The Localization of Cytochrome P450s CYP1A1 and CYP1A2 into Different Lipid Microdomains Is Governed by Their N-terminal and Internal Protein Regions*

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Background: Cytochrome P450s 1A1 and 1A2 are found in different membrane regions despite their high sequence similarity. 

Results: CYP1A chimeric proteins gained or lost the ability to localize in ordered domains. 

Conclusion: Domain localization of CYP1A enzymes was governed by both the early N-terminal region and an internal sequence. 

Significance: Microdomain targeting of P450s may serve as a mechanism for modulating P450 function.

In cellular membranes, different lipid species are heterogeneously distributed forming domains with different characteristics. Ordered domains are tightly packed with cholesterol, sphingomyelin, and saturated fatty acids, whereas disordered domains contain high levels of unsaturated fatty acids. Our laboratory has shown that membrane heterogeneity affects the organization of cytochrome P450s and their cognate redox partner, the cytochrome P450 reductase (CPR). Despite the high degree of sequence similarity, CYP1A1 was found to localize to disordered regions, whereas CYP1A2 resided in ordered domains. We hypothesized that regions of amino acid sequence variability may contain signal motifs that direct CYP1A proteins into ordered or disordered domains. Thus, chimeric constructs of CYP1A1 and CYP1A2 were created, and their localization was tested in HEK293T cells. CYP1A2, containing the N-terminal regions from CYP1A1, no longer localized in ordered domains, whereas the N terminus of CYP1A2 partially directed CYP1A1 into ordered regions. In addition, intact CYP1A2 containing a 206–302-residue peptide segment of CYP1A1 had less affinity to bind to ordered microdomains. After expression, the catalytic activity of CYP1A2 was higher than that of the CYP1A1-CYP1A2 chimera containing the N-terminal end of CYP1A1 with subsaturating CPR concentrations, but it was approximately equal with excess CPR suggesting that the localization of the CYP1A enzyme in ordered domains favored its interaction with CPR. These data demonstrate that both the N-terminal end and an internal region of CYP1A2 play roles in targeting CYP1A2 to ordered domains, and domain localization may influence P450 function under conditions that resemble those found in vivo.

Cytochrome P450 (P450) is a superfamily of heme-containing proteins primarily found in the endoplasmic reticulum (ER). P450s catalyze the oxidative metabolism of a wide variety of endogenous and foreign compounds generally by the insertion of an oxygen atom into the substrate molecule. These monooxygenase reactions require the substrate molecule, molecular oxygen, and electrons. The electrons are relayed from the reduced cofactors, NADPH and NADH, to the P450s by the redox partners, CPR, and cytochrome b5. The lipid bilayer of cell membranes is composed of numerous species of phospholipids. With different physical properties (e.g. hydrophobicity and Tm), the phospholipids are not randomly distributed but are organized in a manner that generates microdomains having unique characteristics. Ordered domains are the most well known example of domains, containing higher concentrations of cholesterol, sphingomyelin, and saturated fatty acids compared with disordered domains that are more loosely packed with unsaturated fatty acids. Ordered domains are not readily solubilized by non-ionic detergents and are also referred to as detergent-resistant membranes, whereas the less ordered regions are readily solubilized. Using non-ionic detergents to solubilize proteins from disordered regions of the membrane, it has been shown that many proteins selectively reside in ordered domains. It has been shown that these ordered domains, particularly those of the plasma membrane, facilitate diverse cellular functions of proteins such as signal transduction and membrane trafficking (1, 2). In our previous studies, we showed that P450 system components were also organized in specific microdomains in the ER (3, 4). In these studies, ordered domains, isolated by detergent extraction with Brij 98 followed by centrifugation on a discontinuous sucrose gradient, contained most of the CYP1A2 and CPR enzymes and were associated with higher levels of cholesterol and sphingomyelin. However, CYP2E1 and CYP1A1 were mostly found in disordered domains, and CYP2B4 distributed equally between both ordered and disordered domains of the ER. The microdomain localization of CYP1A2 and CPR increased CYP1A2 catalytic activity.

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This article contains supplemental Fig. S1.

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2 The abbreviations used are: ER, endoplasmic reticulum; ERF, 7-ethoxyresorufin; CPR, NADPH-cytochrome P450 reductase; MRF, methoxyresorufin; PS, phosphatidylycerine; PNS, post-nuclear supernatant.
activity by enhancing CYP1A2 and CPR complex formation in reconstituted systems (3). Although other groups showed the effect of lipid composition on P450 activity, conformational changes in the proteins, stability of the enzyme, and the rate of protein insertion into the membrane (5–9), these effects were largely attributed to direct effects of the lipid classes on the P450 enzymes and not to their distribution into different membrane domains. However, the formation of anionic lipid domains distinct from detergent-resistant membranes has been implicated by a study using fluorescent lipid probes (10) and may contribute to the above-mentioned functional effects observed in many of the lipid studies involving P450s.

It is well known that the N-terminal region of P450s interacts with the membrane and facilitates their anchoring to the ER (11); however, this is not the only region that associates with the membrane. There are several recent reports suggesting that internal P450 regions also interact with membrane lipids (12–14). Even though it has been suggested that lipophilic and amphiphilic substrates gain access to the P450 active site via the interaction of membrane and catalytic regions, the mechanism is not fully understood (15, 16). Because of increasing evidence indicating the impact of lipids on the P450 function, it is important to understand the organization of P450 in the membrane and to define which regions of the amino acid sequences affect P450 localization. In previous studies, the membrane localization of several P450 enzymes was examined. In this study, we focused on determining the parts of the proteins that were responsible for directing CYP1A proteins to specific membrane microdomains. This was accomplished by comparing the sequences of CYP1A1 and CYP1A2, two closely related proteins that localized in different domains. With most of the sequence variability between the two proteins being in the early N-terminal and catalytic regions, we generated CYP1A1-CYP1A2 chimeras and examined their localization after expression in HEK293T cells. The results demonstrated that both the N terminus and an internal region of these proteins influence the membrane localization of CYP1A proteins.

Experimental Procedures

Reagents—The antibody used for CYP1A2 was provided by Dr. Kristopher Krausz from the National Institutes of Health. Primary antibody against GFP was obtained from Millipore (Billerica, MA). TA cloning kits, Lipofectamine 2000, PBS, and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Invitrogen. pGFP-N2 vector was obtained from BioSignal Packard (Waltham, MA). All restriction enzymes and One TaqDNA polymerase were purchased from New England Biolabs (Ipswich, MA). QIAquick gel extraction kit was purchased from Qiagen (Hilden, Germany). Bicinchoninic acid (BCA) protein assay kit and Supersignal West Pico chemiluminescent substrate were purchased from Thermo Fisher Scientific (Rockford, IL). Brij 98 and all other reagents were obtained from Sigma. 7-Ethoxyresorufin (ERF) and methoxyresorufin (MRF) were purchased from AnaSpec (Freemont, CA).

Isolation of Microsomes—Microsomes were prepared from untreated rabbit livers by differential centrifugation (17, 18). Pellets from the final centrifugation were re-homogenized in 10 mm potassium phosphate buffer (pH 7.25), and the protein concentration was determined using the BCA assay.

Cell Culture—Human embryonic kidney (HEK)-293T cells were purchased from the ATCC (Manassas, VA). Cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 1 × antibiotic antimycotic (Gibco) and were maintained in humidified 5% CO2 incubator at 37 °C. When cells reached 90% of confluence, plasmid DNAs were transfected into cells using Lipofectamine 2000.

Cloning of Rabbit CYP1A1—Total RNA was isolated from 30 mg of lung tissue from an α-naphthoflavone-treated rabbit using RNAeasy protect mini kit (Qiagen), and cDNA was then synthesized from 1 μg of RNA using iScript cDNA synthesis kit (Bio-Rad). A full length of the rabbit CYP1A1 was amplified by PCR using Pfu polymerase (Stratagene, La Jolla, CA) with the following primers: forward 5’-TCA CTT CCA GAG GAG CT-C GG-3’ and reverse 5’-AGG TCA GGC TGC CCA GTT AG-3’. The PCR product was verified in 1% (w/v) agarose gel, and the PCR product extracted from the agarose gel was incubated with 150 μm dNTP, 2 units of Taq polymerase and 1 × Taq buffer (10 mm Tris-HCl, pH 8.3, at 25 °C containing 50 mM KCl and 15 mM MgCl2) for poly(A) tailing at 72 °C for 30 min. Then 5 μl of the reaction mixture was ligated with pCR2.1 vector and was transformed into One Shot cells (Invitrogen). The successful insertion of CYP1A1 into pCR2.1 vector was tested by restriction enzyme treatment and confirmed by sequencing (ACGT Inc.). For expression of CYP1A1 in mammalian cells, CYP1A1 in the pCR2.1 vector was amplified by PCR using forward and reverse primers containing restriction enzyme sites (NheI forward 5’-TTG GCT AGC ATG TTC GAT TT GGA C-3’ and KpnI reverse 5’-GGT ACC ATA GGC CTC GAA GCG-3’) and then was subcloned into pGFP-N2 vector.

Cloning of Chimeric Proteins of CYP1A1 and CYP1A2—For the fusion of CYP1A1 and CYP1A2 proteins, overlap extension PCR was performed, which involves two rounds of PCR (19). Four cut sites were selected (Fig. 1, yellow box), and CYP1A1 and CYP1A2 proteins were fused at each cut site. Briefly, each fragment of CYP1A2 and CYP1A1 was amplified by the first round of PCR in separate tubes with one flanking primer that annealed at the one 5’ or 3’ end o the target sequence and one internal site-specific primer that annealed at the one cut site with overhang complementary sequences to the other target gene as described in Fig. 1 (primers are shown in Table 1). The reaction mixture contained 200 ng of templates, 2.5 units of Pfu polymerase, 1 × polymerase buffer (Stratagene, La Jolla, CA), 1 mm dNTP, and 1 μM each of one flanking and one internal primer in a final volume of 50 μl and was subjected to 40 cycles of denaturation (20 s at 95 °C), annealing (20 s at 59 °C), and extension (45 s at 72 °C). Three minutes was added to the final extension step after the final cycle. The PCR products were analyzed in 1% (w/v) agarose gel and extracted from the gel in 40 μl of distilled water using the QIAquick gel extraction kit (Qiagen, Germantown, MD). The second PCR was carried out with 20 μl of each extracted fragment of CYP1A1 and CYP1A2 and two flanking primers. PCR cycling conditions were the same as those of the first PCR except that the final extension step was extended to 20 min. In the second PCR, two fragments were annealed by overhang complementary sequences

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that allow one strand from each fragment to act like a primer on the other fragment, and the intact CYP1A1-CYP1A2 or CYP1A2-CYP1A1 chimeras were amplified with flanking primers listed in Table 1. After agarose gel verification and gel extraction, two ends of CYP1A1-CYP1A2 chimeric DNA were digested with NheI/BamHI and CYP1A2-CYP1A1 with EcoRI/KpnI. After the restriction enzyme treatment, chimeric constructs were cloned into pGFP2-N2 vector. Because the N terminus is integrated into the membrane, GFP was added at the end of C-terminal regions of the chimeras by a single mutation of stop codons.

Isolation of Post-nuclear Supernatant and Detergent-resistant Membrane—To isolate post-nuclear supernatant, at 48 h post-transfection harvested cells were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, containing 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT and a mixture of protease inhibitors derived from dissolving a commercially available tablet (Roche Applied Science, Mannheim, Germany)) and placed on ice for 30 min (20). Cells were then passed through 30-gauge needle 20 times. Unbroken cells and nuclei were removed by centrifugation at 580 × g, 5 min. Supernatant was saved and was divided into two microcentrifuge tubes. Brij 98 was diluted to a 5% (v/v) stock solution using membrane solubilization buffer (50 mM HEPES, pH 7.25, containing 150 mM NaCl and 5 mM EDTA and the protease inhibitor mixture described above). Either 100 μl of solubilization buffer or 100 μl of 5% (v/v) Brij 98 was added to each tube to a final concentration of 0 or 0.5% (v/v) Brij 98, and both samples were then incubated at 37 °C for 5 min. After the solubilization step, membrane solubilization buffer was added to both samples to obtain a final volume of 3 ml, and then samples were centrifuged using a SW55Ti rotor at 100,000 × g for 1 h. Supernatants were saved, and pellets were resuspended in 3 ml of membrane solubilization buffer. Protein distribution in pellets and supernatants was then analyzed by immunoblotting. In separate experiments, membrane solubilization by Brij 98 at concentrations ranging from 0.25 to 1% (v/v) showed similar localization of CYP1A proteins (supplemental Fig. 1).

Confocal Microscopy—To visualize subcellular localization of CYP1A1 and CYP1A2 proteins tagged with GFP, HEK293T cells were co-transfected with DsRed-ER and either CYP1A1 or CYP1A2. At 48 h post-transfection, cells were fixed in PBS containing 10% (v/v) formalin and 0.02% (v/v) Triton X-100 for 15 min at room temperature and washed three times in PBS. Cells were then mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were acquired with a confocal microscope (Leica DM IRE2) with a ×63 water objective.

Immunoblot Analysis—For analysis of protein levels in each fraction, samples were applied to 10% (w/v) SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad) as described previously with some modifications (21). Membranes were probed using mouse anti-CYP1A1/1A2 and mouse anti-GFP. Horseradish peroxidase-conjugated anti-mouse secondary antibodies were used at 1:4000. Treated blots were visualized using Supersignal West Pico chemiluminescent substrate (Thermo Fisher Scientific).

Enzymatic Assay—The activity of wild type and chimeric proteins was monitored using the substrates, ERF and MRF. The 0.1-ml reaction mixtures contained 0.03 ml of post-nuclear supernatant (PNS) and either 2 μM ERF or 5 μM MRF and were taken to 0.09 ml with buffer containing 0.05 M HEPES, pH 7.5, 15 mM MgCl₂, and 0.1 mM EDTA. Substrates were added from 100-fold stock solutions in DMSO. The reactions were started with the addition of 0.01 ml of 4 mM NADPH. The metabolism of substrates was monitored in real time by fluorescence (excitation, 535 nm/emission, 585 nm). Levels of P450 were determined by immunoblotting with GFP used as a standard. From the results of many preliminary experiments, the GFP fluorescence (excitation, 410; emission, 510) of post-nuclear supernatants was correlated with the expression level of GFP-labeled.
proteins in HEK293T cells (not shown). In experiments measuring catalytic activities of expressed CYP1A enzymes, the rates of metabolism were normalized to protein expression as determined by the GFP fluorescence and reference to the standard curve described above.

**Results**

**Different Microdomain Localization of CYP1A1 and CYP1A2**—
In a previous study, we showed that CYP1A2 and CYP1A1 proteins resided in different microdomains of liver microsomes isolated from pyrazole- or phenobarbital-treated rabbits (4). The data were interesting due to the high sequence similarity between the two forms. As shown in Fig. 2, the sequence alignment shows about 80% sequence identity between the two enzymes. Variable amino acids (uncolored area) are shown mainly in early N-terminal and catalytic regions. Therefore, we hypothesized that unique amino acid sequences in each protein will contain signal sequences responsible for targeting CYP1A proteins to different microdomains. Before testing this hypothesis, we examined the localization of CYP1A1 and CYP1A2 in microsomes from untreated rabbits. Microsomes (2 mg/ml) were solubilized using 1% (v/v) Brij 98, and detergent-sensitive (containing disordered domains) and detergent-resistant (containing ordered domains) membrane fractions were separated by ultracentrifugation. Consistent with our previous study (4), most CYP1A1 proteins were found in the disordered membranes, and CYP1A2 was predominantly found in pellets containing ordered membrane fractions (Fig. 3).

**Localization of CYP1A Family Proteins in the HEK293T Cell Line**—Fig. 3 illustrates the differential localization of CYP1A1 and CYP1A2 in liver microsomes. To test whether the distribution of these two proteins in a cell line is consistent with microsomal data, full-length CYP1A1 and CYP1A2 genes were cloned into a pGFP vector. Using these P450 DNAs, the subcellular localization of CYP1A1 and CYP1A2 in HEK293T cells was confirmed. DsRed-ER (an ER marker) and either pGFP-CYP1A1 or pGFP-CYP1A2 were co-transfected into the cell, and the expression was visualized by confocal microscopy. The expression of CYP1A proteins (green) and DsRed-ER (red) was co-localized (Fig. 4A), indicating that the tagged CYP1A proteins are expressed in the ER. Next, to examine the microdomain localization of CYP1A proteins, HEK293T cells were transfected with either pGFP-CYP1A1 or pGFP-CYP1A2. At 48 h post-transfection, the cells were harvested, and the post-nuclear supernatant (PNS) was collected using hypotonic buffer and low speed centrifugation. Half of the PNS was incubated with 0.5% (v/v) Brij 98, and the other half was incubated without Brij 98 at 37 °C for 5 min. After ultracentrifugation of detergent-treated or -untreated samples, supernatants and pellets...
lets were analyzed by immunoblotting. Without detergent treatment, all intact membrane fractions were found in pellets. However, with detergent treatment most CYP1A1 protein was found in the supernatant, indicating localization in disordered regions, whereas CYP1A2 was found in both supernatant and pellets containing ordered domains (Fig. 4B). These results demonstrate that CYP1A1 and CYP1A2 reside in different regions of the ER membrane and are consistent with the results observed in microsomes (Fig. 3).

**Loss of Ability of CYP1A2 to Reside in Ordered Regions of Membranes by Substitution of N-terminal Sequences from CYP1A1**—Even though CYP1A1 and CYP1A2 proteins have highly conserved sequences, significant variations occur in the early N-terminal and catalytic regions (Fig. 2). In an effort to identify the regions within the CYP1A proteins responsible for the differential localization of CYP1A1 and CYP1A2, CYP1A1-CYP1A2 chimeras were generated, and their localization was compared with that of the intact proteins. P450 proteins generally have conserved fold topology with 12 helices (A–L) and four \( \beta \)-sheets (1–4) (16). The structural core of the proteins containing helices E and I-L as well as \( \beta \)-sheets (1–2) shows most spatial conservation, and the substrate-binding site shows the most diversity (16, 22). To minimize the effects of altered protein folding of chimeras on microdomain localization, four cut sites were selected from loops of CYP1A2 secondary structure (107, 205, 302, and 417). The four points were matched with 109, 207, 306, and 421 in CYP1A1 protein. At each cut site, a part of one CYP1A protein was substituted with sequences from the other protein by overlap extension PCR (16, 23). Briefly, CYP1A1 and CYP1A2 fragments were separately amplified by a conventional PCR, and two fragments were fused in the second PCR step (Fig. 1). Because internal primers covering cut points for fragment A contain at least 15 complementary nucleotides for fragment B (Table 1), fragments A and B are fused by the complementary sequences in the second PCR. The four points were matched with 109, 207, 306, and 421 in CYP1A1 protein. At each cut site, a part of one CYP1A protein was substituted with sequences from the other protein by overlap extension PCR (16, 23). Briefly, CYP1A1 and CYP1A2 fragments were separately amplified by a conventional PCR, and two fragments were fused in the second PCR step (Fig. 1). Because internal primers covering cut points for fragment A contain at least 15 complementary nucleotides for fragment B (Table 1), fragments A and B are fused by the complementary sequences in the second PCR. To examine the role of the N-terminal region on microdomain localization, HEK293T cells were transfected with each plasmid containing CYP1A1-CYP1A2 chimeric constructs illustrated in Fig. 5A. Each full-length DNA of CYP1A1 and CYP1A2 was also transfected into cells as controls. 48 h after transfection, cells were harvested, and PNS was collected as described under “Experimental Procedures.” Each PNS sample was divided into two groups as follows: one group was incubated with 0.5% (v/v) Brij 98, and the other group was incubated without Brij 98 at 37 °C for 5 min. Intact membranes or ordered domains resistant to solubilization by the detergent were collected at the bottom of the centrifuge tubes after centrifugation.

**FIGURE 3. Distribution of CYP1A1 and CYP1A2 in untreated microsomes.** Untreated rabbit microsomes (2 mg/ml) were solubilized by 1% (v/v) Brij 98 followed by ultracentrifugation to separate ordered and disordered fractions. Pellets (P) contained ordered domains, and supernatants (S) contained disordered fractions. The localization of proteins was analyzed by immunoblotting.

**FIGURE 4. Different microdomain localization of CYP1A proteins in HEK293T cells.** A pGFP vector containing either CYP1A1 or CYP1A2 was co-transfected with DsRed ER (ER marker) into HEK293T cells, and the ER localization was analyzed by confocal microscopy (A). To test microdomain localization of CYP1A proteins, cells were transfected with plasmids containing either CYP1A1 or CYP1A2. At 48 h post-transfection, the PNS was collected as described under “Experimental Procedures” and solubilized by 0.5% (v/v) Brij 98. Solubilized PNS was separated into pellet (P) containing ordered regions and supernatant (S) containing detergent-soluble regions by centrifugation at 100,000 × g for 1 h. The GFP-tagged proteins were detected by immunoblotting (IB) (B).

| Chimeras | Cut points | sequence |
|----------|------------|----------|
| CYP1A1-CYP1A2 | N terminus | GAT GCT ACC ATA GGC CTC GAA CCA |
| CYP1A1-CYP1A2 | C terminus | TTT TTC TCT CAG GAT GAT GAT |
| CYP1A1-CYP1A2 | N terminus | GAT GCT ACC ATA GGC CTC GAA CCA |
| CYP1A1-CYP1A2 | C terminus | TTT TTC TCT CAG GAT GAT GAT |

**TABLE 1**

**Oligonucleotides used for generation of CYP1A1 chimeric proteins**

| Chimeras | Cut points | oligonucleotides | Sequence |
|----------|------------|-----------------|----------|
| CYP1A1-CYP1A2 | N terminus | GAT GCT ACC ATA GGC CTC GAA CCA |
| CYP1A1-CYP1A2 | C terminus | TTT TTC TCT CAG GAT GAT GAT |
| CYP1A1-CYP1A2 | N terminus | GAT GCT ACC ATA GGC CTC GAA CCA |
| CYP1A1-CYP1A2 | C terminus | TTT TTC TCT CAG GAT GAT GAT |
Ordered Domain Localization of CYP1A1 Caused by Insertion of N-terminal Regions of CYP1A2—Because CYP1A2 chimeric constructs containing N-terminal sequences of CYP1A1 lost their ability to reside in ordered domains, we hypothesized that attachment of the CYP1A2 N-terminal regions to CYP1A1 would cause the CYP1A1 constructs to relocate from the disordered to the ordered domains. To examine this hypothesis, CYP1A1 chimeric constructs containing CYP1A2 N-terminal sequences of different lengths were transfected into HEK293T cells (Fig. 5). After 48 h post-transfection, post-nuclear supernatants were collected and incubated with 0 or 0.5% (v/v) Brij 98 at 37 °C for 5 min. Chimeras fused at positions 107 and 205 were successfully expressed in membranes and detected in ordered domains after Brij 98 treatment (Fig. 5B). Although chimeras containing longer N-terminal sequences of CYP1A2 were not expressed in HEK293T cells, these results demonstrate that the N terminus of CYP1A2 contains a signal sequence that directs P450 proteins into the ordered microdomains. Like previous experiments, the enzyme activity of the chimera fused at the 107 cut point was similar to the activity of intact CYP1A1 proteins (Fig. 6, D and E).

Catalytic Activities of Wild Type and Chimeric CYP1A Cells from Transfected HEK293T Cells—A potential problem with interpreting the membrane localization of the CYP1A1 chimeras is the possibility that the substitution within the protein at 100,000 × g for 1 h. In contrast, cytosolic or solubilized proteins were found in the supernatant. After separation of disordered and ordered domains, the GFP-tagged proteins were detected by immunoblotting. Without detergent treatment, all chimeric proteins were found in the pellet, indicating all CYP1A1-CYP1A2 chimeras were successfully expressed in cellular membranes. With detergent treatment, wild type (WT) CYP1A1 was found in the supernatant (i.e. disordered domains) and WTCYP1A2 was found in ordered domains. However, CYP1A2 containing the N-terminal region from CYP1A1 failed to localize in the ordered domains (Fig. 5B). Even the CYP1A1-CYP1A2 chimera that was substituted with the shortest CYP1A1 N-terminal sequence was completely solubilized by the detergent treatment. The percent distribution of the chimeras is shown in Fig. 5C. These data indicate that the N-terminal region, especially the early N-terminal sequence of CYP1A2, directs localization of these proteins into ordered domains.
sequence of the P450 rendered a conformational change that affected enzyme function. In other words, any differences in the membrane localization of the wild type and chimeric forms could be an artifact related to the misfolding of the protein. Thus, it was important to measure the catalytic activities of the wild type and chimeric CYP1A proteins. HEK293T cells express very low levels of endogenous CPR. Thus, to compare the functionalities of the P450s, we transfected 1-4 μg of the vectors containing the P450 cDNAs into the HEK293T cells, and we measured the rates of ERF and MRF metabolism by post-nuclear supernatants derived from these cells. When the expression levels of the CPR and different CYP1A proteins were determined by Western blotting and densitometry under these experimental conditions, the CPR/P450 ratios exceeded 4.5:1 in all of the post-nuclear supernatants (shown in Figs. 5, D and E). This is likely due to substitution of active site residues. From Figs. 5 and 6, it would appear that membrane localization does not affect the catalytic activities of the CYP1A enzymes. However, under these conditions of transfection, the CPR was expressed in large excess over that of the P450s. CPR concentrations are extremely limiting in vivo (25, 26). Thus, it was of interest to assess the effect of domain localization on CYP1A catalytic activities when the CPR concentration was limiting. We decided to compare the catalytic activities of the wild type CYP1A2-GFP (which partially localized to ordered domains) and the CYP1A1-CYP1A2-GFP chimera containing the 109 N-terminal amino acids of CYP1A1 (which was observed to reside entirely in the disordered regions of the ER) when the CPR concentration was limiting. To achieve these conditions, we transfected 4 μg of each of the expression plasmids for the CYP1A-GFP enzymes in the

FIGURE 6. Effect of N-terminal sequences of CYP1A2 on localization in ordered domains. The post-nuclear supernatants from HEK293T cells transfected with 2–4 μg of CYP1A-GFP wild type and chimeric constructs (A) were incubated with either 0 or 0.5% (v/v) Brij 98 at 37 °C for 5 min and subjected to centrifugation at 100,000 × g for 1 h to isolate detergent-soluble and insoluble fractions. Each fraction was analyzed by immunoblotting (B). The distribution of the intact and chimeric proteins are shown in C. HEK293T cells were co-expressed with 1 μg of CPR construct and each CYP1A-GFP construct, and the rate of substrate metabolism was determined in isolated PNS as described (D and E). These data represent the mean ± S.E. for three independent determinations. P, pellet; S, supernatant.
HEK293T cells without transfecting any CPR cDNA, and we compared the CYP1A catalytic activities using the post-nuclear supernatants derived from the cells (Fig. 7). The wild type enzyme expressed at higher levels than the chimera. Interestingly, the CPR expressed in proportion to the P450 enzymes, so the post-nuclear supernatants had virtually identical CPR/P450 ratios (1:11). When the rates of metabolism by the wild type and chimeric CYP1A2 proteins were compared under these conditions, the wild type enzyme catalyzed turnover of the substrates at approximately four times those catalyzed by the CYP1A1-CYP1A2 chimera despite the fact that the CPR/P450 ratios were the same for the two cellular extracts.

Is the N-terminal Sequence of CYP1A2 Sufficient to Direct Localization into Ordered Domains?—As mentioned previously, the early N-terminal regions of CYP1A1 and CYP1A2 show some of their greatest variability in amino acid sequence (Fig. 2). Before the first cut site, the variations are contained within 1–30 amino acids in CYP1A2 and 1–32 amino acids in CYP1A1. Therefore, we tested whether these short 30 amino acid sequences can target proteins to different domains. For this experiment, expression plasmids for short N-terminal segments of CYP1A1 (1–32 amino acids) and CYP1A2 (1–30 amino acids) were prepared with GFP tags at the C-terminal ends (Fig. 8A). After 48 h post-transfection, post-nuclear supernatants were collected, and pellets and supernatants from detergent-treated or -untreated samples were separated by high speed centrifugation. Interestingly, even without detergent treatment, 1–32aaCYP1A1 protein were found only in the supernatant. Thus, the protein did not reside in the membrane. However, the short CYP1A2-GFP fusion protein was successfully expressed in the membrane, and after detergent treatment, it was shown to localize in the ordered domains (Fig. 8B). The results demonstrate that the N-terminal region of CYP1A2 contains a signal sequence that is sufficient to target CYP1A2 into the ordered membrane regions.

**Microdomain-targeting Motifs of CYP1A Proteins**

![Graph showing the rate of metabolism for different CYP1A proteins](image)

**Figure 7. Catalytic activities of wild type and chimeric CYP1A proteins in PNS from transfected HEK293T cells when CPR concentrations were limiting.** HEK293T cells were transfected with 4 μg of each construct to express either the wild type CYP1A2-GFP or the chimeric CYP1A2-GFP resulting from substitution of the 109 N-terminal amino acids of CYP1A1. The post-nuclear supernatants were compared for the rates of substrate metabolism as described under "Experimental Procedures." These data represent the mean ± S.E. for three independent determinations (**, p < 0.01; ****, p < 0.0001).

**Figure 8. Microdomain localization of early N-terminal sequences of CYP1A enzymes.** Within the first cut site, most amino acid (aa) variations were found in the early N-terminal sequences. Fusion proteins were constructed that contained the N-terminal 30–32 amino acids of the CYP1A proteins fused to GFP (A). Each short CYP fragment-GFP was transfected into HEK293T cells. PNS was collected 48 h later after transfection. Intact or detergent-insoluble membranes were centrifuged at 100,000 × g for 1 h, following incubation with or without Brij 98 for 5 min at 37 °C. GFP-labeled proteins were detected by immunoblotting (IB) (B). P, pellet; S, supernatant.

**Involvement of Internal Sequences of CYP1A2 in Protein Microdomain Localization**—It is well known that the N-terminal regions of P450 enzymes are responsible for their integration into the ER membrane. However, recent studies suggested that sequences in the internal regions of the proteins, such as the F-G loop, are also important for interaction with the membrane (12, 14). In Figs. 5 and 6, we showed the importance of CYP1A2 N-terminal regions on microdomain localization. Substitution of the N terminus of CYP1A2 with that from CYP1A1 led to the localization of CYP1A2 into the disordered regions. Conversely, when CYP1A1 contained the N-terminal sequence from CYP1A2, some, but not all, of the CYP1A1 was found to localize to the ordered domains. The incomplete relocation of the CYP1A2-CYP1A1 chimeric proteins into ordered regions suggested that other regions of CYP1A2 might also contribute to domain localization of P450 proteins. Therefore, we examined the potential for other regions of CYP1A2 to participate in its microdomain localization. To accomplish this, CYP1A2 was divided into segments based on four cut points (1st, 1–107; 2nd, 108–205; 3rd, 206–302; 4th, 303–417; and 5th, 418–516 amino acids). Because the 1–107-amino acid sequence of CYP1A2 was necessary for microdomain localization (Figs. 5 and 6), the second, third, and fourth segments of CYP1A2 were alternately replaced with a corresponding CYP1A1 fragment. When CYP1A2 contained the 2nd segment from CYP1A1 (amino acids 108–205), no significant difference was observed compared with the distribution of original CYP1A2 proteins (Fig. 9). However, substitution of 3rd fragment of CYP1A2 (amino acids 206–302) with complementary sequence from CYP1A1 caused partial redistribution of the
CYP1A2 chimera to disordered regions (Fig. 9). These data demonstrate that an internal amino acid sequence also influences the membrane domain localization of CYP1A2 in addition to the early N-terminal region of the P450.

Discussion

In our previous study, we observed that CYP1A1 and CYP1A2 localized to different lipid microdomains (4). Because the two P450s share ~80% sequence identity, the regions of the P450s that affected microdomain localization in the endoplasmic reticulum could be elucidated by comparing the localization of CYP1A1-CYP1A2 chimeras. To examine the effects of N-terminal regions of CYP1A proteins on domain localization, N-terminal regions of CYP1A1 and CYP1A2 were switched. CYP1A2 containing the CYP1A1 N terminus was shown to localize to the disordered domains (Fig. 5). In contrast, CYP1A1 containing a CYP1A2 N terminus partially localized in the ordered regions of the ER (Fig. 6). In addition, the short N-terminal domain containing only 1–30 amino acids of CYP1A2 was sufficient for ordered domain localization (Fig. 8). An important role of the N-terminal regions of P450s is to serve as signal sequences for their targeting and insertion into the ER (11, 27). In this study, we have shown that another role of the N-terminal region is to influence microdomain localization of P450s from the CYP1A subfamily.

Even though we showed that the CYP1A2 N terminus was necessary for ordered domain localization of CYP1A2, the N terminus of CYP1A2 only led to a partial relocation of CYP1A1 to ordered domains (Fig. 6). Therefore, we tested the possible involvement of internal regions of the CYP1A proteins on their ability to alter their localization. We did not see a significant difference from replacement of the second internal segment (108–205 amino acids). However, the third segment from CYP1A1 (206–302 amino acids) caused most of CYP1A2 to re-localize into disordered domains (Fig. 8). Based on the secondary structure of human CYP1A2 (28), this fragment contains the F and G helices and F-G loop, regions reported to interact with membrane lipid (14), supporting a role for this region in affecting lipid domain localization. Additionally, part of this region serves as the ceiling for the P450 active site, making it important for substrate binding (15, 16).

One concern with the generation of chimeric proteins is their potential to change their localization characteristics due to misfolding. The fact that the chimeras used in this study retain their metabolic activities in the presence of excess CPR (Figs. 5 and 6) supports the idea that they have similar secondary and tertiary structures to the native enzymes. Interestingly, the NH₂-CYP1A1-CYP1A2 chimera containing only the first hundred N-terminal amino acids of CYP1A1 (i.e. are predominantly CYP1A2) retain CYP1A2 metabolic character, and the NH₂-CYP1A2-CYP1A1 chimeras (i.e. predominantly CYP1A1) metabolize substrates similarly to CYP1A1. Although the activity decreases significantly when the substrate-binding regions in the center of the proteins are switched in the chimera, these activities are not completely eliminated. These data are consistent with the CYP1A chimeras folding in a similar manner to the native enzymes.

FIGURE 9. Effect of cytoplasmic regions of CYP1A2 enzymes on microdomain localization. Based on four cut sites, about 100 amino acids of CYP1A2 (red) were replaced with matched amino acid sequences of CYP1A1 (blue). At 48 h after transfection, isolated PNS was treated with 0.5% (v/v) Brij 98 for 5 min at 37 °C, followed by centrifugation at 100,000 × g for 1 h. Protein distribution was analyzed by immunoblot (A). Relative distribution (%) of proteins was measured by densitometry, and the mean value of each sample from three independent experiments was plotted (B); significant differences from intact CYP1A2 were represented by *, p < 0.01. The rates of metabolism of ERF by the wild type CYP1A2-GFP and the (206–302 amino acids) CYP1A1-CYP1A2-GFP chimera were determined in isolated PNS 48 h after transfecting 2 and 3 μg of each construct, respectively, in HEK cells along with 1 μg of rabbit CPR construct. The rates represent the average and standard errors of three determinations (C). P, pellet; S, supernatant.
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After confirming that the chimeras were not defective catalytically, we tested whether the domain localization might have influenced catalytic activities of the wild type and chimeric CYP1A enzymes when the CPR concentration was rate-limiting. We chose to compare the CYP1A1-CYP1A2 chimera that substituted the 109 N-terminal amino acids of CYP1A1 for those in the CYP1A2 sequence because this substitution caused the complete redistribution of the CYP1A enzyme from ordered domains to the disordered regions of the membrane, and the catalytic activity of the chimera was indistinguishable from that of the wild type when CPR was expressed in excess of the P450. However, when the activities of the CYP1A-GFP enzymes were measured in the presence of a limiting concentration of CPR, the wild type enzyme had 4-fold greater catalytic activity than the chimera (Fig. 7), demonstrating that microdomain localization influences P450 function. In a previous study from our laboratory (3), the binding affinity of CPR and CYP1A2 was shown to be dramatically increased by the presence of ordered membrane domains in lipid-reconstituted systems. Thus, in a membrane environment that simulates that found in vivo, it is likely that membrane domain localization stimulates catalytic activity of CYP1A2 due to the colocalization of CPR and CYP1A2 in the ordered microdomains.

Several studies have shown that P450 function is affected by changes in phospholipid composition. Eberhart and Parkinson (29) showed that CYP3A1 requires phospholipids containing unsaturated fatty acids in order to function optimally. In their study, CYP3A1-dependent testosterone 6β-hydroxylation was undetectable when reconstituted into phosphatidylcholine with saturated fatty acid chains; however, significant activity was observed with phosphatidylcholine containing unsaturated fatty acyl chains as well as natural lipids. Balvers et al. (30) showed that the composition of phospholipids that surround CPR are significantly different from that of the microsomal phospholipids, being enriched in phosphatidylserine (PS) and phosphatidylinositol. The study also showed an increased apparent rate of CYP2B1-dependent pentoxyresorufin-O-dealkylation with reconstituted systems enriched in PS. However, this stimulation was not common to all anionic phospholipids as phosphatidylinositol decreased the reaction rate.

The effect of anionic phospholipids on membrane binding and function of several P450 proteins has been studied by other groups. It has been shown that negatively charged phospholipids increase the rate of reduction of CYP2B4 by CPR by favoring a higher order complex between the enzymes that results in an increase in the proportion of P450 reduced in the fast phase of reduction (31, 32). CYP1A2 also was shown to be more integrally associated with the membrane when reconstituted in the presence of the anionic phospholipids phosphatidic acid, PS, and phosphatidylinositol. These phospholipids also increased monooxygenase activity when reconstituted with CPR (8). The anionic phospholipids phosphatidic acid and cardiolipin were also shown to stimulate CYP1B1-dependent 7-ethoxyresorufin metabolism, although PS was ineffective, demonstrating that the effect of the anionic phospholipids was dependent on the P450 being examined (9). Interestingly, the uncoupling of CYP2E1-dependent activities was diminished in the presence of the anionic phospholipids cardiolipin and PS, without inhibiting the monooxygenase reaction (6). Taken together, these studies and our data show that phospholipid composition has a significant effect on the membrane localization and function of P450 system proteins.

These experiments have focused on potential interactions between P450 system proteins and the membrane; however, there are also potential effects of protein-protein interactions on their microdomain localization. In a recent report, the presence of CYP1A2 was shown to cause CPR to localize to a greater extent in ordered membrane regions in reconstituted systems. In contrast, CPR did not appear to affect CYP1A2 localization (33). The interplay among lipid membranes and multiple P450 system proteins is currently under investigation.

As mentioned, HEK293T cells were used for examining localization of the CYP1A proteins, raising questions regarding how localization might differ from that found in liver cells. Although we have not systematically compared microdomain localization of HEK293T cells with hepatocytes, there are differences that could potentially influence protein localization, including the smaller amount of endoplasmic reticulum in HEK cells, and potential differences in lipid composition. Regardless, the HEK293T cells serve as an effective model for examining P450 localization, having the advantage of not exhibiting high levels of expression of P450 system proteins that could confound our results, particularly when trying to measure enzyme activities. Although not a perfect “mimic” of the hepatocytes, the HEK293T cells serve as a useful model system for examining localization of P450 system proteins.

When comparing the sequences of rabbit and human CYP1A2, we obtained a 90% similarity overall and about 80% for the first 30 amino acids, with a similar result being obtained with CYP1A1 at 88 and 78%, respectively (EMBOSS Matcher). Interestingly, the N terminus of rabbit CYP1A1 and CYP1A2 exhibited 89% sequence similarity, suggesting that subtle substitutions may affect localization of these proteins, which could influence their interactions with other P450 system proteins and as a consequence their catalytic characteristics. The specific amino acids responsible for membrane localization are currently being examined.

Unlike conserved subcellular targeting sequences that are recognized by receptors or signal recognition particles, identified microdomain-targeting sequences from other proteins are highly variable. Those microdomain-targeting sequences were found in all parts of proteins such as transmembrane, extracellular, and cytoplasmic domains (34–37). Besides those data, many studies have shown that association of saturated lipids to proteins during post-translational modification affects microdomain localization. For example, a glycosylphosphatidylinositol anchor, palmitoylation, and myristoylation for proteins during post-translational modification affects microdomain localization. For example, a glycosylphosphatidylinositol anchor, palmitoylation, and myristoylation for proteins increased the affinity for ordered domains and directed the proteins to the domains (38–40). Even though such lipid modifications have not been examined with P450, increasing evidence has shown post-translational modifications of P450, including phosphorylation, nitration, and glycosylation, suggesting that the modifications are involved in facilitating the proper catalytic function of P450 (41). For example, phosphorylation was found in many P450s, including CYP2B1, CYP2E1, and CYP3A4, and the modification resulted in modulation of
enzyme activity, dual targeting of P450s to the ER and mitochondria, or degradation (42–44). Further studies are required to determine whether amino acid sequences within the identified regions are targeted for post-translational modifications that may enhance interaction of P450 with specific membrane regions. Despite the potential for post-translational modifications to further influence localization of P450 proteins, our results clearly demonstrate that specific regions of the CYP1A enzymes lead to their targeting to different regions of the ER membrane.

In conclusion, the data presented here demonstrate not only that CYP1A1 and CYP1A2 reside in different regions of the endoplasmic reticulum but also that their localization is affected by both the N-terminal region and an internal segment of CYP1A2. Further studies will be required to identify the specific residues (or combinations of residues) that direct P450 proteins into particular microdomains and to determine the effects of domain localization on P450 activity, substrate binding, and P450 association with its reduct partners.

Author Contributions—J. W. P. conducted most of the experiments, analyzed the data, and wrote the initial draft of the paper. J. R. R. conducted the experiments examining the functional consequences of microdomain localization of the chimeric proteins and analyzed the data. The manuscript was jointly written by J. W. P., J. R. R., and W. L. B. W. L. B. conceived the idea, and J. W. P. and J. R. R. developed the project. All authors have approved the final published version of the manuscript.

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