The pregnane X receptor (PXR, NR1I2) and constitutive active/androstane receptor (CAR, NR1I3) are involved in the primary response to xenobiotics and endogenous toxins (1, 2).

These receptors respond to ligands by activating the expression of genes encoding enzymes involved in phase I (oxidation) and phase II (conjugation) metabolism as well as proteins involved in the efflux of toxins from the cell (3–5). CAR is normally sequestered in the cytoplasm of untreated liver cells and translocates to the nucleus after exposure to PB and PB-like chemicals (6). The cytoplasmic CAR retention protein (CCRP and designated Dnajc7 in the official gene symbol in the NCBI database) has been shown to maintain the cytoplasmic localization of CAR by forming a complex with CAR and hsp90 (7, 8). Other nuclear receptors, such as the glucocorticoid receptor (GR), vitamin D receptor (VDR), and aryl hydrocarbon receptor also exist as complexes with receptor-specific co-chaperones and hsp90 that play an important role in locating these receptors in the cytoplasm of unstimulated cells (9). These co-chaperones, including immunophilin-FK506-binding proteins and hepatitis B virus protein X-associated protein 2, contain multiple tetra-tricopeptide repeat (TPR) motifs, which are 34 amino acid sequences that form a pair of anti-parallel α helices that mediate protein-protein interactions and the assembly of multi-protein complexes (10).

The movement of proteins between the nucleus and the cytoplasm is an energy-dependent process determined by both nuclear localization sequence (NLS) and nuclear export sequence on the protein (11). The typical NLS consists of a single or repeat cluster of basic amino acids that associate with factors such as importins, which carry the proteins into the nucleus through a nuclear pore complex. The typical nuclear export sequence is a leucine-rich region and has been identified on a number of proteins, including the aryl hydrocarbon receptor (12, 13). After binding to ligand, the nuclear receptor GR moves along cytoskeletal tracks toward the nucleus by connecting with dynein motors (reviewed in Ref. 13). The C-terminal AF2 domain is required for ligand-dependent transcriptional activation by steroid hormone and many other nuclear receptors (14), and it is displaced upon the binding of ligand, allowing it to interact with co-activators. The removal of the AF2 domain prevents the nuclear translocation of the VDR and GR (15, 16). However, the AF2 domain is not required for the nuclear translocation of CAR following PB exposure (17). The nuclear translocation of CAR following drug exposure is dependent on the xenochemical response sequence (XRS), a leucine-rich sequence near the C terminus conserved as (L/M)XXLXX (17) in mouse and human CAR and PXR. However, mutations in the key leucine residues in the XRS did not affect the formation of heterodimers between CAR and RXR or the co-activation of CAR by SRC-1. This suggests that the XRS regulates the translocation but not the activation of CAR.

In contrast to CAR, PXR is generally thought to be primarily retained in the nucleus where it activates the expression of genes such as Cyp3a11 after binding ligands such as PCN. Thus, the possibility that co-chaperones could regulate the
cellular localization of PXR has not been investigated. However, recent results of the immunostaining of mouse liver sections using a commercially available antibody suggest that mPXR may be located in the cytosol of untreated liver cells (18); however, additional supporting experiments, such as Western blot analysis, were not presented. Moreover, in transformed cells, human hPXR (SXR) is located primarily in the nucleus, and mutation of specific amino acids in the so-called NLS region of the DNA binding domain of hPXR resulted in cytoplasmic localization of the receptor in transformed cells (18). However, the role of the NLS as well as other specific motifs (AF2 and XRS) in the nuclear translocation of mPXR in the liver following drug treatment remains unexplored.

Our goal was to use more definitive methods to determine whether mPXR was located in the cytoplasm of untreated liver and translocated to the nucleus only after exposure to xenobiotics. To achieve this, we produced specific antibodies against the hinge region of mPXR and used these antibodies in the Western blotting of liver nuclear extracts prepared from control mice and those treated with PCN. We also investigated the interaction of mPXR with CCRP in the cytosol to determine whether CCRP played a role in maintaining mPXR in the cytosol. We then looked at the NLS, XRS, and the AF2 domain, which may be involved in the nuclear translocation of mPXR in response to drug treatment, to determine whether these components also affected the association of mPXR with CCRP. Finally, we investigated the role of endogenous CCRP in the activation of mPXR in response to drug treatment.

**Experimental Procedures**

**Antibodies**—Anti-mPXR antibody was raised in rabbits using the hinge region peptide NH2-CSNAAVEQRLIRIRKRE-COOH conjugated to keyhole limpet hemocyanin as the antigen. The antibody was purified from serum by affinity chromatography using the peptide coupled to SulfoLink gel (Pierce). Rabbit anti-CAR antibody and rabbit anti-CCRPR antibody were produced as described previously (7, 19). Anti-hsp90 monoclonal antibody was purchased from Affinity Biosciences, and anti-V5-horseradish peroxidase antibody was purchased from Promega. Anti-lamin B was from Santa Cruz Biotechnology (sc-20682, Santa Cruz, CA).

**Plasmids**—cDNAs encoding full-length mPXR, mPXRΔAF2 (residues 1–416), hPXR, mCAR, and CCRP were PCR-amplified using the appropriate primers and cloned into pcDNA3.1/V5-His-TOPO (Invitrogen) to produce pcDNA3.1/mPXR-V5-His, pcDNA3.1/mPXRΔAF2-V5-His, pcDNA3.1/mCAR-V5-His, pcDNA3.1/pcDNA3.1/CCRPR/V5-His, mPXR and CCRP were also amplified using 5′ primers containing an in-frame XhoI site and 3′ primers containing an EcoRI site and cloned into pEYFP-C1 or pECFP-C1 expression vectors (BD Biosciences) to produce a N-terminal fusion with green fluorescent protein. The XREM-3A4-Luc (p3A4–362/7836/720InsI) reporter plasmid was kindly provided by Dr. Bryan Goodwin (Gliaxoz-Smith/Klein) (20). The following mutants were constructed using the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) and the appropriate primers: pcDNA3.1/mPXR-V5-His or pEYFP-mPXR containing either the mutations M391A, L394A, or L397A in the XRS region and either the mutations M391A, L394A, or L397A in the XRS region and mutation of specific amino acids in the so-called NLS region of the DNA binding domain of hPXR resulted in cytoplasmic localization of the receptor in transformed cells (18). However, the role of the NLS as well as other specific motifs (AF2 and XRS) in the nuclear translocation of mPXR in the liver following drug treatment remains unexplored.

Our goal was to use more definitive methods to determine whether mPXR was located in the cytoplasm of untreated liver and translocated to the nucleus only after exposure to xenobiotics. To achieve this, we produced specific antibodies against the hinge region of mPXR and used these antibodies in the Western blotting of liver nuclear extracts prepared from control mice and those treated with PCN. We also investigated the interaction of mPXR with CCRP in the cytosol to determine whether CCRP played a role in maintaining mPXR in the cytosol. We then looked at the NLS, XRS, and the AF2 domain, which may be involved in the nuclear translocation of mPXR in response to drug treatment, to determine whether these components also affected the association of mPXR with CCRP. Finally, we investigated the role of endogenous CCRP in the activation of mPXR in response to drug treatment.

**Preparation of Mouse Liver Nuclear Extracts**—Nuclear extracts were prepared based on published methods (24, 25). Liver was homogenized in 1 ml of 10 mM HEPES buffer, pH 7.6, containing 10% glycerol, 10% EGTA and 10% FBS. The homogenate was added to 20-fold diluted HEPES buffer, pH 7.6, containing 0.4 M NaCl and centrifuged at 10,000 × g for 10 min at 4 °C to obtain a clear cytosolic fraction for use in immunoprecipitation. The homogenate was washed twice with phosphate-buffered saline, and the supernatant was centrifuged at 17,800 × g for 10 min to obtain a clear cytosolic fraction for use in immunoprecipitation. The homogenate was layered over a cushion of the same buffer and centrifuged at 50,000 × g for 60 min at 4 °C. The pellet was suspended in 1 ml of lysis buffer (as described above), mixed at 4 °C for 30 min in the presence of 0.4 M NaCl, and centrifuged at 100,000 × g for 30 min. The supernatant was added to 20-fold diluted HEPES buffer, pH 7.6, containing 20% glycerol, 0.2 mM EDTA, 1 mM Na2VO4, 1 mM dithiothreitol, and Complete protease inhibitor. The resulting suspension was mixed at 4 °C for 12 h in the presence of 0.4 M NaCl and centrifuged for 10 min at 4 °C to obtain a clear nuclear extract.

**Immunoprecipitation**—Anti-hsp90 (5 μl) or IgM (1 μg), as a control, was added to 2 μg of cytosolic protein from HepG2 cells and incubated overnight at 4 °C. Twenty μl of a 50% slurry of protein L-Sepharose (Immunopure immobilized protein L, Pierce) previously washed in 2% BSA was added, and the mixture was incubated for 1 h at 4 °C. The resin was recovered by centrifugation and washed five times with 1 ml of 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 20 mM Na2MoO4, and 0.2% Nonidet P-40. The immunoprecipitated proteins were extracted with NuPAGE LDS sample buffer (Invitrogen) and subjected to Western blotting.

**Western Blotting**—Proteins were separated either on a NuPAGE 4–10% BisTris gel in NuPAGE MOPS SDS running buffer (Invitrogen) or a 10% SDS gel in Tris-glycine buffer and transferred to nitrocellulose membrane using the SemiPhor semi-dry transfer unit (Hoefer Scientific Instruments, San Francisco, CA). The membrane was then incubated for 1 h in a mixture of TBS and 0.1% Tween 20 containing 5% Blotto milk powder followed by 1 h of incubation with primary antibody (rabbit anti-mPXR, rabbit anti-mCAR) in the same medium and 1 h with secondary antibody (donkey anti-rabbit IgG horseradish peroxidase conjugate, Santa Cruz Biotechnology). For the detection of V5-tagged proteins, the nitrocellulose membranes were incubated with anti-V5-horseradish peroxidase conjugate in a mixture of TBS and Tween 20 with 5% Blotto. The protein bands were visualized on the membranes using Lumigen PS-5 ECL detection reagent (Amersham Biosciences). In some cases, the immunoblots were stripped with Restore Western blot stripping buffer (Pierce) and reprocessed with antibodies.

**GST Pull-down Assay**—The GST-CCRPR fusion protein was expressed and purified using glutathione-Sepharose 4B (Amersham Biosciences) (7). 35S-labeled mPXR and mCAR were produced from pcDNA3.1/mPXR-V5-His and pcDNA3.1/mCAR-V5-His using the Tnt Quick coupled transcription/translation system (Promega) along with 35S-labeled methionine. GST-CCRPR or GST coupled to the glutathione-Sepharose 4B was incubated with the 35S-labeled mPXR or mCAR in 50 mM HEPES buffer, pH 7.5, containing 0.1 M NaCl and 0.1% Triton X-100 for 20 min at room temperature. The resin was then recovered by centrifugation and washed three times in the same buffer.

Proteins were extracted from the resin by heating for 10 min at 70 °C in NuPAGE LDS sample buffer (Invitrogen) and separated on a NuPAGE 4–10% BisTris gel in NuPAGE MOPS SDS running buffer (Invitrogen) and blotted to nitrocellulose. The membrane was then incubated for 1 h in a mixture of TBS and 0.1% Tween 20 containing 5% Blotto milk powder followed by 1 h of incubation with primary antibody (rabbit anti-mPXR, rabbit anti-mCAR) in the same medium and 1 h with secondary antibody (donkey anti-rabbit IgG horseradish peroxidase conjugate, Santa Cruz Biotechnology). For the detection of V5-tagged proteins, the nitrocellulose membranes were incubated with anti-V5-horseradish peroxidase conjugate in a mixture of TBS and Tween 20 with 5% Blotto. The protein bands were visualized on the membranes using Lumigen PS-5 ECL detection reagent (Amersham Biosciences). In some cases, the immunoblots were stripped with Restore Western blot stripping buffer (Pierce) and reprocessed with antibodies.
mPXr Accumulates in the Nucleus of Liver Cells after Drug Treatment—To determine the cellular localization of endogenous mPXr in mouse liver in vivo, Western blotting of liver nuclear extracts was performed using an antibody raised against the hinge region of mPXr (Fig. 1A). Levels of mPXr were lowest in nuclear extracts from untreated mice or mice treated with MeSO vehicle and increased following PCN treatment but not after PB treatment. When the same blot was stripped and then immunostained using antibody against mouse CAR, levels of CAR were highest in nuclear extracts from PB-treated mice, with no effect of PCN treatment. The results obtained from the present Western blot analysis have now established that mPXr accumulates in the nucleus of liver cells following PCN treatment. This nuclear accumulation of mPXr occurred in a time-dependent manner. Livers were removed from untreated mice and, at various times up to 40 h after treatment with PCN and nuclear extracts, were prepared and used for Western blotting using anti-mPXr antibody. The results (Fig. 1B) show that levels of mPXr in the nucleus increased rapidly after treatment with PCN and were already maximal at the first time point of 3 h after treatment. Levels of mPXr remained high in the nuclear extracts at 8 h after PCN treatment and decreased by the 17-h time point. To compare the time course for gene activation with the accumulation of mPXr in the nucleus after treatment with PCN, levels of CYP3A11 mRNA were measured in the livers by real-time PCR. Levels of CYP3A11 mRNA increased to a maximum at the 17-h time point after PCN treatment and remained elevated over untreated control values at 40 h after treatment. This indicates that activation of the CYP3A11 gene occurred after mPXr accumulates in the nucleus in response to PCN treatment. Thus, mPXr moves into the nucleus in response to PCN treatment, activates transcription of responsive genes, and then is cleared from the nucleus.

Structural Components of mPXr That Affect Nuclear Translocation—We investigated the cellular localization of mPXr by in vivo transfection of YFP-tagged mPXr in mouse liver. In untreated liver, YFP-mPXr was localized throughout the cytoplasm of the liver cell (Fig. 2A). However, in liver sections from mice that had been treated with PCN, YFP-mPXr was present predominantly in the nucleus. This cytoplasmic retention and nuclear translocation of YFP-mPXr are reminiscent of what
occurs with the endogenous receptor. To identify the structural features of mPXR that affect nuclear translocation in vivo, we expressed mPXR and its various mutants as YFP fusion proteins in mouse livers. These mPXR mutants contained substitutions in the XRS region or the NLS region in the DNA binding domain or had the C-terminal AF2 region deleted (Fig. 2B). We then measured the percentage of cells with mPXR expressed predominantly in the cytoplasm, the percentage with equal nuclear and cytoplasmic localization, and the percentage with primarily nuclear localization of mPXR (Fig. 2A). Wild type YFP-mPXR was located primarily in the cytoplasm in untreated mouse liver and translocated to the nucleus after treatment with PCN. However, mutations in key amino acid residues in the XRS or NLS region or deletion of the AF2 domain prevented nuclear translocation in vivo in response to treatment with PCN. The distribution of the NLS and XRS mutants remained almost entirely cytoplasmic, whereas the AF2 deletion mutant had slightly more nuclear distribution, which was not affected by PCN treatment. These results suggest that the XRS, NLS, and AF2 regions are all required for

**FIG. 2.** Cellular localization of wild type mPXR, XRS mutants, NLS mutants, and mPXRΔAF2 in mouse liver before and after drug treatment. Plasmids encoding mPXR and its various mutants as YFP fusion proteins were injected via the tail vein using the TransIT in vivo gene delivery system. A, mice were either untreated (Control) or injected intraperitoneally with 15 mg/kg PCN and killed 6 h later. Liver sections were prepared and examined by microscopy for YFP expression (in yellow) and Hoechst 33342 (0.5 μg/ml) staining for nuclei (in blue). Liver sections from untreated mice showing cytoplasmic expression of YFP and from mice injected intraperitoneally with PCN showing nuclear localization of YFP. The percentage of cells with PXR expressed predominantly in the cytoplasm (C > N), the percentage with equal nuclear and cytoplasmic localization (C ≈ N), and the percentage with primarily nuclear localization of PXR (C < N) were determined. At least 50 cells from four different sections were examined for each treatment, and the results are expressed as mean ± S.D., using open and closed bars for control and PCN treatments, respectively. WT, pEYFP-mPXR; M391A, pEYFP-mPXR M391A; L394A, pEYFP-mPXR L394A; R63A/R64A/R85A/R86A, pEYFP-mPXR R63A/R64A/R85A/R86A; R63A/R64A/R88A/R89A, pEYFP-mPXR R63A/R64A/R88A/R89A; mPXRΔAF2, pEYFP-mPXRΔAF2 with the C-terminal AF2 region deleted to include amino acid residues 1–416. B, schematic representation of NLS, XRS, and AF2 in mPXR molecule. DBD, DNA binding domain; LBD, ligand binding domain.
FIG. 3. Association of mPXR and CCRP. A, GST pull-down assay. 35S-labeled mPXR and mCAR were produced by in vitro translation and incubated with equal amounts of either GST-CCRP or GST coupled to glutathione-Sepharose 4B, and the proteins bound to the resin were examined by SDS-PAGE and autoradiography. The input represents 5% of the amount of 35S-labeled mPXR and mCAR used in the pull-down assay. B, CCRP forms a cytoplasmic complex with mPXR in HepG2 cells. HepG2 cells were transfected with 8 μg of pcDNA3.1/mPXR-V5-His and with from 0 to 16 μg of pcDNA3.1/CCRP-V5-His. Cytosols were prepared and used for immunoprecipitation (IP) using anti-hsp90 antibody or normal IgM and Western blotting stained with anti-V5 antibody. The input represents 0.5% of the amount of cytosol used for the immunoprecipitation.

the nuclear translocation of mPXR after PCN treatment in vivo.

Role of CCRP in Cytoplasmic Localization of mPXR—CCRP is involved in maintaining the cytoplasmic localization of CAR (7). We hypothesized that CCRP could also bind to mPXR and was involved in the retention of mPXR in the cytoplasm. As a first step, we used a GST pull-down assay to demonstrate that GST-CCRP can bind to mPXR as well as binding to CAR (Fig. 3A). Next we expressed mPXR-V5 and CCRP-V5 in HepG2 cells and found that they formed a complex in the cytosol with hsp90 that can be immunoprecipitated by anti-hsp90 antibody (Fig. 3B). Furthermore, when the ratio of CCRP to mPXR in transfected HepG2 cells is increased, the retention of mPXR in the cytosol is also increased. We then did a FRET analysis of mouse liver sections after co-expression of CFP-CCRP and YFP-mPXR. The results indicated that CCRP and mPXR are closely associated in vivo to allow FRET to occur. Before photobleaching of YFP, excitation at 458 nm produced a clear FRET signal from CFP-CCRP to increase the signal observed from YFP-mPXR (Fig. 4A). After photobleaching of YFP, this FRET did not occur, and the CFP signal intensity increased. The CFP and YFP signal intensity was measured before and after photobleaching in four different regions of the cell (Fig. 4B). After photobleaching, the YFP signal intensity decreased rapidly with a concomitant increase in the signal intensity of CFP. Measurements of FRET efficiency in seven different cells ranged from 49 to 66%, which suggested that CFP-CCRP and YFP-mPXR were within an estimated distance of 4.69 ± 0.23 nm of each other. Taken together, these results demonstrated that mPXR forms a complex with CCRP that may promote the retention of mPXR in the cytoplasm of liver cells.

Given that mPXR and CCRP could form a complex with hsp90, we expressed mPXR-V5 and its various mutants in HepG2 cells along with CCRP-V5 to investigate whether the signature motifs (NLS, XRS, and AF2) were involved in the formation of the mPXR-CCRP-hsp90 complex. Immunoprecipitation of cytosols with hsp90 antibody demonstrated that the various mPXR mutants can all form a complex with CCRP and hsp90 in the cytosol with approximately equal efficiency (Fig. 5). Thus, the extent of binding of mPXR mutants to CCRP in the cytosolic complex is not correlated with the amount of the mPXR mutant that translocates to the nucleus after PCN treatment, as shown in Fig. 2A. Thus, the CCRP-mediated retention of mPXR in the cytosol of HepG2 cells does not fully explain the mechanism of nuclear translocation of mPXR in liver in vivo.

Role of CCRP in Regulating the Activity of PXR—We next transfected HepG2 cells with pcDNA3.1/mPXR-V5-His and pcDNA3.1/CCRP-V5-His and immunoprecipitated the CCRP-mPXR complex with anti-hsp90 antibody. When the Western blot was stained with anti-CCRP antibody, the endogenous CCRP that was immunoprecipitated along with the CCRP-V5 could be clearly seen, indicating that the endogenous CCRP also forms a complex with endogenous hsp90 and the transfected mPXR in HepG2 cells (Fig. 6A). Immunoprecipitation experiments using anti-V5 antibody also confirmed that hsp90 was precipitated along with mPXR-V5 and CCRP-V5 (data not shown). We then wanted to determine whether CCRP affects the activation of PXR in response to drug treatment. When siRNA for CCRP was transfected in HepG2 cells, a clear decrease in endogenous CCRP protein was observed by Western blotting (Fig. 6B), whereas there was no effect on levels of mPXR in the nucleus. The decrease in CCRP expression by siRNA treatment resulted in an attenuated response in a reporter assay for mouse PXR activity, with a more dramatic response observed with 50 μM PCN than with 10 μM PCN. In addition, the response of human PXR to activation by rifampicin was also attenuated by treatment with siRNA for CCRP. This clearly demonstrated that CCRP plays a role in the activation of both mouse and human PXRs following treatment with their activators.

DISCUSSION

We produced an antibody directed against the hinge region of mPXR and used this antibody in Western blots to show that the levels of mPXR initially increase in the nucleus of mouse liver cells after PCN treatment, followed by activation of a mPXR-responsive gene, and then levels of mPXR decrease in the nucleus. Expression of YFP-mPXR in mouse liver in vivo revealed that mPXR is expressed in the cytoplasm of untreated liver cells and is translocated in the nucleus after PCN treatment. These data definitively demonstrate that nuclear translocation of mPXR occurs, as with other nuclear receptors such as CAR, GR, and VDR, as a primary step in the activation of mPXR-responsive genes.

PXR transfected into transformed cells normally accumulates spontaneously in the nucleus without drug treatment. Using this model, Kawana and his associates (18) identified a NLS region in hPXR (SXR) that, when mutated, resulted in...
accumulation of the receptor in the cytosol of transformed cells. They concluded that the NLS is essential in the ligand-independent translocation of human PXR in HeLa cells. They also found that hPXR with the AF2 domain deleted was accumulated in the nucleus, suggesting that the AF2 domain was not necessary for nuclear translocation. The results of our experiments, in which various mutants of mPXR were expressed in mouse liver \textit{in vivo}, demonstrate that the PCN-dependent nuclear translocation requires not only the NLS but also the AF2 domain. In addition to the NLS and the AF2 domain, mPXR contains the XRS motif that is known to regulate the nuclear translocation of both mouse and human CAR in mouse liver \textit{in vivo} (17). Our experiments indicate that the XRS motif is also required for the nuclear translocation of mPXR to occur in response to PCN treatment. The differences between the findings of Kawana and associates (18) and the present work may be due to differences between hPXR and mPXR, cell types used, and/or differences between the model of spontaneous accumulation of PXR in the nucleus and nuclear translocation of PXR in response to drug treatment. Because all of the NLS, XRS, and AF2 domains are required for nuclear translocation of mPXR \textit{in vivo}, they may all function in the mechanism of nuclear translocation of mPXR.

In contrast to mPXR, hCAR does not need either the NLS or the AF2 domain for nuclear translocation, requiring only the ligand binding domain containing the XRS region. CAR lacking its AF2 domain or everything except the C-terminal half of the LB domain (residues 181–348) translocated to the nucleus in mouse liver \textit{in vivo} following treatment with CAR activators (17, 21). The NLS region of CAR may not be effective for

**Fig. 4. Association of mPXR and CCRP FRET assay.** pEYFP-mPXR and pECFP-CCRP were injected via the tail vein using the TransIT \textit{in vivo} gene delivery system, and liver sections were prepared for confocal laser scanning microscopy. A, CFP and YFP protein images before and after bleaching of YFP-mPXR. Before the bleaching of YFP, FRET from CFP increases the signal intensity from YFP and decreases the signal from CFP. After the bleaching of YFP, FRET does not occur, therefore, that signal from CFP is increased. The thickness of the closed arrows in the schematic drawing represents intensity of fluorescence. B, quantification of CFP and YFP intensities from three ROIs (regions of interest) of a cell (boxes 1, 2, and 3) and the whole cell overall (ROI 4) over time. The graphs are showing YFP and CFP fluorescent intensity changes by the photobleaching YFP in each ROI. Photobleaching began after recording the base-line CFP and YFP intensities twice.
nuclear translocation because of the substitution of a highly conserved lysine by serine in human, rat, and mouse CAR compared with PXR and VDR (18). In fact, a chimeric hPXR containing the NLS of hCAR underwent nuclear translocation as observed with hCAR. The AF2 domain regulates receptor function and is activated by the direct binding of ligand. The direct binding of agonistic ligand initiates GR and VDR to translocate into the nucleus (16). In contrast, the mechanism of nuclear translocation of CAR may be distinct, because the CAR activator PB does not bind to CAR, and the AF2 domain is not required for translocation of CAR. Because PCN binds directly to mPXR and the translocation requires the AF2 domain, the regulatory mechanism for nuclear translocation may be similar to that of GR and VDR.

CCRP (a mouse ortholog of human TPR2 (26)), a co-chaperone of the class III TPR family, which includes proteins that are involved in antiviral interferon response, the stress response, and protein import, was identified as a CAR binding protein by yeast two-hybrid screening of a mouse liver cDNA library using CAR as bait. CCRP, acting as a co-chaperone, mediates the formation of the CAR-CCRP-hsp90 complex in the cytosol of HepG2 cells (7). We have shown here that mPXR can also bind to the co-chaperone CCRP to form a complex within the cytosol along with hsp90, increasing the retention of mPXR in the cytosol of HepG2 cells. In addition, our FRET analysis indicates that CCRP and mPXR are closely associated in mouse liver in vivo. CCRP has also been reported to bind to the GR and is required at a narrowly defined expression limit for maximal activation of the GR (27). Similar to the GR activity, we have found that the mPXR-mediated trans-activation of genes is modulated by CCRP in HepG2 cells. These observations suggest that CCRP may play a role not only in the cytoplasmic retention of the receptors, but also in their activation in the nucleus. The role of hsp90 and TPR proteins in the formation of complexes with signaling proteins, such as steroid receptors, and their movement to the nucleus has recently been

---

**Figure 5.** Immunoprecipitation of CCRP and mPXR mutants from HepG2 cells with anti-hsp90 antibody. HepG2 cells were transfected with pcDNA3.1/CCRP-V5-His and various mutants of pcDNA3.1/mPXR-V5-His. Cytosols were prepared and used for immunoprecipitation (IP) using anti-hsp90 antibody or normal IgM and Western blotting using anti-V5 antibody. The input represents 0.5% of the amount of cytosol used for the immunoprecipitation. M391A, pcDNA3.1/mPXR M391A V5-His; L394A, pcDNA3.1/mPXR L394A V5-His; L397A, pcDNA3.1/mPXR L397A V5-His; R63A/R64A/R85A/K86A, pcDNA3.1/mPXR R63A/R64A/R85A/K86A V5-His; R63A/R64A/R88A/R89A, pcDNA3.1/mPXR R63A/R64A/R88A/R89A V5-His; mPXR AF2, pcDNA3.1/mPXR AF2 V5-His with the C-terminal AF2 region deleted to include amino acid residues 1–416.

**Figure 6.** Effects of endogenous CCRP on PXR in HepG2 cells. A, immunoprecipitation (IP) of endogenous CCRP. HepG2 cells were transfected with 8 μg of pcDNA3.1/mPXR-V5-His and 0, 4, or 8 μg of pcDNA3.1/CCRP-V5-His. Cytosols were prepared and used for immunoprecipitation using anti-hsp90 antibody or normal IgM and Western blotting and stained with anti-CCRP antibody. The input represents 0.5% of the amount of cytosol used for the immunoprecipitation. B, effect of treatment with siRNA on expression of CCRP. HepG2 cells were transfected with pcDNA3.1/mPXR-V5-His along with siRNA for CCRP or siRNA scramble control. Total cell extract was prepared for Western blotting and stained with anti-CCRP antibody or anti-PXR antibody. C, reporter assay showing the fold induction of PXR activity due to drug treatment (solid bars) compared with untreated controls (open bars). HepG2 cells were transfected with pcDNA3.1/mPXR-V5-His or pcDNA3.1/hPXR-V5-His and XREM-3A4-Luc reporter. Values are expressed as mean ± S.D.

---

2 T. Sueyoshi and M. Negishi, unpublished observation.
reviewed (14). The GR forms a complex with hsp90 and immunophilins, which are TPR proteins that link the complex to dynein motor proteins for retrograde movement along microtubules to the nucleus. However, the mechanism by which CCRP regulates the retention and activation of these receptors remains an interesting target for further investigations.

In summary, we have shown that mPXR is located in the cytoplasm of untreated liver cells and is concentrated in the nucleus following drug treatment. mPXR forms a complex with CCRP and hsp90 that maintains the receptor in the cytosol. The formation of the mPXR-CCRP-hsp90 complex is not dependent on the XRS, NLS, or AF2 domains in mPXR. However, these regions are all required for the nuclear translocation of mPXR. Thus, although CCRP is involved in maintaining mPXR in the cytosol, the binding of mPXR to CCRP does not regulate the nuclear translocation of mPXR in response to PCN treatment. However, CCRP does modulate the activation of mPXR in response to drug treatment. This demonstration of the cytoplasmic localization of mPXR in untreated liver and the role of CCRP should stimulate future research on the mechanism of nuclear localization and activation of PXR following drug treatment.

REFERENCES
1. Goodwin, B., Redinbo, M. R., and Kliewer, S. A. (2002) Annu. Rev. Pharmacol. Toxicol. 42, 1–23
2. Sueyoshi, T., and Negishi, M. (2001) Annu. Rev. Pharmacol. Toxicol. 41, 123–143
3. Honkakoski, P., Sueyoshi, T., and Negishi, M. (2003) Ann. Med. 35, 172–182
4. Sonoda, J., Rosenfeld, J. M., Xu, L., Evans, R. M., and Xie, W. (2003) Curr. Drug Metab. 4, 59–72
5. Swales, K., and Negishi, M. (2004) Mol. Endocrinol. 18, 1589–1598
6. Kawamoto, T., Sueyoshi, T., Zelko, I., Moore, R., Washburn, K., and Negishi, M. (1999) Mol. Cell. Biol. 19, 6318–6322
7. Kobayashi, K., Sueyoshi, T., Inoue, K., Moore, R., and Negishi, M. (2003) Mol. Pharmacol. 64, 1–7
8. Yoshimura, K., Kobayashi, K., Moore, R., Kawamoto, T., and Negishi, M. (2003) FEBS Lett. 548, 17–20
9. Pratt, W. B., and Toft, D. O. (1997) Endoer. Rev. 18, 306–360
10. D’Andrea, L. D., and Began, L. (2003) Trends Biochem. Sci. 28, 655–662
11. Hood, J. K., and Silver, P. A. (1999) Curr. Opin. Cell Biol. 11, 241–247
12. Ikuta, T., Eguchi, H., Tachibana, T., Yoneda, Y., and Kawajiri, K. (1998) J. Biol. Chem. 273, 2895–2904
13. Berg, P., and Pongratz, I. (2001) J. Biol. Chem. 276, 43231–43238
14. Pratt, W. B., Galipigniana, M. D., Harrell, J. M., and DeFranco, D. B. (2004) Cell. Signal. 16, 857–872
15. Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997) Curr. Opin. Cell Biol. 9, 222–232
16. Race, A., and Barsony, J. (1999) J. Biol. Chem. 274, 19352–19360
17. Zelko, I., Sueyoshi, T., Kawamoto, T., Moore, R., and Negishi, M. (2001) Mol. Cell. Biol. 21, 2838–2846
18. Kawana, K., Ikuta, T., Kobayashi, Y., Gotoh, O., Takeda, K., and Kawajiri, K. (2003) Mol. Pharmacol. 63, 524–531
19. Honkakoski, P., Zelko, I., Sueyoshi, T., and Negishi, M. (1998) Mol. Cell. Biol. 18, 5652–5658
20. Goodwin, B., Hodgson, E., and Liddle, C. (1999) Mol. Pharmacol. 56, 1329–1339
21. Sueyoshi, T., Moore, R., Pascussi, J-M., and Negishi, M. (2002) Methods Enzymol. 357, 205–213
22. Harpur A. G., Bastiaens P. I. H. (2001) in Molecular Cloning: A Laboratory Manual, (Sambrook, J., and Russell D. W., eds) 3rd Ed., pp. 18.69–18.95, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
23. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
24. Gorski, K., Carneiro, M., and Schibler, U. (1986) Cell 47, 767–776
25. Sueyoshi, T., Kobayashi, R., Nishio, K., Aida, K., Moore, R., Wada, T., Handa, H., and Negishi, M. (1995) Mol. Cell. Biol. 15, 4158–4166
26. Murphy, A. E., Bernards, A., Church, D., Wasmuth, J., and Gusella, J. F. (1996) DNA Cell Biol. 15, 727–735
27. Brychzy, A., Rein, T., Winkhofer, K. F., Hartl, F. U., Young, J. C., and Obergmann, W. M. J. (2003) EMBO J. 22, 3613–3623
Cytoplasmic Localization of Pregnane X Receptor and Ligand-dependent Nuclear Translocation in Mouse Liver
E. James Squires, Tatsuya Sueyoshi and Masahiko Negishi

J. Biol. Chem. 2004, 279:49307-49314.
doi: 10.1074/jbc.M407281200 originally published online September 2, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407281200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 26 references, 10 of which can be accessed free at http://www.jbc.org/content/279/47/49307.full.html#ref-list-1