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Human Liver Mitochondrial Cytochrome P450 2D6: Individual Variations and Implications in Drug Metabolism

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Summary

Constitutively expressed human cytochrome P450 2D6 (CYP2D6) is responsible for the metabolism of approximately 25% of drugs in common clinical use. It is widely accepted that CYP2D6 is localized in the endoplasmic reticulum of cells; however, we have identified this enzyme in the mitochondria of human liver samples and found that extensive inter-individual variability exists in the level of the mitochondrial enzyme. Metabolic assays using 7-methoxy-4-aminomethylcoumarin as a substrate show that the human liver mitochondrial enzyme is capable of oxidizing this substrate and that the catalytic activity is supported by mitochondrial electron transfer proteins. Here we show that CYP2D6 contains an N-terminal chimeric signal that mediates its bimodal targeting to the endoplasmic reticulum (ER) and mitochondria. *In vitro* mitochondrial import studies using both N-terminal deletions and point mutations suggest that the mitochondrial targeting signal is localized between residues 23-33 and that the positively charged residues at positions 24, 25, 26, 28, and 32 are required for mitochondrial targeting. The importance of the positively charged residues was confirmed by transient transfection of a CYP2D6 mitochondrial targeting signal mutant in COS-7 cells. Both the mitochondria and the microsomes from a CYP2D6 stable expression cell line contain the enzyme and both fractions exhibit bufuralol 1′-hydroxylation activity, which is completely inhibited by CYP2D6 inhibitory antibody. Overall these results suggest that the targeting of CYP2D6 to mitochondria could be an important physiological process that has significance in xenobiotic metabolism.

Keywords

human CYP2D6; mitochondrial targeting; bimodal targeting signal; liver mitochondrial CYP2D6 content; bufuralol 1′-hydroxylase

Cytochrome P450 2D6 (CYP2D6) is a constitutively expressed enzyme in hepatic and brain tissues and accounts for the metabolism of 20-25% of all drugs in clinical use [1]. This enzyme is of particular interest because it shows a high degree of interindividual variability due to extensive genetic polymorphism that influences both its expression and function. The substrates of CYP2D6 include a wide spectrum of antiarrhythmics, antihypertensives,

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antidepressants, antipsychotics, analgesics, and β-adrenergic blocking agents, in addition to some physiological substrates [2;3]. Since its discovery as a polymorphic enzyme, at least 112 allelic variants have been described (http://www.imm.ki.se/CYPalleles/cyp2d6.htm) and can be categorized into four general phenotypes: poor metabolizers (PMs), who lack the functional enzyme; intermediate metabolizers (IMs), who are heterozygous for one deficient allele or have two alleles causing reduced activity; extensive metabolizers (EMs), who have two normal alleles; and ultrarapid metabolizers (UMs), who have multiple gene copies that are inherited in a dominant manner [4].

Many pharmacogenetic studies suggest that polymorphisms in CYP2D6 can significantly affect the activity of the enzyme and, therefore, serve as an important guideline for determining the dose of antidepressant drugs and preventing drug-induced toxicity [2-6]. A large majority of studies on the biochemical and genetic properties, pharmacological and toxicological roles, and clinical relevance of CYP2D6 are based on the steady-state levels and activity of the enzyme associated with the microsomal fraction of liver and brain tissues [7;8].

Recent studies from our laboratory have shown that a number of xenobiotic inducible CYPs including CYP1A1, 2B1, and 2E1 are bimodally targeted to both the microsomal and mitochondrial fractions of hepatic, brain, and lung tissues and also in cultured cells induced to express these proteins [9-13]. These studies gave rise to the concept of a new family of N-terminal targeting signals, termed “chimeric signals,” which facilitate the bimodal targeting of the protein. The chimeric signals consist of a cryptic mitochondrial targeting signal immediately adjacent to the ER targeting and transmembrane domains of the apoproteins. Our results also showed that the cryptic mitochondrial targeting signals require activation either by endoproteolytic processing by a cytosolic protease, as in the case of CYP1A1 [9;14], or protein kinase A (PKA) mediated protein phosphorylation at Ser residues located approximately 100 amino acids downstream of the cryptic mitochondrial targeting signal, as in the case of CYP2B1 and 2E1 [11;13]. The mitochondrial targeted CYPs physically and functionally associate with adrenodoxin (Adx) and adrenodoxin reductase (AdxR), the components of mitochondrial matrix electron transport system, and efficiently catalyze drug metabolism [10;15;16]. Some of the mitochondrial targeted forms exhibit altered substrate specificity when compared to the microsomal enzymes. P450 MT2 (N-terminal truncated CYP1A1) has been shown to catalyze the N-demethylation of erythromycin, lidocaine, morphine, and various other neuroactive drugs [17]. Interestingly, these reactions are not catalyzed by the microsome-associated intact CYP1A1 in reactions supported by microsomal NADPH-cytochrome P450 reductase (CYPR) [10;18].

In this study we show that CYP2D6 is present in the mitochondria of human liver samples and that mitochondria isolated from the liver samples are active in the metabolism of 7-methoxy-4-(aminomethyl)coumarin (MAMC), a substrate for microsomal CYP2D6. We also demonstrate that CYP2D6 is targeted to the mitochondrial compartment in isolated mitochondria and in COS-7 cells transiently or stably expressing the human protein. Mutation of the putative mitochondrial targeting signal eliminates this targeting mechanism *in vitro*. Mitochondria isolated from the stable expression cell line are active in the 1′-hydroxylation of bufuralol, a probe substrate for the microsomal CYP2D6. This activity is inhibited by CYP2D6 inhibitory antibody. These results suggest that the mitochondrial localization of CYP2D6 may be an important physiological process that may have a role in drug metabolism and drug-induced toxicity.

Results

Localization of CYP2D6 in Human Liver Mitochondria

Mitoplast and microsomal isolates from 20 human liver samples were analyzed by immunoblot analysis using polyclonal antibody to human CYP2D6. The blots were also co-developed with antibody to a mitochondrial specific marker protein, mtTFA, and a microsome specific marker protein, CYPR. Representative immunoblot profiles for eight such samples are presented in Fig. 1A. The microsomal isolates from six human liver samples (HL132, 134, 136, 137, 139, and 140) contained relatively high CYP2D6 content while two samples (HL131 and 141) demonstrated moderate levels of CYP2D6, as indicated by the intensity of the 50 kDa antibody reactive band (Fig. 1A). The mitoplasts, on the other hand, showed a marked variability in CYP2D6 contents ranging from a relatively high level in HL134 and 137 to a moderate level in HL136, low levels in HL132, 139, and 140 and almost undetectable levels in HL131 and 141 (Fig. 1A). Densitometry measurements were used to calculate the subcellular distribution of the CYP2D6 protein in the microsomal and mitochondrial fractions (Fig. 1A). HL134 had almost equal levels of CYP2D6 in mitochondria and microsomes, whereas nearly all (97 to 99%) of the CYP2D6 in HL131 and 141 was associated with the microsome (Fig. 1A). HL137 and HL136 had 34% and 20% of the protein, respectively, in the mitochondrial fraction (Fig. 1A). The immunoblots also showed that the 78 kDa CYPR protein was detectable in the microsomal isolates but not significantly in the mitochondrial membrane isolates. Similarly, the 29 kDa mtTFA protein was seen mostly in the mitochondrial isolates but sparingly in the microsomal isolates. As in our previous studies [9;10;17] mitochondrial isolates were routinely analyzed for microsomal contamination by assaying for rotenone insensitive NADPH-cytochrome *c* reductase. By using this marker assay we found that the mitochondrial isolates contained < 1% microsomal contamination (results not presented).

The immunoblot (Fig 1B) shows the results of a control experiment, which assessed the relative resistance or sensitivity of human liver microsome- and mitochondria-associated CYP2D6 to limited digestion with trypsin. Proteins localized in the mitochondrial matrix or intermembrane space are expected to be resistant to limited trypsin treatment under these conditions, while those adventitiously adhering to the outer mitochondrial membrane and microsomal fragments should be sensitive. In all three microsomal isolates tested (HL126, 130, and 141), the antibody-reactive CYP2D6 was sensitive to trypsin treatment. In contrast, mitochondria-associated CYP2D6 in samples HL126 and 130 was resistant to trypsin treatment. This result suggests that CYP2D6 is localized within the mitochondrial membrane compartment. In sample HL141, which contained no significant mitochondrial CYP2D6, the trypsin-treated mitochondria also did not show detectable antibody reactive protein.

Metabolic Activity of Mitochondrial CYP2D6

The ability of mitochondrial CYP2D6 to metabolize substrates was investigated using MAMC, a known substrate of microsomal CYP2D6 (Figs. 2A and 2B). Mitoplasts from five randomly selected human liver samples were tested for their ability to oxidize MAMC. Because of the known ability of other CYPs, especially CYP1A2, to oxidize this compound, various inhibitors were used to assess the activity mediated by mitochondrial CYP2D6 (Fig. 2A). All five samples tested yielded varying activity ranging from moderate (samples HL139 and HL140) to high (HL129, HL111 and HL130) activity for MAMC O-demethylation. The activities of both HL129 and HL111 were inhibited by approximately 53% and 50%, respectively, by addition of 10 μM quinidine, a CYP2D6 specific inhibitor.[2] When these mitoplasts were pre-incubated with antibody to Adx, an essential protein in the mitochondrial electron transfer system, the activity was reduced by 83% and ~ 100%,

respectively. The activities of HL139 and 140 liver mitochondria were reduced by 94% and 84% respectively following incubation with Adx antibody. A CYP2D6 specific inhibitory antibody was also used to further investigate the role of CYP2D6 in this activity. Samples HL139 and 140 both showed a considerable reduction in metabolic activity following pre-incubation with CYP2D6 antibody. The activity was reduced by 75% and 94%, respectively. Sample HL127 had a moderately high activity, which was reduced by approximately 52% following addition of CYP2D6 antibody. MAMC is known to be oxidized by both CYP2D6 and CYP1A2 [19-21] and an inhibitory antibody to CYP1A2 inhibited the activity of HL127 liver mitochondria by about 52% by CYP1A2 antibody. The specificity of the antibody inhibition was tested by incubating HL130 mitochondria with either nonspecific mouse IgG or specific CYP2D6 inhibitory antibody. The nonspecific IgG had virtually no effect on the MAMC metabolizing activity, whereas the CYP2D6 inhibitory antibody reduced the activity by approximately 62%. Finally, a general P450 inhibitor, SKF-525A, reduced the activity by 94% and 100%, respectively, in mitochondria from HL129 and 111 livers Fig. 2A). The remaining human liver sample mitoplasts were capable of oxidizing MAMC; however, there were significant inter-individual differences in the level of activity (Fig. 2B).

Characterization of Mitochondrial Targeting Signal of CYP2D6

The N-terminal signal sequence and the phosphorylation domains of CYP2B1 and 2E1 were compared with the amino acid sequence of human CYP2D6 (Fig. 3A). The N-terminal amino acid sequence of CYP2D6 bears resemblance to the chimeric signal sequences identified in CYP2B1 and CYP2E1. The sequence contains a 22-amino acid region with a hydrophobic helical structure that is thought to act as both an ER targeting and membrane anchor domain [22;23]. There is an immediately adjacent putative mitochondrial targeting signal composed of a stretch of positively charged residues including a His at position 24 and Arg residues at positions 25, 26, 28, and 32, followed by the Pro-rich domain beginning at position 34 and a potential PKA target phosphorylation site at Ser135, similar to those reported for CYP2B1 and CYP2E1. The putative signal domain of CYP2D6 contains five positively charged residues, compared to two positively charged residues in CYP2E1 and four in CYP2B1. CYP2D6 also has a putative PKC phosphorylation site adjacent to the PKA target site.

In order to map the mitochondrial targeting signal domain of CYP2D6, a series of constructs were generated with N-terminal truncations and point mutations in the putative mitochondrial targeting signal and used for *in vitro* import into isolated mitochondria. Intact wild-type CYP2D6 (WT 2D6) was imported at a moderate level into mitochondria (Figs. 3B, 3C). Deletion of two N-terminal domains, ER targeting domain and mitochondrial targeting signal (+34/2D6), or all three N-terminal domains -- ER targeting signal, mitochondrial targeting signal, and the Pro-rich domain (+40/2D6) -- reduced import by approximately 95% as compared to the wild-type protein (Fig. 3B). Furthermore, point mutations in the putative mitochondrial targeting domain also significantly disrupted the mitochondrial import of CYP2D6 (Fig. 3C). Substitution of Arg at positions 25, 26, and 28 with neutral Asn (ArgM 2D6) in the putative mitochondrial targeting signal reduced the level of mitochondrial import by approximately 50% as compared to the wild-type protein. Additionally, mutation of all five positively charged residues in the putative mitochondrial targeting signal to Ala residues (MitoM 2D6) reduced the mitochondrial import of CYP2D6 by approximately 90% as compared to the wild-type protein (Fig. 3C).

[1]A concentration of 1 μM quinidine is generally sufficient to inhibit CYP2D6 in a system using purified microsomes; however, the sensitivity of CYP2D6 to quinidine within the mitochondrial compartment is unknown.

Su-9 DHFR was used as a positive control for the *in vitro* import experiments (Fig. 3D). In this construct, the pre-sequence of subunit 9 of *Neurospora crassa* F_0F_1-ATPase has been fused to DHFR. This is a classic mitochondrial targeting signal that gets cleaved following entry into mitochondria [24]. In this *in vitro* system, only the cleaved protein (27 kDa) is present following import and trypsin treatment (Fig. 3D). DHFR, a cytosolic protein, was used as a negative control for these experiments. There was no detectable entry of this protein into mitochondria (Fig. 3D). Additional controls were performed to determine whether the import of WT CYP2D6 into mitochondria is energy dependent. Mitochondria were incubated with carbonyl cyanide 3-chlorophenyl hydrazone (CCCP), which disrupts the mitochondrial membrane potential, and oligomycin, which disrupts the mitochondrial ATP pool, prior to import. The level of import of WT CYP2D6 into mitochondria was significantly reduced by incubation with both CCCP and oligomycin (Fig. 3E). The relatively lower level of binding and import of WT CYP2D6 in Figs. 3C and 3E as compared to Fig. 3B probably reflects natural variation in mitochondrial activity between different rat livers.

Mitochondrial Targeting of CYP2D6 in Transiently Transfected COS-7 Cells

Mitochondrial and microsomal fractions isolated from cells transiently transfected with WT CYP2D6 demonstrate almost equal levels of CYP2D6 in mitochondria and microsomes (Fig. 4A). In contrast, when cells were transfected with ArgM CYP2D6, the level of mutant CYP2D6 in microsomes was two times higher than that in mitochondria (Fig. 4A). Limited trypsin digestion eliminated both WT and ArgM CYP2D6 from the microsomal fraction, but the mitochondria associated CYP2D6 was resistant to trypsin treatment, suggesting that the protein is localized inside the mitochondrial membranes (Fig. 4B). As expected, the level of mitochondrial outer membrane anchored protein TOM20 was markedly reduced by trypsin digestion (Fig. 4B). COS cells had a low level of endogenous CYP2D6 in the microsomal fraction that was sensitive to trypsin digestion, while there was no detectable CYP2D6 in mitochondria (Figs. 4A, 4B). Role of PKA-mediated phosphorylation in mitochondrial targeting of CYP2D6. Our previous studies have shown that mitochondrial targeting of CYP2E1 and 2B1 was facilitated by PKA-mediated phosphorylation at Ser 129 and Ser128 of the protein, respectively [11;13]. Analysis of CYP2D6 using NetPhosK 1.0 [25], which predicts phosphorylation sites, revealed the presence of a high consensus (score 0.85) PKA site (RRFSV) at Ser135 in addition to two other lower consensus sites at Ser 148 and Ser217. The Ser135 site was positionally similar to the Ser128 and Ser129 PKA sites of CYP2B1 and CYP2E1, which were shown to be functionally important for mitochondrial import [11;13]. For this reason, we tested the importance of the Ser135 PKA site for the mitochondrial import of CYP2D6 by mutagenesis at this site (see Fig 5A) and *in vitro* import of the protein. *In vitro* import of wild-type CYP2D6 increases by approximately 23% when the nascent protein is pre-incubated with PKA and ATP (Fig. 5B). Interestingly, the PKA phosphorylation site mutant (PKAM2D6) was imported at a much lower level than WT protein under basal conditions (Fig. 5B). Pretreatment with PKA and ATP increased the import of the mutant protein; however, the overall level of increase was nearly half that of the wild-type protein subjected to PKA treatment (Fig. 5B). These results suggest that PKA phosphorylation contributes to the mitochondrial transport of human CYP2D6. The precise reason for the PKA-mediated increase in the import of mutant PKAM2D6 remains unclear. It is, however, likely that other putative PKA sites (Ser148 and Ser217) also contribute to mitochondrial import and mutation in the S135 site only partly affects protein import.

Mitochondrial Localization of Human CYP2D6 in a Stable Expression Cell Line

To assess the role of mitochondrial CYP2D6 in drug metabolism we generated cell lines expressing human CYP2D6 under the regulation of a doxycycline (DOX) inducible promoter. Mitochondria and microsomes isolated from DOX induced cells were analyzed

using immunoblot analysis (Fig. 6). CYP2D6 was present in both the mitochondria and the microsomes following induction with DOX, although the level in mitochondria is significantly lower than that in the microsomes. There was no expression of CYP2D6 in the absence of DOX induction. The immunoblots were co-developed with CYPR and TOM20 antibodies demonstrating that there is minimal cross-contamination between the two subcellular fractions. Additionally, analysis of CO-difference spectra indicated that there is 172 pmol P450/mg protein in microsomes and 146 pmol P450/mg protein in mitochondria in this cell line.

Bufuralol 1′-Hydroxylation Activity of Mitochondrial CYP2D6

Mitochondria and microsomes isolated from the stable cell line were assayed for their bufuralol 1′-hydroxylation activity (Fig. 7). Bufuralol is a classic probe substrate for CYP2D6 activity [26;27]. Mitochondria and microsomes were both active in the 1′-hydroxylation of bufuralol. Mitochondrial CYP2D6 oxidized bufuralol at a rate of 30.2 ± 0.53 pmol/min/nmol P450, while microsomal rate was 27.7 ± 0.73 pmol/min/nmol P450. Pre-incubation of both mitochondria and microsomes with CYP2D6 inhibitory antibody almost completely eliminated the oxidation of bufuralol (Fig. 7). These results confirm that mitochondria-localized CYP2D6 is active in bufuralol metabolism.

Discussion

We reported previously that a number of CYPs including CYP1A1, 2B1, and 2E1 are bimodally targeted to mitochondria in addition to their well-established ER destination. In the case of CYP1A1, endoprotease-mediated processing at the N-terminus of the nascent protein activates the mitochondrial targeting signal [9;14]. In contrast, intact CYP2B1 and 2E1 are targeted to mitochondria. In the present study we investigated the mitochondrial targeting of constitutively expressed CYP2D6 and found that it is also targeted to mitochondria. We not only show the presence of CYP2D6 in human liver mitochondria but also that a marked interindividual variation exists in the mitochondrial content of this protein. Furthermore, we have mapped the mitochondrial targeting signal domain of human CYP2D6 and demonstrate metabolic activity of the mitochondrial enzyme. Immunoblot analysis identified CYP2D6 in both the mitochondria and microsomes of human liver samples and indicated that the level of the mitochondrial enzyme varies significantly among individuals (Fig 1A). The mitochondrial enzyme was relatively resistant to trypsin digestion, indicating localization inside the mitochondrial membranes, as opposed to the high sensitivity of microsomal CYP2D6 (Fig. 1B).

Many CYP2D6 substrates contain a basic nitrogen atom, an aromatic moiety, and an oxidation site separated by 5-7Å from the basic nitrogen atom [27-31], with some exceptions [31]. The highly hydrophobic nature of these substrates permits their entry into mitochondria and metabolism by mitochondria targeted CYP2D6. Our results suggest that the mitochondrial enzyme is active in the oxidation of MAMC and that there is significant inter-individual variability in this activity (Figs. 2A, 2B). The catalytic activity is supported by the mitochondrial electron transfer protein Adx as tested by antibody inhibition (Fig. 2A). In most cases, the activity was predominantly mediated by CYP2D6 because there was significant inhibition with either quinidine (10 μM) or CYP2D6-specific antibody. In some samples, e.g. HL127, only part of the activity was inhibited by CYP2D6 antibody, while CYP1A2 antibody inhibited the remaining activity (Fig. 2A) suggesting contribution by both enzymes in human liver mitochondria. Limited tissue availability has precluded a more in-depth analysis of the contribution of CYP1A2.

In all metabolic assays, Adx and Adr purified from bovine adrenal glands were added to the reaction mixture. This is mainly to compensate for any loss of Adx during mitochondrial

isolation and digitonin treatment. Previous studies from our lab have shown that ferredoxin (Fdx), a 12 kDa soluble protein, and other small soluble proteins are lost in significant amounts during the preparation of mitochondria or mitoplasts from liver tissue [33]. The mitochondrial content of a larger soluble protein such as ferredoxin reductase (Fdr, 53 kDa) was also appreciably decreased in the mitoplast preparations [33]. While CYP2D6 is similar in size to Fdr, it is less likely to be released during mitochondrial isolation because of its predicted association with the mitochondrial inner membrane. Previous studies from our lab have shown that mitochondrial CYP1A1, CYP2B1 and CYP2E1 are associated with the inner membrane in a membrane extrinsic manner and require high salt or detergent treatment for releasing these proteins from the inner membrane [34-36].

In vitro import studies were used to investigate the putative mitochondrial targeting signal domain of CYP2D6. Our results suggest that CYP2D6 contains a chimeric signal at its N-terminus that is analogous to that identified in CYP2B1 and CYP2E1 [11;13]. *In vitro* import studies using N-terminal deletions suggest that the mitochondrial targeting signal is localized between residues 23-33 and that the positively charged residues are required for mitochondrial targeting (Fig. 3B). This was further confirmed by results showing that point mutations at the positively charged residues within the putative signal sequence (residues 23 to 33) markedly reduced import (Fig. 3C).

The localization of the mitochondrial targeting signal and the importance of the positively charged residues were further confirmed by transient transfection of WT CYP2D6 and ArgM CYP2D6, a construct in which three positively charged Arg residues are mutated to neutral Asn residues. WT CYP2D6 targets to mitochondria at a significantly higher level than ArgM CYP2D6 and is resistant to trypsin treatment (Figs. 4A, 4B). This suggests that the positively charged residues in the mitochondrial targeting signal are required for targeting of CYP2D6 to mitochondria. The mitochondrial protein appears to have the same mobility as the microsomal protein, with an apparent molecular weight of 50 kDa, suggesting that CYP2D6 is targeted to mitochondria as a full-length protein (Fig 4A). This finding is further substantiated by the *in vitro* import experiments in which the protein that was imported into mitochondria appears to be the same size as the translation product (Figs 3B, 3C).

Generation of a tetracycline-inducible stable cell line expressing WT CYP2D6 permitted further investigation of the mitochondrial targeting. CYP2D6 targets to the mitochondria in this stable cell line (Fig. 6) and the mitochondrial enzyme is active in the 1′-hydroxylation of bufuralol, a probe substrate of microsomal CYP2D6 (Fig. 7). This activity is consistent with that reported earlier for human lymphoblastoid microsomes expressing human CYP2D6 [38]. The bufuralol 1′-hydroxylation activity was clearly mediated entirely by CYP2D6 because pre-incubation with CYP2D6 inhibitory antibody almost completely eliminated activity for both mitochondria and microsomes.

The cAMP-regulated targeting of various cytochrome P450 enzymes to the mitochondria could have evolved as a mechanism to protect the mitochondria against chemical or oxidative damage. Thus, PKA mediated phosphorylation at Ser135 and possibly at other PKA sites (Ser148 and Ser217) may have implications in the observed variations in mitochondrial contents of CYP2D6 in human liver samples. Targeting of CYP2D6 to mitochondria could certainly be protective since the enzyme is capable of detoxifying and eliminating many hydrophobic substrates that can enter mitochondria. However, the spectrum of drugs and chemicals to which the average individual is exposed has increased exponentially over time, and so it is also possible that CYP2D6 could convert certain substrates into reactive species within the mitochondria, thereby inducing toxicity.

The exact reason for the high level of inter-individual variability in the level of the mitochondrial enzyme remains unclear; however, given the highly polymorphic nature of CYP2D6, it is tempting to speculate that the presence of mutations in the targeting signals and the possible involvement of other physiological factors, e.g. phosphorylation, may determine the level of mitochondrial CYP2D6. A majority of studies on the biochemical and genetic properties, pharmacological and toxicological roles, and clinical relevance of CYP2D6 have been based on the enzyme associated with the microsomal fraction of the liver [7;8]. The present study suggests that mitochondrial CYP2D6 may also contribute to drug metabolism and detoxification in human liver.

Materials and Methods

Isolation of Mitochondria and Microsomes from Frozen Human Liver Samples

Liver samples were obtained through Tennessee Donor Services, Nashville, TN and used in accord with Vanderbilt Institutional Board guidelines. Mitochondria and microsomes were isolated from human liver samples using a modification of a method described previously [39;40]. Briefly, livers were washed in ice cold saline and homogenized in 10 volumes of sucrose-mannitol buffer (20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (pH 7.5) containing 70 mM sucrose, 220 mM mannitol, 2 mM EDTA, and 0.5 mg/ml bovine serum albumin (BSA)). Mitochondrial and microsomal fractions were isolated from the homogenates using a differential centrifugation method [9]. Mitochondria were pelleted at 8,000 g for 15 min. Crude mitochondrial fractions were washed twice in the above buffer and layered over 0.8 M sucrose. The fractions were centrifuged at 14,000 g for 30 min, and the mitochondrial pellet was washed twice in sucrose-mannitol buffer. Mitoplasts were prepared by suspending the crude mitochondrial pellet in sucrose-mannitol buffer at a concentration of 50 mg/ml and treating with digitonin (75 μg/mg protein, Calbiochem, San Diego, CA) at 4°C. The resulting mitoplast pellet was washed twice in sucrose-mannitol buffer. Microsomes were isolated from the post-mitochondrial supernatant by centrifugation at 100,000 g for 1 h at 4°C. All final subcellular membrane preparations were resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride.

Immunoblot Analysis of Human Liver Subcellular Fractions

Protein estimation was carried out using the method of Lowry *et al* [41]. Mitoplast and microsomal proteins (50 μg protein each) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Polyclonal antibody against CYP2D6 was used at a dilution of 1:1000 (antibody raised to *Escherichia coli* recombinant CYP2D6 [42]). Blots were co-developed with antibodies to CYPR (1:1,500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) and mitochondrial transcription factor A (mtTFA, 1:3,000 dilution, gift from Dr. David Clayton, Howard Hughes Medical Institute, Janelia Farm, Ashburn, VA). Immunoblots were developed with chemiluminescence Super Signal Ultra Kit (Pierce, Rockford, IL) and image analysis was performed using a Versa-Doc imaging system (Bio-Rad). Digital image analysis was performed using Quantity One v.4.5.

Limited Trypsin Digestion of Mitochondria and Microsomes

Mitochondrial and microsomal fractions (100 μg protein each) isolated from human liver samples or transiently transfected COS cells were subjected to trypsin digestion on ice in 50 μl of sucrose-mannitol buffer (20 mM HEPES (pH 7.5) containing 70 mM sucrose, 220 mM mannitol, and 2 mM EDTA). Human liver subcellular fractions were incubated with 150 μg trypsin/mg protein for 20 min, while transfected COS cell subcellular fractions were incubated with 100 μg trypsin/mg protein for 30 min. The mitochondrial reactions were terminated by addition of soybean trypsin inhibitor (1.5 mg/mg protein, Sigma, St. Louis,

MO) and then the mitochondria were washed two times in sucrose-mannitol buffer. The final mitochondrial pellet was resuspended in an equal volume of 2X Laemmli sample buffer [43]. The microsomal reactions were terminated by addition of soybean trypsin inhibitor (1.5 mg/mg protein) and an equal volume of 2X Laemmli sample buffer. For both mitochondria and microsomes, one-half of the final suspension in Laemmli sample buffer was loaded onto the gel. Proteins were denatured by incubation at 95°C for 5 min, resolved by electrophoresis on 12% SDS-polyacrylamide gels and transblotted onto nitrocellulose membranes (Bio-Rad) for immunoblot analysis. Blots were developed with CYP2D6 antibody (1:1,000 dilution) and/or TOM20 antibody (1:1,000 dilution).

Spectrofluorometric Assay of MAMC Demethylation

Mitoplasts isolated from human liver samples were assayed for O-demethylation activity using MAMC as a substrate [20]. Incubations were performed in a Photon Technology International 814 PMT spectrofluorometer (Birmingham, NJ) with the excitation wavelength set at 405 nm and emission set at 480 nm. The mitoplasts were first permeabilized by incubation in hypotonic buffer (10 mM sodium phosphate, pH 7.4) for 10 minutes on ice. The reactions were performed in a final volume of 1 ml of 25 mM Tris-HCl buffer (pH 7.6) containing 20 mM $MgCl_2$, 200 μg mitoplast protein, 0.2 nmol of purified adrenodoxin (Adx), 0.02 nmol adrenodoxin reductase (AdxR), and 16 μM MAMC. Reactions were initiated by addition of 120 μM NADPH and the fluorescence was recorded for 20 min while the samples were stirred at 37°C. Inhibition studies were performed using 10 μM quinidine (Sigma), 1 mM proadifen-HCl (SKF-525A, Sigma), 5 μl CYP2D6 inhibitory monoclonal antibody (10 mg/ml, BD Gentest, Bedford, MA), 5 μl CYP1A2 inhibitory antibody (10 mg/ml, BD Gentest), 5 μl mouse IgG (10 mg/ml), and 10 μl Adx antibody (gift from Dr. Michael Waterman, Vanderbilt University). The reactions were performed as described above except that permeabilized mitoplasts were pre-incubated at 37°C with quinidine or proadifen hydrochloride for 10 min or Adx antibody for 30 min before addition to reaction mixture. CYPD6 and CYP1A2 inhibitory antibodies, and mouse IgG were pre-incubated with permeabilized mitoplasts for 10 min on ice before addition to reaction mixture. For assays used to compare mitochondrial CYP2D6 activities between the various human liver samples, reactions were performed in 500 μl volume in a shaking water bath at 37°C for 20 min and terminated by addition of 0.5 ml of 100 mM glycine (pH 10.2). Insolubles were sedimented by centrifugation at 10,000 *g* for 10 min and the supernatant containing 7-hydroxy-4-aminomethylcoumarin (HAMC) was measured fluorometrically.

Construction of WT and Mutant CYP2D6 cDNAs

Human wild-type (WT) CYP2D6 cDNA was amplified from human liver by RT-PCR. Total RNA was isolated from human livers using TRIzol reagent as per the supplier's protocol (Invitrogen, Carlsbad, CA). Reverse transcription was performed with 20 μg total RNA and the appropriate antisense primer. PCR was performed to amplify the full-length 1.5 kb sequence. The intact WT cDNA was used as a template to generate N-terminal deletions by polymerase-chain reaction (PCR) using the appropriate sense and anti-sense primers. ArgM 2D6 cDNA with internal mutations Arg25Asn, Arg26Asn, Arg28Asn; MitoM 2D6 cDNA with internal mutations His24Ala, Arg25Ala, Arg26Ala, Arg28Ala, and Arg32Ala; PKAM 2D6 cDNA with internal mutations Ser135Ala, were all generated using overlap PCR.

In vitro Transport of ^{35}S Labeled Protein into Isolated Mitochondria

cDNA constructs in pGEM7zF and PCR TOPO II (Invitrogen) vectors were used as templates in Sp6 or T7 polymerase-coupled rabbit reticulocyte lysate (RRL) transcription-translation systems (Promega) in the presence of [^{35}S]Met as described before [9]. Import of ^{35}S-labeled translation products in RRL was carried out using the system described by Gasser et al. [44] and modified by Bhat et al. [45] and Addya et al. [9] using freshly isolated

rat liver mitochondria. For some control experiments, mitochondria were pre-incubated with carbonyl cyanide 3-chlorophenyl hydrazone (CCCP, 50 μM, Sigma) or oligomycin (50 μM, Sigma) at 37°C for 20 min prior to initiating the import reaction. In experiments with PKAM 2D6, translation products were phosphorylated according to a protocol by Koch and Waxman [46]. Translation products were pre-incubated with the catalytic subunit of protein kinase A (PKA, Sigma, St. Louis, MO), 2.5 U/50 μl reaction and 100 μM ATP for 30 min at 37°C, prior to import. Following import, trypsin digestion (150 μg/ml) of mitochondria was performed for 20 min on ice. Mitochondria from both trypsin-treated and untreated samples were re-isolated by pelleting through sucrose, 0.8 M, and the proteins were subjected to SDS-PAGE followed by fluorography.

Transient Transfection of WT and mutant CYP2D6 in COS-7 cells

COS-7 cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and gentamycin (50 μg/ml). Cells were transiently transfected with FUGENE HD (Roche) transfection reagent using DNA purified with the Universal Mega Plasmid Preparation kit (Boston Bioproducts). The transfection reagent/DNA ratio was 3:2. After 48 hours the cells were harvested, washed in 1X phosphate buffered saline (PBS), and subjected to subcellular fractionation.

Isolation of Mitochondria and Microsomes from COS-7 Cells

Cell pellets were resuspended in sucrose-mannitol buffer (20 mM HEPES (pH 7.5) containing 70 mM sucrose, 220 mM mannitol, and 2 mM EDTA) and homogenized using a glass/Teflon Potter Elvehjem homogenizer for approximately 20 strokes or until approximately 80% cell lysis was achieved. The homogenate was centrifuged twice at 600*g* for 10 min to remove nuclei and cell debris. The supernatant was then centrifuged at 7000*g* for 15 min to sediment the crude mitochondrial fraction. The pellet was resuspended in sucrose-mannitol buffer, layered over 0.8M sucrose and centrifuged at 14,000*g* for 20 min to purify the mitochondria. The supernatant fraction was centrifuged at 100,000*g* to pellet microsomes. Following purification through the sucrose cushion, the mitochondrial pellet was washed in sucrose-mannitol buffer two times and mitochondria were pelleted at 7,000*g* for 10 min. Final preparations of mitochondria and microsomes were resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride.

Generation of Tetracycline-Inducible CYP2D6 Expression Cell Line

WT human CYP2D6 was cloned into a tetracycline inducible lentivirus vector LVPT-tTRKRAB [47] to replace GFP. Lentivirus was produced by transfection of three plasmids (Gag-pol, VSV-G, lentivirus 2D6 target vector) in 293T cells. Cells were harvested 48 hours post-transfection and filtered to collect viral particles. COS-7 cells were seeded in a 100 mm cell culture dish as single cells (about 100 cells per dish) 12 hours prior to infection. Lentivirus infection was conducted for 16 hours in the presence of 6 ug/ml polybrene. After infection, cells were cultured in 90% DMEM, 10% FBS, 1% penicillin and streptomycin, for several weeks to allow for expansion. Single cell colonies were selected and cultured, and immunoblot analysis was used to detect CYP2D6 expression in the presence of doxycycline (1 μg/ml) and confirm that there is no CYP2D6 expression in the absence of doxycycline. When culturing cells for subcellular fractionation, doxycycline was added 16 hours after plating and the cells were harvested 72 hours later.

CO Difference Spectral Analysis

The CYP content of stable cell mitochondria and microsomes was measured by the difference spectra of CO treated and dithionite reduced samples as described by Omura and

Sato [48] with modifications as described by Guengerich [49], using a dual-beam spectrophotometer (Cary 1E, Varian, Walnut Creek, CA). Mitochondrial or microsomal (0.5 mg) proteins were solubilized in potassium phosphate buffer (0.1 M, pH 7.4) containing 1 mM EDTA, 20% glycerol (v/v), sodium cholate (0.5%, w/v), and Triton N-101 (0.4%, w/v). Sodium hydrosulfite was added and the baseline was recorded. The solution in the sample cuvette was then bubbled gently with CO (60 s). The spectrum was recorded between 400 and 500 nm.

Bufuralol Oxidation Assay

Standard bufuralol oxidation reactions were conducted as described by Hanna et al. [50] with modifications. Briefly, the reactions were performed in 250 μl final volumes of 0.1 M potassium phosphate buffer (pH 7.4) containing 250 μg mitochondria or microsomal protein isolated from WT CYP2D6 stable cell lines, and 0.1 mM bufuralol. For the mitochondrial reactions, mitochondria were frozen and thawed five times to permeabilize the membranes before adding to the reaction mixtures. The mitochondrial reactions were supplemented with 0.2 nmol of purified adrenodoxin (Adx) and 0.02 nmol adrenodoxin reductase (AdxR) in order to compensate for any loss of these small soluble proteins during mitochondrial isolation. The mixtures were pre-incubated for 3 min at 37°C and then the reactions were initiated by addition of 120 μM NADPH. The incubations were carried out for 10 min and then quenched by addition of 25 μl of 60% $HClO_4$. The reaction mixtures were centrifuged at 3,000 g for 10 min to sediment precipitated proteins and salts and the supernatants were used for LC/MS analysis. Inhibition studies were performed using 10 μl CYP2D6 inhibitory monoclonal antibody (10 mg/ml, BD Gentest, Bedford, MA), and 10 μl Adx antibody. The reactions were performed as described above except that mitochondria were pre-incubated with CYPD6 inhibitory antibody for 10 min on ice, or Adx inhibitory antibody for 30 min at 37°C, before addition to reaction mixtures.

1′Hydroxybufuralol was measured using LC-MS according to the method of Yu et al. [30], utilizing a ThermoFisher TSQ instrument coupled with and an HPLC system with a ProntoSIL C18-ace-EPS octadecylsilane colume (3 μm, 4.6 mm × 150 mm). A flow rate of 250 μl/min was used with solvents A (0.1% HCO_2H in H_2O, v/v) and B (0.1% HCO_2H in CH_3CN) and the following gradient: t 0-1 min, 100% A; t 1 min; t 1-16 min, 0 to 100% B; t 16 to 20 min, hold at 100% B; t 20-20.5 min, 0% A to 100% A); t 20.5 to 25 min, hold at 100% A. The transitions *m/z* 278—>150 and 262—>157 were used to monitor 1′-hydroxybufuralol and bufuralol, respectively, and the internal standard dextromethorphan (*m/z* 258—>157). The limit of detection was 0.1 pmol of 1′-hydroxybufuralol.

Acknowledgments

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Abbreviations

Adx	adrenodoxin
AdxR	adrenodoxin reductase
CCCP	carbonyl cyanide 3-chlorophenyl hydrazone
CYP	cytochrome P450 (EC 1.14.14.1)
CYPR	NADPH-cytochrome P450 reductase (EC 1.6.2.4)
DHFR	dihydrofolate reductase (EC 1.5.1.3)
DOX	doxycycline
ER	endoplasmic reticulum
HAMC	7-hydroxy-4-aminomethylcoumarin
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

HL	human liver sample
MAMC	7-methoxy-4-(aminomethyl)coumarin
mtTFA	mitochondrial transcription factor A
PKA	protein kinase A (EC 2.7.11.11)
RRL	rabbit reticulocyte lysate
TOM20	translocase of outer mitochondrial membrane 20
WT	wild-type

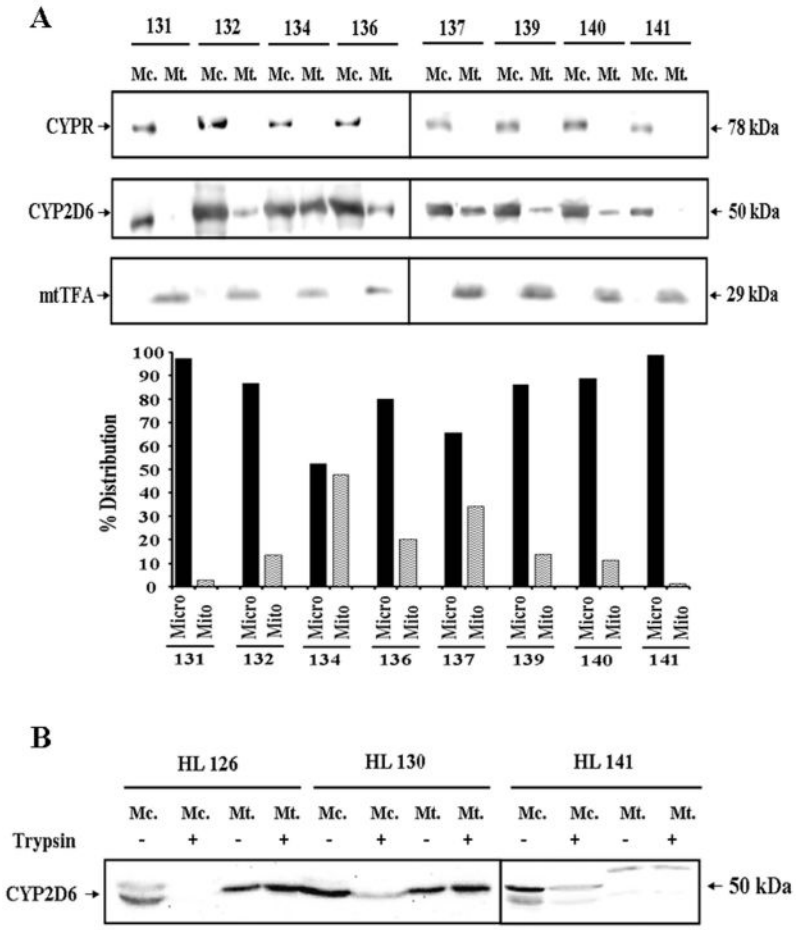

Fig. 1.

Localization of CYP2D6 in the mitochondria of human liver samples. (A) Immunoblot analysis of mitoplast and microsome (50 μg protein each) fractions isolated from human liver samples. “Mc” represents the microsomal fraction. “Mt” represents the mitoplast fraction. Densitometric analysis was performed to determine the distribution of CYP2D6 between mitochondria and microsomes in each liver sample analyzed. (B) Immunonlot analysis of human liver mitochondria and microsomes subjected to limited trypsin digestion (150 μg/mg protein, 20 min on ice). Blots were developed with polyclonal antibodies to CYP2D6 (1:1,000) and mtTFA (1:3,000) and monoclonal antibody to CYPR (1:1,500).

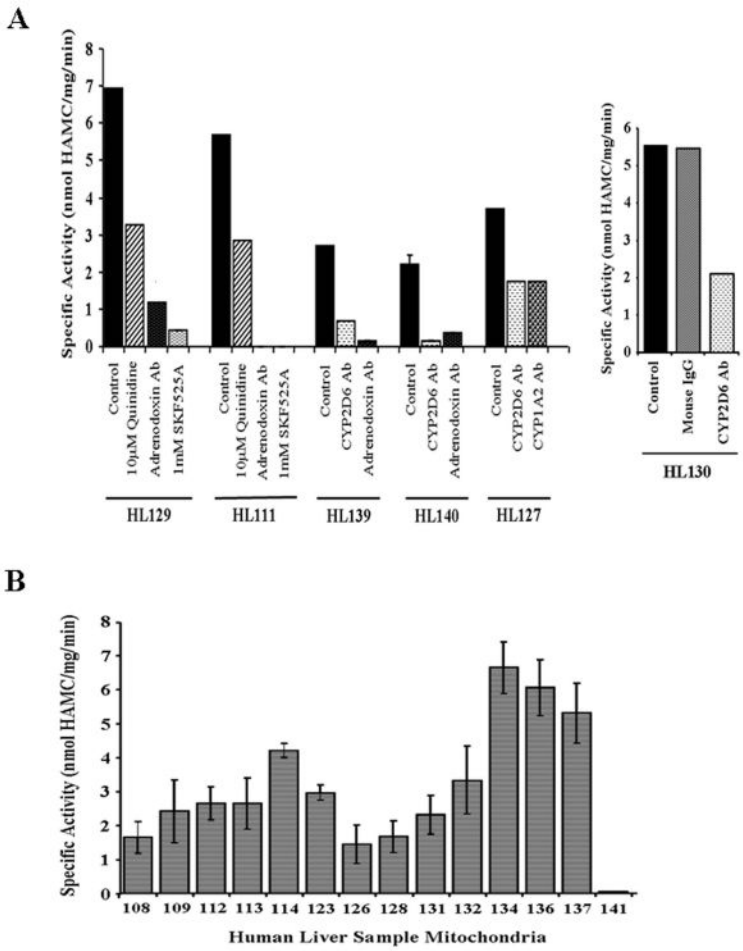

Fig. 2.

Metabolic activity of human liver mitochondrial CYP2D6. (A and B) Mitoplasts isolated from human liver samples were assayed for *O*-demethylation activity using the substrate MAMC. Assays were performed as described in “Material and Methods.” (A) Mitoplasts from five human liver samples were tested for MAMC oxidizing activity and various inhibitors were used to establish if the activity is mediated by mitochondrial CYP2D6. Mitochondria were pre-incubated with inhibitors as described in “Materials and Methods.” “Control” refers to activity in the absence of any inhibitors. The control activity for sample HL140 represents an average ± S.E.M for three separate estimates. The control activities for samples HL129, 139, and 127 represent an average from two separate estimates. All other values represent single assay points. (B) MAMC *O*-demethylation activity was compared between mitoplasts isolated from the remaining fourteen human liver samples using the protocol described in “Materials and Methods.” The activities in all cases represent an average ± S.E.M from three separate estimates.

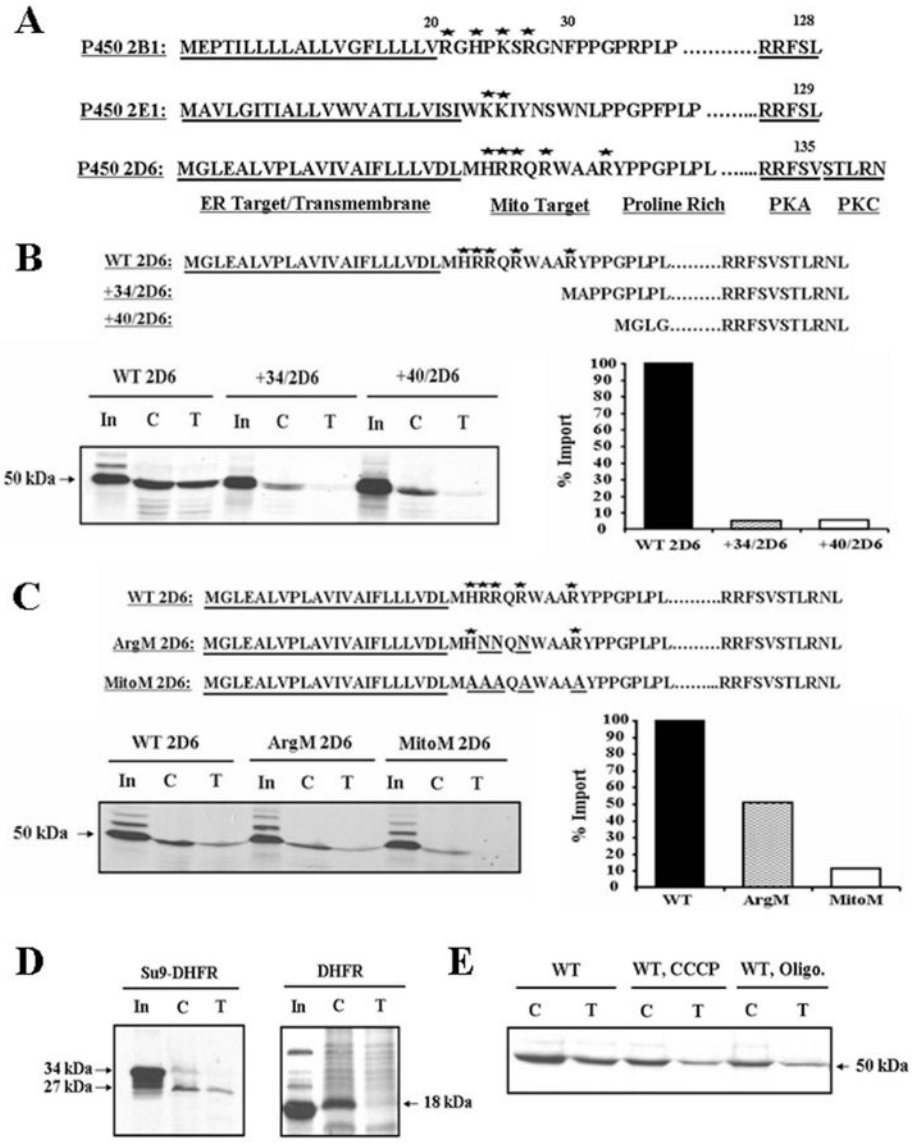

Fig. 3.

Localization of mitochondrial targeting signal of CYP2D6. (A) Alignment of CYP2D6 N-terminal sequence with chimeric signal sequences of CYP2B1 and CYP2E1. (B-D) *In vitro* import of [^{35}S]-labeled translation products in isolated rat liver mitochondria. CYP2D6 WT (B, C and E), N-terminal truncation mutants (B), and mitochondrial targeting signal mutants (C) were generated in the rabbit reticulocyte lysate system (RRL). (D) Su9-DHFR, in which the pre-sequence of subunit 9 of *N. crassa* F_0F_1-ATPase has been fused to DHFR, and DHFR were translated in RRL and used as positive and negative controls respectively. In (E) mitochondria were pre-incubated with CCCP (50 μM) or oligomycin (oligo, 50 μM) for 20 min at 37°C prior to initiating the import reaction. In all experiments, trypsin digestion (150 μg/ml) of mitochondria was performed for 20 min on ice. Proteins (200 μg each) were subjected to SDS-PAGE and fluorography. “C” represents control experiments in which total protein bound and imported into mitochondria is present, “T” represents trypsin treated mitochondria in which only the protein imported into mitochondria is present. In the lanes marked “In,” 20% of the counts used as input for the import reactions have been loaded. In (B and C) densitometric analysis was performed to analyze the level of import for each

construct following trypsin treatment. The level of import of the wild-type protein was considered to be 1 for calculating the relative import of various deletion and point mutations.

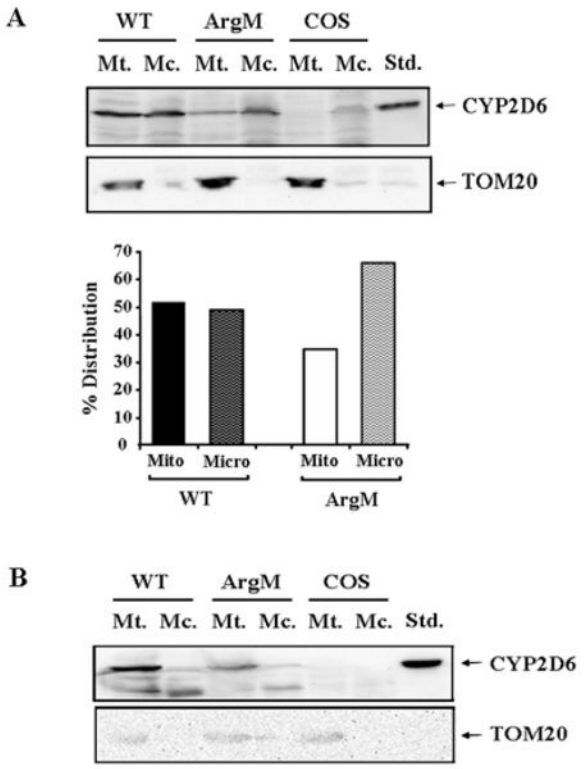

Fig. 4.

Role of Arg residues from the putative signal region for the mitochondrial targeting of CYP2D6 in COS-7 cells. Immunoblot analysis of mitochondria and microsomes isolated from COS-7 cells transiently transfected for 48 hours with WT and ArgM CYP2D6 cDNA. (A) Mitochondria and microsome fractions before trypsin treatment. (B) Mitochondria and microsome fractions following limited trypsin digestion (100 μg/mg protein, 30 min on ice). Blots were co-developed with polyclonal antibodies to CYP2D6 (1:1,000) and TOM20 (1:1,000). In (A) densitometric analysis was performed and the % distribution in the mitochondrial and microsomal fractions were based on the aggregate values (mitochondria + microsome) considered as 100%.

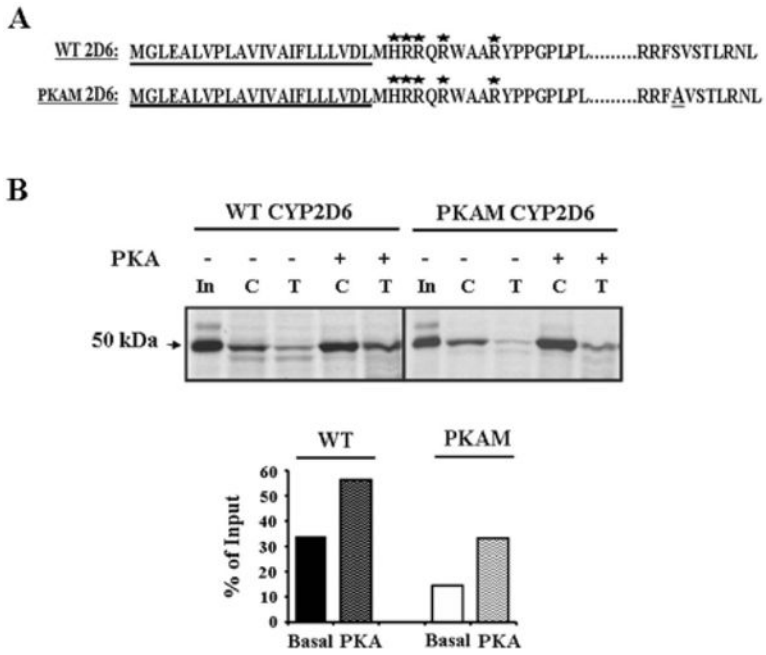

Fig. 5.

Role of PKA-mediated phosphorylation in mitochondrial targeting of CYP2D6. CYP2D6 WT and PKA phosphorylation site mutant (PKAM) constructs were translated in the rabbit reticulocyte lysate (RRL) system in the presence of [^{35}S]-Met. In some cases, translation products were pre-incubated with protein kinase A (PKA) and ATP for 30 min at 37°C, prior to import. Labeled proteins were imported into isolated mitochondria as described in "Materials and Methods." "C" represents control experiments in which total protein bound and imported into mitochondria is present, "T" represents trypsin treated mitochondria in which only the protein imported into mitochondria is present. In the lanes marked "In," 20% of the counts used as input for the import reactions have been loaded. Densitometric analysis was performed to determine the extent of import following trypsin treatment for each construct in the presence and absence of phosphorylation. The values were expressed as % of input of each WT and mutant protein.

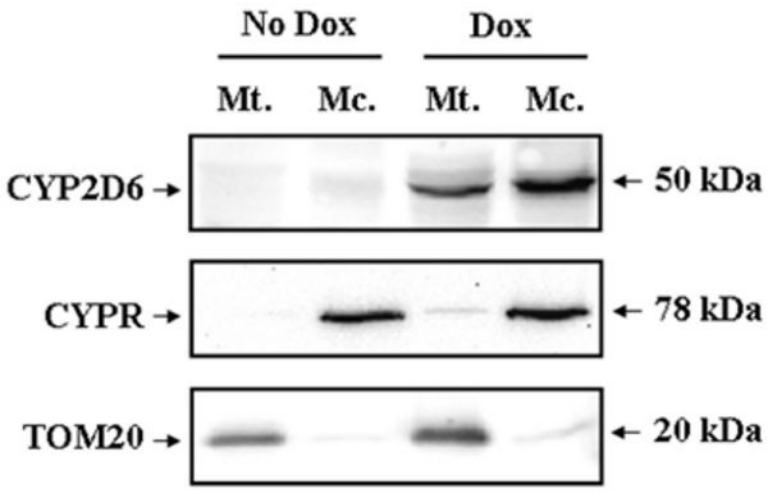

Fig. 6.

Mitochondrial localization of CYP2D6 in a doxycycline (DOX)-inducible stable cell line. Immunoblot analysis of mitochondria and microsomes isolated from a DOX-inducible CYP2D6 stable cell line. Cells were cultured for 72 hours in the absence (No Dox) or presence (Dox) of doxycycline (1 μg/ml). Blots were co-developed with polyclonal antibodies to CYP2D6 (1:1,000) and TOM20 (1:1,000), and monoclonal antibody to CYPR (1:1,500).

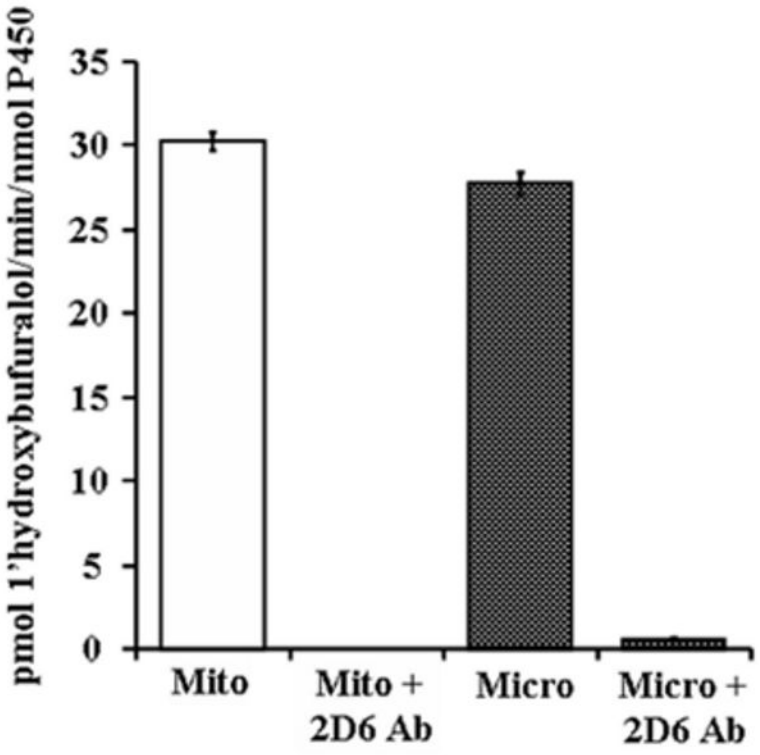

Fig. 7.

Bufuralol 1′hydroxylation activity of mitochondrial CYP2D6. Mitochondria and microsomes isolated from a DOX-inducible CYP2D6 stable expression cell line were assayed for bufuralol 1′hydroxylation activity. Assays were performed as described in "Materials and Methods." The activity values represent an average ± S.E.M for three separate estimates. In the case of mitochondria pre-incubated with CYP2D6 inhibitory antibody, three estimates were performed but two of the activity levels were below the level of detection for this assay (0.1 pmol).