bimodal transport of cytochromes P450 (CYPs) to endoplasmic reticulum and mitochondria (MT), reported previously by us, likely represents one mode of non-canonical protein targeting to MT. Herein, we have studied the mechanism of mouse MT-CYP1A1 targeting to gain insight into the regulatory features and evolutionary conservation of bimodal targeting mechanism. Mouse MT-CYP1A1 consists of two NH2-terminal-truncated molecular species, +91A1 and +331A1. Mutations Pro-2 → Leu and Tyr-5 → Leu, which increase the signal recognition particle (SRP) binding, diminished MT targeting of the protein in intact cells. By contrast, mutations Leu-7 → Asn and Leu-17 → Asn, which decreased SRP-binding affinity, enhanced MT targeting, thus suggesting that SRP binding is an important regulatory step that modulates bimodal targeting. Protein kinase C (PKC)-mediated phosphorylation of nascent chains at Thr-35 vastly decreased affinity for SRP binding suggesting an important regulatory step. In support of these results, COS cell transfection experiments show that phosphomimetic mutation Thr-35 → Asp or induced cellular PKC caused increased CYP1A1 targeting to MT and correspondingly lower levels to the endoplasmic reticulum. Results suggest evolutionary conservation of chimeric signals and bimodal targeting of CYP1A1 in different species. The mouse MT-CYP1A1 is an extrinsic membrane protein, which exhibited high FDX1 plus FDXR-mediated N-demethylation of a number of tricyclic antidepressants, painkillers, anti-psychotics, and narcotics that are poor substrates for microsomal CYP1A1.

Proteins targeted to mitochondria contain either NH2-terminal or internal signals, which include amphipathic α-helices, β-sheet, and random structures with spaced positively charged residues (1–3). A majority of the NH2-terminally located mitochondrial-targeting signals are cleaved by the mitochondrial matrix metalloprotease following their import, although in a number of cases the signals remain uncleaved (1–4). Recent proteomic studies indicate that yeast mitochondria contain as many as 800 proteins while the rodent heart and liver mitochondria may contain well over 1500 proteins (5–8). It is estimated that >50% of mitochondria-associated proteins in both yeast and mammalian cells lack canonical MT targeting signals, and the precise mode of targeting of these proteins as well as mechanism of their translocation across the MT membranes remain unclear. The bimodal targeting of CYPs to ER and MT, Alzheimer amyloid precursor protein to plasma membrane and MT, and translocation of cytosolic Glutathione S-transferases to MT (9–14) may represent one mode of targeting of non-canonical signal containing proteins to the MT compartment.

Recent studies from our laboratory showed that different xenobioc-inducible CYPs such as rat CYP1A1, CYP2E1, and CYP2B1 and others that are widely recognized as microsomal proteins (MC-CYPs) are also targeted to varying degrees to mitochondria (9–11). These studies led to the concept of a new family of chimeric non-canonical-targeting signals, which function both as ER-targeting and MT-targeting signals, under different physiological conditions. We proposed that bimodal targeting of these CYP proteins is facilitated by the cryptic MT-targeting signal domain (residues 29–40), located between the transmembrane helical domain (residues 1–30) and the Pro-rich domain (residues 39–44) in various members of the CYP1, CYP2, and CYP3 families. We have also shown that the cryptic MT-targeting signals of different CYPs are activated by two distinct mechanisms: (a) endoproteolytic cleavage of the NH2-

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‡ Both authors contributed equally to this work.

§ Present address: INSERM, Unite 481, Faculte de Medecine Xavier Bichat, 75018 Paris, France.

3 To whom correspondence should be addressed: Dept. of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St., Philadelphia, PA 19104. Tel.: 215-898-8819; Fax: 215-573-6651; E-mail: narayan@vet.upenn.edu.

4 The abbreviations used are: MT, mitochondrial; BNF, β-naphthoflavone; ER, endoplasmic reticulum; FDX1, (adrenodoxin) ferredoxin-1; FDXR, ferredoxin reductase; CC01, mitochondrial genome coded cytochrome c oxidase subunit 1; OAA, α-octylamine agarose; PEG, polyethylene glycol (average molecular weight 8000); POR, NADPH-P450 oxidoreductase; RRL, rabbit reticulocyte lysate; CYP, cytochrome P450; CYP1A1, full-length translated protein from the mouse or rat Cyp1a1 gene; MC-CYP1A1, microsomal (ER)-targeted CYP1A1 protein; MT-CYP1A1, mitochondrial (MT)-targeted CYP1A1 protein; PKC, protein kinase C; SRP, signal recognition particle; CMV, cytomegalovirus; PMA, phorbol 12-myristate 13-acetate.
terminal 4th and 32nd amino acids of the protein by a cytosolic endoprotease, which exposes the MT-targeting signal, as reported for rat CYP1A1 (9), and (b) protein kinase A-mediated phosphorylation of the nascent chains at Ser-128 in CYP2B1 or Ser-129 in CYP2E1, which activates the cryptic MT-targeting signal. Activation of a cryptic signal by either of the two mechanisms causes vastly diminished ER targeting, increased MT targeting, increased affinity of the nascent protein for binding to cytosolic HSP70/HSP90 chaperones, and increased binding to the mitochondrial outer-membrane and inner-membrane translocator proteins, TOM and TIM, respectively (10, 11). However, the precise mechanism, by which CYP and amyloid precursor proteins that are normally translated on the ER-bound ribosomes end up in the soluble protein pool and become candidates for MT translocation, remains unclear.

In the present study we have purified and characterized MT-CYP1A1 from livers of BNF-treated mice with three specific objectives: (a) to study the factors that regulate SRP binding to nascent chains and proteolytic processing of CYP1A1 for bimodal targeting to the ER and MT; (b) to provide further supporting evidence for our hypothesis on the occurrence of chimeric signals that drive the bimodal protein targeting; and (c) to determine the evolutionary conservation of the endoproteolytic-processing mechanism for the activation of the cryptic MT-targeting signal in another animal besides the rat. Our results show that the mouse MT-CYP1A1 is truncated just beyond the 8th and 32nd amino acids and the processing site close to residue 32 is conserved in many species. Mutational analysis indicates that PKC-mediated phosphorylation of a newly identified target site at Thr-35 not only modulates SRP-binding affinity of nascent chains but also determines the level of ER or MT targeting of CYP1A1. The nascent polypeptide pool that escapes SRP binding essentially becomes the substrate for endoprotease processing and activation of cryptic mitochondrial-targeting signal. The results also show that mouse MT-CYP1A1 actively metabolizes erythromycin, as well as a number of psychotropic drugs, including anti-depressants, anti-psychotics, and anesthetics in an FDX1 plus FXDR-supported system, thus exhibiting substrate specificity quite distinct from the full-length MC-CYP1A1 (ER-associated).

**MATERIALS AND METHODS**

**Treatment of Animals and Tissue Fractionation**—All animal procedures were carried out in compliance with National Institutes of Health guidelines for the use of vertebrate animals. Swiss-Webster mice (30 g, Harlan Sprague-Dawley Inc., Indianapolis, IN) received intraperitoneal injections of BNF (80 mg/kg body weight in corn oil) once daily for 5–10 days, as described (12). The control mice received equivalent volumes of corn oil. Animals were killed by CO2 asphyxiation 24 h after the last injection and perfused transcardially with ice-cold saline. The resultant mitoplast pellet was washed twice and resuspended in sucrose-mannitol buffer containing 0.1 mM phenylmethylsulfonyl fluoride. The purity of the mitochondrial preparation was checked routinely by assaying the marker enzyme activities and by immunoblot analysis, as described before (13). CYP content of mitoplasts was measured by the sodium dithionite-reduced CO-binding difference spectrum (15) in a buffer system containing 100 mM KH2PO4, pH 7.4, 20% glycerol (v/v), 0.5% sodium cholate (w/v), and 0.06% Triton N-101 (v/v).

**Solubilization and Purification of Mouse Liver MT-CYP1A1**—Mitoplasts were suspended in 100 mM KH2PO4 buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, and 1 mM diethiothreitol, at a final concentration of 20 mg of protein/ml and sonicated for 3 min (30-s pulse followed by 60 s of standing on ice) at setting 6 of a Branson sonifier. CYP protein was solubilized by adding sodium cholate to a final concentration of 0.5% and fractionated with PEG, as described previously (16). The 0–20% PEG precipitate was collected by centrifugation at 100,000 × g for 1 h, and the pellet was suspended in 100 mM KH2PO4, pH 7.4, containing 20% glycerol, 1.0 mM EDTA, 1.0 mM diethiothreitol, and 0.5% sodium cholate. The proteins were dissolved by homogenization with a glass homogenizer, followed by stirring the suspension for 30 min at 4 °C. The solubilized protein fraction (10 mg of protein/ml) was subjected to OAA column chromatography, as described (16). The column was first washed until the A280 of the eluant reached <0.05, and the protein bound to the column was eluted successively with ten column volumes of buffer A containing 0.1% Triton N-101. Fractions rich in CYP (>0.1 A at 417 nm) were pooled and concentrated by ultrafiltration through Amicon filters. The concentrated samples were dialyzed overnight against three changes of buffer containing 10 mM K2HPO4, pH 7.7, 20% glycerol, 0.5% sodium cholate, and 0.06% Triton N-101, and subjected to further purification by DEAE-Sepharose chromatography (16).

**Construction of cDNA Expression Plasmids**—The mouse CYP1A1 cDNA (17) was amplified using a 5′ primer containing a HindIII site and a 3′ primer containing an XbaI site, cloned into the pCMV4 mammalian expression vector and the pGEM7zf bacterial plasmid vector. Deletion mutants were generated by PCR amplification of specific cDNA regions and cloning into the appropriate vectors. The various point mutations at the ER-targeting and MT-targeting signal domains were generated by overlap PCR. All constructs were engineered to contain an ATG codon preceded by a Kozak consensus sequence, as previously described (18).

**In Vitro Import of Labeled Proteins into Mitochondria**—Freshly isolated rat liver mitochondria were used for in vitro import, using a system described before (9, 10). In vitro translation products were generated in the rabbit reticulocyte lysate (RRL) system (Promega, Madison, WI) in the presence of added [35S]Met (40 μCi/50 μl reaction, PerkinElmer Life Sciences), according to the manufacturer’s recommended protocol. The import assays were carried out in 200-μl final volumes using sucrose gradient (9), washed twice, and resuspended in the above buffer at a concentration of 45–50 mg/ml. In some experiments, mitochondria were treated with digitonin (Calbiochem) at a final concentration of 75 μg/mg of protein at 4 °C. The resultant mitoplast pellet was washed twice and resuspended in sucrose-mannitol buffer containing 0.1 mM phenylmethylsulfonyl fluoride. The purity of the mitochondrial preparation was checked routinely by assaying the marker enzyme activities and by immunoblot analysis, as described before (13). CYP content of mitoplasts was measured by the sodium dithionite-reduced CO-binding difference spectrum (15) in a buffer system containing 100 mM KH2PO4, pH 7.4, 20% glycerol (v/v), 0.5% sodium cholate (w/v), and 0.06% Triton N-101 (v/v).
Post-translational Processing of Mouse CYP1A1 Protein

100 μg of mitochondrial protein, as described before (9), and was treated with 125 μg/ml Pronase for 20 min on ice. Pronase-treated and untreated samples were mixed with protease inhibitor mix, and mitochondria were recovered by sedimentation through 1.0 M sucrose, as described (9). Mitochondrial proteins were solubilized in 2× Laemmli sample buffer for 10 min at 75 °C and analyzed by SDS-PAGE (19) and fluorography.

Transient Transfection of COS Cells with CYP1A1 cDNAs—Various cDNAs cloned in pCMV4 vector were transfected into COS-7 cells using FuGENE 6, a non-liposomal transfection reagent (Roche Molecular Biochemicals), according to the manufacturer’s recommended protocol. Cells from 10 plates (100 mm), after 60–70 h of transfection, were washed with phosphate-buffered saline (20 mM KH2PO4 (pH 7.4) plus 150 mM NaCl) and homogenized in the Sucrose-Mannitol buffer containing protease inhibitor mixture, with a Teflon-fitted glass homogenizer (~40 strokes). Isolation of mitochondria by differential centrifugation and further purification by sucrose density banding, as well as isolation of microsomes, were described before (9, 10).

Isolation of Rat Cytoplasmic Fraction Rich in Endoprotease—Rat liver cytoplasmic fraction was isolated by homogenization with 0.1 M Na2CO3 (pH 11), separa-

tion of soluble and insoluble protein fractions, and immunoblot analysis of proteins were carried out as described (13). Briefly, proteins were translated in rabbit reticulocyte lysate (RRL) in the presence of 2 units of unwashed canine pancreatic ER membranes/25 μl of reaction (10). Reactions were stopped by adding 1 mM cycloheximide, and membranes were extracted with 0.1 M Na2CO3 (pH 11) for 1 h on ice and fractionated into soluble (200,000 × g supernatant) and insoluble membrane (200,000 × g pellet) fractions, as described (23). Both the membrane pellet and the soluble protein fractions were resuspended in 40 μl of 10 mM Tris-HCl, pH 7) containing 2% SDS and subjected to SDS-PAGE.

Membrane Integration Assay—Freshly isolated mitochondria and microsomes were subjected to Pronase digestion as described (13). Mitochondria and microsomes were suspended at 10 mg of protein/ml in 50 mM potassium phosphate buffer containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and subjected to trypsin digestion (30 μg/mg of protein) for 30 min at room temperature. The reaction was stopped by adding 10× molar excess of trypsin inhibitor and an equal volume of 2× Laemmli sample buffer (19). The samples were incubated at 95 °C for 5 min and subjected to 12% SDS-PAGE for immu-

noblots analysis.

Immunofluorescence Microscopy—Cells grown on coverslips were transfected with the cDNA constructs as described above. The cells were fixed in 2% paraformaldehyde 60 h post-transfection and permeabilized with 0.1% Triton X-100. The cells were blocked with 5% goat serum for 1 h at 37 °C followed by incubation with primary CYP1A1 (anti-rabbit) and CcO1 (anti-
mouse) antibodies at 1:100 dilutions, each for 1 h at 37 °C. The cells were then labeled with 1:100 dilution of Alexa 488–conjugated anti-rabbit antibody and Alexa 594-conjugated anti-mouse goat IgG (Molecular Probes, Inc., Eugene, OR), respec-

tively (1:100 dilution each). Coverslips were washed repeatedly with phosphate-buffered saline to remove unbound antibodies and were mounted on slides using Antifade mounting medium (Molecular Probes). Slides were viewed through a Zeiss Meta 510 confocal microscope.

Metabolism of Xenobiotic Substrates—The N-demethylation of various substrates was measured by assaying the rate of form-
aldehyde formation (24, 25). Reactions with mitoplasts and microsomes were carried out in 500–μl final volumes in a buffer containing 50 mM Tris-HCl, pH 7.4, 20 mM MgCl2, 200 μg of mitochondrial or microsomal protein, and 100 μM substrate. Purified FDX1 (0.1–0.2 nm) plus FDXR (0.01–0.02 nm) were added in some reactions with mitoplast preparations, as indi-
cated. Reconstitution of enzyme activity with purified MT-CYP1A1 was carried out essentially as described before (16) in dialyryl-phosphatidylcholine vesicles, using the same
buffer system described above for intact membranes. The final reaction volume was 200 μl, and contained 50 pmol of CYP, 0.2 nmol of FDX1, 0.02 nmol of FDXR, or 0.1 nmol of POR and 100 μM substrate. After 3 min of preincubation at 37 °C, the reaction was initiated by the addition of 1 mM NADPH, and the reaction was continued at 37 °C for another 20 min in a shaking water bath. Reactions were terminated by adding 0.5 volume of ice-cold trichloroacetic acid (10% w/v). The insoluble material was pelleted by centrifugation at 10,000 × g at room temperature. An aliquot of the supernatant was mixed with an equal reaction volume was 200 μl, and contained 50 pmol of CYP, 0.2 nmol of FDX1, 0.02 nmol of FDXR, or 0.1 nmol of POR and 100 μM substrate. After 3 min of preincubation at 37 °C, the reaction was initiated by the addition of 1 mM NADPH, and the reaction was continued at 37 °C for another 20 min in a shaking water bath. Reactions were terminated by adding 0.5 volume of ice-cold trichloroacetic acid (10% w/v). The insoluble material was pelleted by centrifugation at 10,000 × g at room temperature. An aliquot of the supernatant was mixed with an equal volume of Nash reagent (250 or 100 μl) and further incubated at 55 °C for 15 min. The reaction product, formaldehyde, was measured according to the method of Nash (24). A notable drawback of this assay is a generally high background value, which can be controlled by using freshly prepared Nash reagent and limiting the heating step during color development to 55 °C for 15 min. Typically, we obtained ΔAbs values in the range of 0.01–0.014 for control samples without added enzyme, and all significant N-demethylation activities reported here correspond to >3.5-fold of this background reading.

RESULTS

Localization of NH2-terminal Truncated Forms of CYP1A1 in Mouse Liver Mitochondria—Localization of CYP1A1 in BNF-treated mouse liver mitochondria was ascertained by limited protease digestion of MT and MC preparations. Immunoblot in Fig. 1A shows that both MT and MC from untreated mouse liver contain undetectable CYP1A1. These same fractions from 10 days of BNF-treated mouse livers show a distinct 54-kDa band. Quantification of immunoblots indicates that the MT-CYP1A1 content at this stage of BNF induction is ~20–25% more than the MC-CYP1A1 content (results not shown). Fig. 1A also shows that the MT-CYP1A1 is relatively resistant to trypsin digestion, whereas the MC-CYP1A1 protein is completely degraded by added trypsin. Additionally, MT preparations contained no detectable MC marker proteins suggesting very low microsomal contamination. Mitochondria of comparable purity were used in all the experiments presented in this report.

Digitonin-stripped MT, containing <0.5% MC contamination as judged by marker enzyme assays, were used for enzyme purification. Mouse liver MT-CYP1A1 was purified by PEG fractionation followed by chromatography on OAA and DEAE-Sephrose columns (Fig. 1B). Digitonin-stripped mitoplasts contained 1.67 nmol of CYP/mg of protein, whereas the cholate extract contained 2.29 nmol of CYP/mg of protein (not shown). The 20% PEG precipitate represented half the total input CYP and specific activity of ~3.8 nmol/mg of protein. Most of the input CYP was eluted from the OAA column with 0.1% Triton N-101; this fraction, representing 33% of input CYP and containing 3.8 nmol of CYP/mg of protein, was used for further purification by DEAE-Sephrose column chromatography. The DEAE-Sephrose-purified protein contained 13.75 nmol of CYP/mg of protein.

The Coomassie Blue R-stained gel pattern (Fig. 1B), and the immunoblot (Fig. 1C), show that a protein of ~55 kDa is enriched progressively at different stages of protein purification, with the OAA and DEAE-Sephrose column frac-

![FIGURE 1. Characterization of mouse MT-CYP1A1. Hepatic mitochondria and microsome were isolated from mice treated with BNF for 5 days by sucrose density banding as described under "Materials and Methods." In A, mitochondria were digested with trypsin (30 μg/mg of protein) for 30 min at room temperature. Proteases were inactivated by adding trypsin inhibitor, and the proteins were resolved by 12% SDS-PAGE. Proteins electroblotted to membranes were subjected to immunoblot analysis using rat CYP1A1 antibody (1:2000 dilution). The blots were stripped and reprobed with antibodies to TIM44 and careticulin (1:1500 each, both from Santa Cruz Biotechnology) B, MT-CYP1A1 from digitonin-treated mitoplasts was purified using a combination of cholate extraction, OAA, and DEAE-Sephacel chromatography. Protein fractions at various stages of purification were subjected to electrophoresis on a 12% SDS-PAGE. Lane M, molecular weight markers; lane 1, 40 μg of total mitochondrial protein; lane 2, 30 μg of cholate-solubilized fraction; lane 3, 30 μg of 20% PEG precipitate; lane 4, 30 μg of OAA-column fraction, eluted with 0.1% Triton N-101; lane 5, 1 μg of protein eluted from DEAE-Sephrose column (fractions 34–42). Protein bands were visualized by staining with Coomassie Brilliant Blue. C, a companion blot, same as in B, was subjected to immunoblot analysis with anti-rat CYP1A1 antibody (1:2000 dilution). D, 10 μg of purified mouse liver MT-CYP1A1 (B, lane 5) was resolved on a 10–18% gradient gel and transblotted to a Sequi blot polyvinylidene difluoride membrane and stained with Coomassie Brilliant Blue. Proteins from the two indicated bands were subjected to NH2-terminal sequencing.](https://example.com/figure1.png)
Post-translational Processing of Mouse CYP1A1 Protein

**A**

| Mouse CYP1A1 |
|--------------|
| --- |
| --- |
| 55 kDa |

**B**

| WT CYP1A1 | HE-SRP Mut1 | HE-SRP Mut2 | LE-SRP Mut3 | T35L-Mut | T35D-Mut | +91A1 | +91A1-39 Mut | +91A1-39/34 Mut |
|-----------|-------------|-------------|-------------|----------|----------|-------|-------------|---------------|
| MYSGLPAFVSATELALLAVTFCLGFVVVRATRTWPKGTLKTPGWGL | MLYSMGPAPAFVSATELALLAVTFCLGFVVVRATRTWPKGTLKTPGWGL | MLYSMGPAPAFVSATELALLAVTFCLGFVVVRATRTWPKGTLKTPGWGL | MYSGLPAFVSATELALLAVTFCLGFVVVRATRTWPKGTLKTPGWGL | MYSGLPAFVSATELALLAVTFCLGFVVVRATRTWPKGTLKTPGWGL | MYSGLPAFVSATELALLAVTFCLGFVVVRATRTWPKGTLKTPGWGL | MAFVSATELALLAVTFCLGFVVVRATRTWPKGTLKTPGWGL | MAFVSATELALLAVTFCLGFVVVRATRTWPKGTLKTPGWGL | MAFVSATELALLAVTFCLGFVVVRATRTWPKGTLKTPGWGL |

**FIGURE 2.** Functional domains of mouse CYP1A1 for bimodal targeting and conservation of endoprotease processing by a cytosolic protease. A, the in vitro translated $^{35}$S-labeled CYP1A1 from mouse was incubated with partially purified rat cytosolic protease (+ = 1.5 μg of enzyme and ++ = 3.0 μg of enzyme/10 μl of RRL). Aprotinin, when added, was at the 5 μM level. B, designation of various functional domains was based on sequence comparisons with the rat CYP1A1, CYP2B1, and CYP2E1 that have also been extensively characterized for MT targeting. Indicated point mutations and NH2-terminal deletions were generated, as described under “Materials and Methods.”

Post-translational Processing of Mouse CYP1A1 Protein—As previously shown for the rat enzyme (9), the mouse CYP1A1 is processed into a faster migrating component by adding rat cytosolic protease under in vitro conditions (Fig. 2A). The intensity of the faster migrating component is increased with increasing amounts of protease added, suggesting a concentration dependence for processing. Furthermore, the processing is inhibited by serine protease inhibitor, aprotinin (5 μM). These results show that, similar to what has been shown for the rat CYP1A1, the mouse CYP1A1 protein is also processed by partially purified rat cytosolic protease (9). Although the precise site of processing of mouse CYP1A1 has not been characterized, the in vitro-processed protein product may be +331A1. Our previous results with various inhibitors suggested that the cytosolic endoprotease may be a member of serine protease family (9), and the processing site at residues 30–35 resembles the consensus for a “trypsin family” protease (9). A computer analysis of CYP1A1 sequences from various species (see supplemental Fig. S1) indicates that a trypsin-like consensus cleavage site exists at about residue 30–40 in almost all species examined, although in the cat and dog, a substantial sequence divergence is seen. Furthermore, in the human and Rhesus monkey, the processing site appears to be located between residues 25 and 31. Based on this, it is likely that endoprotease

tions showing more intense cross-reactivity to anti-rat P4501A1 antibody (see Fig. 1C). As seen from Fig. 1B (lane 5) the DEAE-Sepharose-purified MT-CYP1A1 shows a major band of ~55 kDa. As shown later (Fig. 1D), this band resolved into two components on a high resolution gel. Based on the gel scan, we estimated ~65% purity. The partially purified ~55-kDa protein in lane 5 also cross-reacted with the anti-rat CYP1A1 antibody (see Fig. 5C). Electrophoresis of partially purified mouse MT-CYP1A1 on a polyacrylamide gradient gel yielded two closely migrating bands (Fig. 1D), both of which cross-reacted with the anti-rat CYP1A1 antibody (results not shown). The two protein species were subjected to NH2-terminal sequencing by Edman degradation. The upper band (Fig. 1D) yielded the sequence of AFVSATELLL, suggesting that it is CYP1A1, which lacks the first 8 amino acid residues (hereafter called +91A1). The lower band yielded the sequence of TRTWVPK, suggesting that it is CYP1A1 devoid of the first 32 amino acid residues (hereafter called +331A1).

**Interspecies Conservation of NH2-terminal Endoprotease Processing Site on CYP1A1—**As previously shown for the rat enzyme (9), the mouse CYP1A1 is processed into a faster migrating component by adding rat cytosolic protease under in vitro conditions (Fig. 2A). The intensity of the faster migrating component is increased with increasing amounts of protease for the activation of cryptic MT-targeting sequence of the CYP1A1 protein is an evolutionarily conserved mechanism.

**Cryptic Mitochondrial Targeting Signal of the Mouse CYP1A1 Protein**—Fig. 2B shows that similar to the previously analyzed rat CYP1A1, CYP2B1, and CYP2E1 proteins, the mouse CYP1A1 contains a putative ER-targeting domain (residues 1–10), a transmembrane domain (up to about residue 32), a cryptic MT-targeting domain containing three positively charged residues (amino acids 32–42), and a Pro-rich domain (residues 44–50). Previously, we proposed that the CYP1A1 protein translated in the free form served as a putative substrate for processing by a cytosolic endoprotease (9, 25). In the present study we have tested this hypothesis by targeted mutations at the NH2-terminal 10–15 amino acid sequence region that increased or decreased the affinity for SRP binding and how these mutations affected mitochondrial targeting. For this purpose we designed ER-domain mutations by using the ExPASy (Expert Protein Analysis System) program (Swiss Institute of Bioinformatics); this algorithm was used to design mutations that increase α-helicity and hydrophobicity within the NH2-terminal 10–15 amino acid residues with a predicted increase in the affinity for SRP binding and ER targeting. Alternatively reduced α-helicity and hydrophobicity within the first 15-amino acid region is expected to reduce affinity for SRP binding.
and ER targeting. Mutation Pro-2 → Leu (marked as HE-SRP-Mut1 in Fig. 2B) predicted a 15–20% higher ER targeting potential, whereas mutation Pro-2 → Leu plus Tyr-5 → Leu (marked as HE-SRPMut2 in Fig. 2B) further increased the potential by 30%, compared with the wild-type CYP1A1 protein. Additionally, Leu-7 → Asn and Leu-17 → Asn (marked as LE-SRPMut3 in Fig. 2B), which disrupts α-helicity and reduces hydrophobicity, also predicted reduced ER targeting potential by 50% of the wild-type CYP1A1 protein. As shown in Fig. 2B, the three ER-targeting signal mutations (HE-SRPMut1, HE-SRPMut2, and LE-SRPMut3) were generated for testing our predictions, and mutations at the putative MT targeting domain (+91A1–39Mut and +91A1–34/39Mut) were generated for confirming the presence of chimeric signal in the mouse CYP1A1. Furthermore, Thr-35 from the trypsin-like protease processing site is also a part of PKC consensus phosphorylation site, and this site was mutated to T35L (T35L-Mut) or T35D (T35D-Mut) to determine the role of PKC in bimodal targeting of CYP1A1 to mitochondria and ER.

The effects of mutations in the ER-targeting domain on SRP binding and ER targeting were tested by two approaches. In the first approach, we assessed the effects of saturating levels of added SRP on the translation efficiencies of wild-type versus mutant proteins. It is known that SRP binding arrests chain elongation until the NH$_2$ termini of nascent chains are docked onto the ER receptors. In this regard, the rate of inhibition of translation by SRP is considered as a measure of the SRP-binding affinity of the ER-targeting domain. The rate of in vitro translation of wild-type protein was diminished by 50% (Fig. 3), whereas, in keeping with the computer-based predictions, the translation rates of HE-SRPMut1 and HE-SRPMut2 proteins were decreased by 53–94%. The translation of LE-SRPMut3 was inhibited only marginally (7.6%), suggesting inefficient SRP binding. The translation of preprolactin, a bona fide secretory protein, was inhibited by ~94%.

In the second approach, we translated the wild-type and mutant proteins in the presence of increasing levels of ER membrane (Fig. 4, A and C), and the extent of membrane integration was assessed by alkaline Na$_2$CO$_3$ extraction. The $^{35}$S-labeled CYP1A1 in the Na$_2$CO$_3$-soluble and -insoluble fractions were quantified by SDS-gel electrophoresis and radiometric imaging. A representative gel profile of proteins translated with 1 unit of added ER membrane and quantitation of gel pattern are presented in Fig. 4 (A and B, respectively). In keeping with the inhibition of translation by SRP, nearly 63% of the translated CYP1A1 was resistant to alkaline extraction, suggesting integral membrane association. About 37% of the translation products were detected in the soluble fraction, reflecting the extrinsic membrane organization. In the case of the HE-SRPMut1 and HE-SRPMut2 proteins, the percent membrane integration increased to 70 and 93%, respectively. Correspondingly, the CYP protein in the soluble pool was decreased to ~30% and >10%, respectively. In the case of LE-SRPMut3, however, >80% of the protein was detected in the Na$_2$CO$_3$-soluble fraction, whereas <20% was partitioned in the insoluble membrane fraction. Additionally, +91A1 lacking part or most of ER-targeting signal was partitioned nearly completely in the soluble fraction (Fig. 4B). The kinetic data in Fig. 4C show that the extent of membrane association is essentially saturated between 2 and 3 units of added ER membrane per reaction and reaches a maximum of 30% for the SRP mutant protein, LE-SRPMut3, with added 8 units of ER membrane per reaction. These results indeed confirm that the affinity of nascent chains for binding to SRP likely determines the extent of ER targeting of mouse CYP1A1.

**Efficient Import of NH$_2$-terminal Truncated CYP1A1 into Isolated Mitochondria**—We studied the extent of transport of $^{35}$S-labeled WT and the SRP-binding domain mutant proteins into mitochondria using an in vitro mitochondrial transport system. Resistance to limited digestion with Pronase was used as a criterion for the transport of proteins into the mitochondrial membrane compartment. We have previously shown that intact rat CYP1A1 is poorly imported into rat liver mitochondria (9), whereas the NH$_2$-terminal truncated form, +331A1 (P450MT2), was efficiently imported. As shown in Fig. 5A intact mouse CYP1A1 was imported into rat liver mitochondria rather inefficiently (3–5% of input cpm, Fig. 5A and quantitation in C), whereas the +91A1 protein was imported with a high efficiency of ~30% input (see Fig. 5, B and D). On the contrary, all three mutant proteins (HE-SRP-Mut1, HE-SRP-Mut2, and LE-SRPMut3), exhibiting varying efficiencies for SRP binding, were imported at efficiencies similar to the wild-type CYP1A1 (Fig. 5, A and C). These results suggest that polypeptide chains with intact NH$_2$ termini, irrespective of their hydrophobicity or hydrophilicity, are imported poorly under these in vitro conditions. As shown for the rat CYP1A1, a mutation targeted to one of the positive residues in the putative MT-targeting signal domain (+91A1–39Mut) outside the putative PKC target site markedly decreased mitochondrial import (Fig. 5B, lanes 4–6). Mutations of both Arg-34 and Lys-39 (+91A1–34/39Mut) sites reduced the mitochondrial import to near undetectable levels (Fig. 5B, lanes 7–9). Mutation at the Arg-34, but not at Lys-39, affected both processing by cytosolic endoprotease and PKC-mediated phosphorylation. These results suggest not only the need for activation of a cryptic MT-targeting signal by protease processing but also that the positive residues at 39, and also possibly at 34, positions are a critical part of the MT-targeting signal.
Inverse Relationship of SRP Binding and Mitochondrial Targeting Efficiencies of CYP1A1 Mutant Proteins in Intact Cells—

The in vivo targeting of WT and SRP mutant CYP1A1 to mitochondria was investigated using COS cells transfected with various cDNA constructs cloned into pCMV4. After 62 h of transfection, mitochondria and microsomes were isolated and analyzed by immunoblot analysis. As previously described (9, 13), the cell fractionation procedure yielded mitochondrial preparations containing >0.5% microsomal contamination, as tested by marker enzyme assays (results not shown). Immunoblot analysis of MT and MC protein fractions from transfected cells is shown in Fig. 6. It is seen that mock-transfected cells do not show a significant CYP1A1 antibody cross-reactive ~55-kDa band (Fig. 6, A and C). Both the MT and MC protein fractions from cells transfected with WT1A1 cDNA (Fig. 6A) showed an antibody cross-reactive band of ~55-kDa. However, the band intensity with the MC fraction was about twice as intense as with the MT protein fraction. Transfection with HE-SRPMut1 and HE-SRPMut2 constructs also yielded both the MT and MC 55-kDa species, although the two mutants showed progressively reduced MT levels (20 and 6%, respectively). In contrast, the LE-SRP Mut3 protein, with diminished affinity for SRP binding, was poorly targeted to MC, but at a markedly higher level (85%) to the MT compartment (Fig. 6B). These data together with the results of SRP-mediated translation inhibition in Fig. 4 suggest that the relative efficiency with which the nascent chains bind to SRP might determine the availability of nascent CYP1A1 protein for targeting to the mitochondrial compartment.

As expected, transfection with the +91A1 cDNA yielded markedly increased mitochondrial targeting and diminished microsomal contents (Fig. 6C). In keeping with the results of the in vitro import in Fig. 5, mutations targeted to the two positively charged residues (+91A1–39Mut and +91A1–34/39Mut) showed vastly decreased MT targeting.

The markedly different patterns of subcellular targeting of LE-SRP Mut3 and HE-SRP Mut2 proteins was further verified by immunohistochemical analysis of transfected COS cells. As shown in Fig. 6D, in cells transfected with WT 1A1 cDNA, only a small fraction of A1-specific antibody stain co-localized with mitochondria-specific CcO1 stain. In the case of cells transfected with +91A1 a large part of A1 antibody stain co-localized with CcO1-specific stain indicating predominantly mitochondrial localization. LE-SRP Mut3 protein with lower affinity for SRP binding co-localized extensively with CcO1-stained membranes. HE-SRP Mut2 protein with higher affinity for SRP binding, on the other hand, co-localized with mitochondrial membranes very poorly. These results are consistent with the immunoblot data in Fig. 6 (A and C).
The need for endoprotease processing of CYP1A1 for efficient MT transport was further tested using in vitro import assays with or without added partially purified cytosolic protease. Fig. 6E shows that in the presence of added cytosolic protease, both HE-SRP-Mut2, which is more preferentially targeted to MC in cell transfection assays, and LE-SRP-Mut3, which is more preferentially targeted to MT, are imported by isolated mitochondria at comparable levels of ~39–45% of input protein. This level of in vitro import is comparable to the import of WT CYP1A1 (~40%) with added cytoplasmic protease fraction. These results suggest that nascent chains, that escape SRP binding and are translated as membrane-free proteins, are potential substrates for endoprotease processing and activation of cryptic MT-targeting signal.

PKC-mediated Phosphorylation Modulates the Affinity of CYP1A1 for SRP Binding—The mouse CYP1A1 contains a 70–80% consensus PKC site (RTWVPK), which is conserved in almost all vertebrate CYP1A1 proteins. We therefore investigated if this site is indeed phosphorylated, and if so, whether phosphorylation plays a role in SRP binding using multiple approaches. Fig. 7A shows that SRP addition to an in vitro translation mix caused 35–40% inhibition of translation. Surprisingly, the SRP-mediated inhibition was nearly completely reversed by added PKC. Similar was the case with HE-SRP Mut2 protein, which otherwise binds SRP with very high efficiency. Notably, the translation inhibition of T35L mutation targeted to the PKC domain was not affected by added PKC. Furthermore, the translation of phosphomimetic mutant T35D was not affected by added SRP with or without addition of PKC (not shown). These results support the possibility that phosphorylation at the T35 site causes a reduction in affinity of nascent chains for SRP binding.

In vitro import assays in Fig. 7B show that mitochondrial import of WT1A1 was markedly increased by adding cytosolic protease, which was further increased by added PKC. Results also show that both cytosolic protease and PKC had very little effect on the mitochondrial import of R34A/R39A mutant CYP1A1 protein confirming the role of the positive residues in mitochondrial targeting. These results suggest that, in addition to modulating SRP binding efficiency, PKC may have additional yet uncharacterized stimulatory effects on the mitochondrial import of CYP1A1.

The role of PKC-mediated phosphorylation on mitochondrial targeting of CYP1A1 under in vivo condition was investigated by cDNA transfection in COS cells, followed by immunoblot analysis of subcellular fractions. Immunoblot in Fig. 7C (top panel) shows that mitochondrial localization of WT1A1 was increased by ~2.5-fold by the PKC inducer PMA. As expected, the PKC-mediated increase was nearly completely reversed by the PKC inhibitor Go6850. The mitochondrial targeting of HE-SRPMut2 (Fig. 7C, middle panel) was vastly increased by PMA treatment. The MC content of this protein was correspondingly reduced by PMA. However, the PKC inhibitor Go6850 markedly reversed the MT targeting of the protein. The mitochondrial content of LE-SRP Mut3, which is predominantly targeted to mitochondria under normal conditions, was not significantly affected by PMA treatment. The PKC inhibitor, however, drastically reduced the mitochondrial content of this protein, providing additional support for the possibility that PKC has a positive modulatory effect on the mitochondrial transport system.

If PKC-mediated protein phosphorylation indeed interferes with SRP binding to CYP1A1 and supports increased MT targeting, we reasoned that the MC- and MT-CYP1A1 may con-
FIGURE 6. Bimodal targeting of mouse CYP1A1 to the mitochondria and microsomes. COS-7 cells were transfected with 10 μg/plate of the various wild-type cDNA, mutant cDNA constructs, or pCMV4 vector without any insert (mock-transfected). Cells from 10 companion plates were used to isolate the mitochondrial and microsomal membrane fractions. Protein (25 μg) from each of the cell fractions was resolved by electrophoresis on 12% PAGE and subjected to immunoblot analysis, using anti-rat CYP1A1 antibody. A and C, transfections with wild-type and point mutants of full-length CYP1A1. B, transfections with wild-type and mutant constructs of NH2-terminal truncated CYP1A1. The blots were probed with an antibody to the mitochondria-specific marker protein CcO1 (A and B) or mitochondrial transcription factor A (mtTFA) (C) and microsome-specific marker protein POR (A and B) or calreticulin (C). The percent distribution in the mitochondrial and microsomal fractions was calculated based on a 1:2 recovery of mitochondrial and microsomal proteins from total cell homogenates. D, immunohistochemical analysis of COS cells transfected with the indicated cDNA constructs. Cell transfection, staining with primary and secondary antibodies, and confocal imaging of cells were as described under "Materials and Methods." E, in vitro import assays were run as in Fig. 5 and under "Materials and Methods." Using 35S-labeled WT and SRP Mut proteins preincubated with (10 μg/50 μl of RRL) or without partially purified cytosolic protease fraction. The values at the bottom of the gel pattern indicate percent radioactive protein bound to mitochondria (Pronase tubes) and % imported into mitochondria (plus Pronase tubes). The values at the bottom of A–D represent averages of two separate assays. There was <10% variability between each experiment.
tain vastly different levels of phosphoryl residues. This was investigated by immunoprecipitation of MC- and MT-CYP1A1 and partially purified enzyme from these two membrane compartments and probing with antibody to Thr phosphate. The immunoblot in Fig. 7D shows that CYP1A1, immunoprecipitated from total mitochondrial protein or partially purified MT-CYP1A1, contains a high level of Thr phosphate residues. In contrast, the MC-CYP1A1 is phosphorylated at very low to negligible levels. These results provide further support for the possibility that PKC-mediated phosphorylation may regulate the extent of CYP1A1 targeting to the two membrane compartments.

The modulatory role of Thr-35 phosphorylation on MT targeting of CYP1A1 was further investigated by transfection of COS cells with T35L and T35D mutant cDNAs and analyzing the MT and MC 1A1 protein contents and the levels of phosphorylation. MT and MC protein fractions (200 μg each) from transfected cells were immunoprecipitated with CYP1A1 antibody, and the immunoprecipitates were probed with CYP1A1 antibody (upper panel) or Thr phosphate antibody (lower panel). The immunoblot in Fig. 7E (upper panel) shows that the PKC phosphorylation site-mutated (T35L) protein was targeted to MT very poorly. In contrast, the phosphomimetic mutant, T35D, was more preferentially targeted to MT, and at 3- to 4-fold reduced level to MC. The immunoblot developed with anti-Thr phosphate antibody (bottom panel) shows that only wild-type MT-Cyp1A1 was phosphorylated, whereas none of the mutant CYP1A1s associated with MC and MT compartments were phosphorylated. These results confirm that CYP1A1 most likely contains a single PKC target phosphorylation site at Thr-35.

Altered Substrate Specificity of Mouse Liver MT CYP1A1—

The demethylase activities of mitoplasts from control and BNF-
Post-translational Processing of Mouse CYP1A1 Protein

TABLE 1
Metabolism of various substrates by liver mitoplasts from BNF-treated mice

Lever mitoplasts from control mice and mice treated with BNF for 10 days were assayed for N-demethylation of various resorufins (20 μM each case) and erythromycin (100 μM). FDX1 (0.02 nM) and FDXR (0.2 nM) were added to counter the possible loss of these soluble proteins during isolation and gradient banding. Average values ± S.E. were calculated from three to four separate assays.

| Cell fraction and treatment | Specific activitya | EROD | MROD | PROD | BROD | ERND |
|----------------------------|------------------|------|------|------|------|------|
| Control mitoplasts (FDX1 plus FDXR) | 9.38 ± 0.15 | 0.68 ± 0.01 | 0.58 ± 0.03 | 0.15 ± 0.01 | 260 ± 0.02 |
| BNF mitoplasts (FDX1 plus FDXR) | 22.20 ± 0.22 | 1.08 ± 0.03 | 0.88 ± 0.03 | 0.52 ± 0.02 | 720 ± 0.06 |

a EROD, ethoxyresorufin O-demethylase; MROD, methoxyresorufin O-demethylase; PROD, pentoxyresorufin O-deethylase; BROD, benzoxyresorufin O-dealkylase; ERND, erythromycin N-demethylase.

induced mouse liver were assayed with various resorufins and erythromycin as substrates (Table 1). As shown in Fig. 8A, a considerable amount of FDX1 was lost during mitoplast preparation from both control and BNF-treated livers. Digitonin treatment appears to cause the loss of small soluble proteins from mitochondrial matrix fraction. Reconstitution of enzyme activity with mitoplast preparations were therefore carried out with added FDX1 and FDXR. Control mitoplasts yielded activities ranging from 0.15 to 9.3 pmol/min/mg of protein for different resorufins and 260 pmol/min/mg of protein with erythromycin. Mitoplasts from BNF-treated mice yielded highest ethoxyresorufin O-demethylase activity (in the range of 22 pmol/min/mg of protein) and relatively lower activities (in the range of 0.5 to 1.0 pmol/min/mg of protein) for benzoxyresorufin O-dealkylase, pentoxyresorufin O-deethylase, and 7-alkoxyresorufin O-deethylase. Mitoplasts from BNF-treated mice also yielded relatively high erythromycin N-demethylase activity of (720 pmol/min/mg of protein). These results are in line with our previous observations of the activity patterns of mitoplasts from BNF-induced rat liver.

Table 2 shows the activities of partially purified mouse MT CYP1A1 reconstituted in lipid vesicles in the presence of the mitochondrial (FDX1 plus FDXR) versus microsomal (POR) electron-donor system. Similar to that observed with the rat enzyme, the mouse MT CYP1A1, in an FDX1 plus FDXR-supported system, showed relatively high activity for both ethoxyresorufin O-demethylase (137 pmol/min/nmol of P450) and erythromycin N-demethylation (1.4 nmol/min/nmol of P450); also, both activities were inhibited <85% by CYP-specific inhibitors, CO and SKF525A. The activity was also dependent on added NADPH as the electron donor, because omission of this cofactor vastly lowered the activity. Furthermore, as previously reported for the rat enzyme, the mouse MT CYP1A1 exhibited a significantly higher activity with both substrates in the presence of the FDX1 plus FDXR electron-donor system, as compared with POR, suggesting a preference for the former system. In addition, the FDX1 plus FDXR-supported ethoxyresorufin O-demethylase and erythromycin N-demethylation activities were inhibited by anti-rat CYP1A1 antibody, as well as an antibody to FDX suggesting the specificity of enzyme assays.

As shown in Fig. 8B partially purified mouse liver MT-CYP1A1 showed high activity, in the range of 2.5-4.0 nmol/nmol of CYP, for the metabolism of a spectrum of neuroactive drugs, including aminopyrine, benzphetamine, diazepam, haloperidol, imipramine, lidocaine, and morphine, in an FDX1 plus FDXR-supported system. As observed with other substrates (Table 2), the
POR electron-donor system yielded markedly lower activity, in the range of 0.8 – 1.5 nmol/nmol of CYP, with these substrates. Additionally, the CYP1A1 antibody inhibited the activity with all substrates by >90% (data not shown); this confirms that the activity is indeed supported by mouse MT-CYP1A1. CO and SKF-525A also inhibited the metabolism of all substrates (not shown). In addition to showing altered substrate specificity of the MT-CYP1A1, as compared with that of MC-CYP1A1, these results support the possibility that MT CYP1A1, in the rodent and other species including human, may play an important role in the detoxication of various psychotropic drugs and pain suppressants.

DISCUSSION

Bimodal targeting of some of the ER (CYP1A1, CYP2B1, and CYP2E1) and plasma membrane proteins to mitochondria by virtue of their chimeric-targeting signals requires the activation of cryptic MT-targeting signals. The BNF-inducible rat CYP1A1 is targeted to the MT compartment of liver, brain, and cultured glioma C6 cells (9, 16, 25, 26), and the MT-CYP1A1 is composed of the NH2-terminal truncated form (9, 25). In extension of these studies, we now show that the extent of CYP1A1 targeting to the ER and MT is a regulated process. Our results suggest that SRP binding to the nascent CYP1A1 represents an important point of control of its bimodal targeting and that PKC-mediated phosphorylation at Thr-35 of the protein represents a key factor affecting the affinity of the nascent chains for SRP binding. Results of this study also show that the bimodal targeting mechanism and the activation of cryptic signal by proteolytic processing by a cytosolic endoprotease are conserved in different metazoan species.

Our previous study with the rat CYP1A1 showed that nearly 30% of the nascent chains translated in membrane-free form probably represented the candidate pool for MT transport (9). Additionally, with prolonged BNF treatment (up to 10 days) of rats or cultured C6 glioma cells, we observed preferential accumulation of MT-CYP1A1 and correspondingly reduced MC-CYP1A1 (25). These results suggested the modulatory nature of CYP1A1 targeting to these two membrane compartments, although the precise molecular basis of this regulation remained unclear. In the present study we used a mutational strategy for addressing this question. Substitutions of Pro at the second and Tyr at the fifth positions with more hydrophobic Leu vastly increased the affinity of the protein for SRP binding as seen from higher extent of SRP-mediated, and or, ER membrane-mediated translation inhibition, as well as the ER membrane association. In contrast, substitution of Leu at the 7th and 17th positions with Asn yielded a protein with dramatically reduced affinity for SRP binding and reduced membrane association in an in vitro system. Predictably, HE-SRPMut1 and HE-SRPMut2 proteins with higher affinities for SRP were more preferentially targeted to the ER and correspondingly at lower levels to MT in COS cells. The LE-SRPMut3 protein exhibiting a lower SRP-binding affinity, on the other hand, showed an opposite pattern in that it was more abundantly targeted to MT and less abundantly to the ER. These results provide direct evidence that SRP-binding affinity is an important regulatory step in the bimodal targeting. Results also shed light on the evolutionary aspects of the bimodal targeting mechanism.

Unpublished results in our laboratory showed that chronic BNF treatment for <76 h induced PKC activity in C6 glioma cells that had some direct or indirect role in increased CYP1A1 targeting to the MT compartment. These results prompted us to investigate the possible role of PKC-mediated phosphorylation of a single target site at Thr-35, albeit with partial consensus, on the affinity of nascent chains for SRP binding and bimodal targeting. Interestingly, addition of PKC to the translation mix vastly reduced the affinity of HE-SRPMut2 protein for SRP, leading to reduced membrane association of the nascent chain. The modulatory role of PKC-mediated phosphorylation was supported by three different lines of experiments: First, PMA, an inducer of intracellular PKC activity, caused increased MT transport of HE-SRP Mut2 protein, which is otherwise transported to the ER in a preferential manner. Second, both partially purified and total MT-CYP1A1 contained higher levels of Thr phosphorylation, whereas the MC-CYP1A1 contained very low or negligible phosphorylation. Third, phosphomimetic mutant protein T35D not only showed reduced affinity for SRP binding but also was more preferentially targeted to MT in COS cell transfection. Although results are not shown, PKA exerted no significant effect on the SRP-binding affinity of CYP1A1. Additionally, we were unable to detect significant Ser phosphate residues on MT- or MC-CYP1A1. Based on the computer analysis for consensus PKC sites and biochemical analysis of mutant proteins in Fig. 7, we propose that phosphorylation at the single PKC site (Thr-35) causes reduced affinity of the ER-targeting signal for SRP binding. Our in vitro import and cell

### TABLE 2
Reconstitution of mouse mtCYP1A1 enzyme activity with various electron donor moieties and the effects of inhibitors

| Electron donor moieties | Inhibitor | Activities | EROD (pmol/min/nmol CYP) | ERND (pmol/min/nmol CYP) |
|-------------------------|-----------|------------|--------------------------|--------------------------|
| FDX1 plus FDXR plus NADPH | None      | 136.8 ± 5.22 | 1359 ± 162.00 |
| FDX1 plus FDXR minus NADPH | None      | 2.3 ± 0.05   | 8.5 ± 1.56   |
| FDX1 plus FDXR plus NADPH | CO        | 11 ± 0.01    | 19.9 ± 1.40  |
| FDX1 plus FDXR plus NADPH | SKF525-A  | 5.05 ± 0.04  | 49.01 ± 5.60 |
| FDX1 plus FDXR plus NADPH | Anti-rat P450MT2 | 2.05 ± 0.02 | 39.02 ± 5.30 |
| FDX1 plus FDXR plus NADPH | Anti-FDX1 antibody | 8.08 ± 0.03 | 29.51 ± 2.0 |
| POR plus NADPH | None      | 670 ± 6.50   | 502.21 ± 40.05 |
| POR plus NADPH | Anti-POR  | 60.06 ± 0.04 | 55 ± 8.40   |

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transfection studies also suggest that PKC may have additional modulatory effects on mitochondrial import of CYP1A1. It is likely that phosphorylation affects the binding of CYP1A1 to cytosolic chaperones HSP70, and or HSP90, which is an important step in the pathway for mitochondrial protein transport. Alternatively, it is possible that PKC modulates the activity or function of mitochondrial translocation complexes.

The positively charged residues from the canonical MT-targeting signals play an important role in binding to peripheral TOM receptors, specifically TOM20 (1, 2). In keeping with this, mutational analysis and chemical cross-linking studies suggested that the positively charged residues from the +29 to +39 sequence regions of CYP1A1, -2B1, and -2E1 (9–11) represent important components of cryptic MT-targeting domains. In the case of both rat and mouse CYP1A1, because Arg-34 is part of the PKC consensus site, it is likely that mutation at this site may affect phosphorylation, and or, endoprotease processing, which may be reasons for the reduced MT targeting of the protein (Fig. 7E). However, mutation at Lys-39, which is outside the PKC target and protease-processing site, also affected MT protein transport (Figs. 5B and 6B) confirming its possible role as part of MT-targeting signal.

All three ER-targeting domain mutants (HE-SRP Mut1, HE-SRP Mut2, and LE-SRP Mut3) as well as the WT CYP1A1 are translocated to isolated mitochondria at the same low basal level, probably because of a lack of endoproteolytic activity in the reticulocyte lysate system. Addition of partially purified cytosolic protease to the translation mix increased the extent of in vitro transport of all these proteins suggesting that the endoprotease-processing step is important for the efficient MT targeting of CYP1A1. It remains to be determined if this step is also an important regulatory step that determines the extent of MT targeting. It is becoming increasingly apparent that mitochondrial proteolysis is growing and includes NADPH-cytochrome b5 reductase (27), transforming growth factor-β (28), mitochondrial SLIT3, cytoplasmic interleukin 15 (IL15), cathepsin L, glutathione S-transferases (14, 29–31), plasma membrane-targeted amylloid precursor protein (32), ER-targeted CYP proteins (9–11), and the prion protein (33). In the case of various CYP and glutathione S-transferase proteins, detailed analysis of targeting mechanisms shows a need for the activation of chimeric MT-targeting signals by physiological factors (9–11, 14). These results on bimodal targeting are also supported by recent proteomic data showing a large number of proteins identified as part of mitochondrial proteomes are also localized to extramitochondrial compartments, including the cytosol, peroxisomes, and ER membrane (5–8). It is therefore highly likely that the bimodal targeting, driven by chimeric NH2-terminal signals as shown for various CYP and glutathione S-transferase enzymes, represents a precisely evolved physiological process with functional significance. We also propose that this mechanism is important in the mitochondrial targeting of a number of proteins lacking canonical mitochondrial signals.

The mouse MT-CYP1A1 protein was readily extractable with alkaline Na2CO3 buffer, suggesting an extrinsic membrane location, as opposed to the transmembrane orientation of the MC-CYP1A1 (Fig. 4B). Furthermore, MT-CYP1A1 showed a preference for mitochondrial soluble electron transport protein FDX1 plus FDXR suggesting subtle structural difference from the MC-CYP1A1. MC-CYP1A1 is responsible for the metabolic activation and detoxication of a variety of xenobiotics, including some polycyclic aromatic hydrocarbons (34, 35). Erythromycin N-demethylase activity is predominantly catalyzed by microsomal CYP3A enzymes (36, 37); interestingly, both mitoplasts from BNF-treated mouse liver and purified MT-CYP1A1 show erythromycin N-demethylase activity, which is not a marker for parent ER-targeted CYP1A1. Purified MT-CYP1A1 showed nearly 2-fold higher activity in an FDX1 plus FDXR-supported system (Table 2 and Fig. 8), as compared with the activity in a POR-supported system, suggesting a preference for the former electron donor system. The erythromycin N-demethylase and ethoxyresorufin O-deethylation activities of the purified enzyme represent a true monooxygenase activity, based on inhibition by CO, SKF525-A, CYP1A1 antibody, and dependence on NADPH, as well as the requirements for the electron-transfer proteins (Table 2). Ethoxy- and methoxyresorufins are markers for the CYP1 family (38, 39). Ethoxyresorufin O-demethylase activity is commonly used to provide relative estimates of functional CYP1A1 protein content (38). These results confirm and extend our previous observations with rat liver P450MT2 (13, 40, 26) that NH2-terminal truncation and the new mitochondrial environment affect both substrate-binding specificity and electron-transfer-protein binding capacity of the enzyme possibly through altered conformation. Indeed, CD spectral analysis showed that MT-CYP1A1 and -2B1 showed markedly reduced helical content and increased β-sheet structure indicating a more unfolded state of the protein inside the mitochondrial compartment (9–11).

An intriguing finding of this study is that MT-targeted mouse liver CYP1A1 exhibited N-demethylase activity for a number of psychotropic drugs. Imipramine is a widely used tricyclic antidepressant. This monoamineoxidase inhibitor is also known to affect serotonin uptake (41, 42). Diazepam is an anticonvulsant drug, which facilitates γ-aminobutyric acid (GABA) uptake, acting through benzodiazepine receptors (42); these compounds are known to be metabolized by microsomal CYP2C and CYP3A enzymes (42). Microsomal CYP2B enzymes are implicated in the metabolism of morphine and lidocaine (43). Aminopyrine is a pain killer and antipyretic and is used in the treatment of acute leukemia; in humans, it is mainly metabolized by microsomal CYP2C19, CYP2C8, and CYP2D6 (44). Benzphetamine is an anorexiant used in the treatment of narcolepsy, obesity, and depression; this is metabolized principally by microsomal CYP4F enzymes (45). Haloperidol is an antipsychotic and neuroleptic drug, metabolized mainly by CYP2D6 (46). It is surprising that MT-CYP1A1, but not MC-CYP1A1, shows high N-demethylase activities for all eight of these drugs. Based on the relative activities and mitochondrial CYP content, we
estimate that, after the chronic 10-day induction by BNF, the mitochondrial activity for the metabolism of these psychotropic substrates represents ~85% of total tissue activity. Results presented in this study therefore suggest an important physiological function for mammalian MT-CYP1A1 in modulating the pharmacokinetic and pharmacodynamics of a family of antidepressants, anticonvulsants, opiates, and other psychotropic drugs.

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