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NCOA6 differentially regulates the expression of the *CYP2C9* and *CYP3A4* genes

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Abstract

CYP2Cs and *CYP3A4* sub family of enzymes of the Cytochrome P-450 super family metabolize clinically prescribed therapeutics. Constitutive and induced expressions of these enzymes are under the control of HNF4 α and rifampicin activated PXR. In the present study, we show a mechanism for ligand dependent synergistic cross talk between PXR and HNF4 α . Two-hybrid screening identified NCOA6 as a HNF4 α interacting protein. NCOA6 was also found to interact with PXR through the first LXXLL motif in GST pull down and mammalian two hybrid assays. NCOA6 enhances the synergistic activation of *CYP2C9* and *CYP3A4* promoter activity by PXR and HNF4 α in the presence of rifampicin. However silencing NCOA6 abrogated the synergistic activation and induction of *CYP2C9* by PXR-HNF4 α but not of *CYP3A4*. ChIP analysis revealed that NCOA6 could bridge HNF4 α and PXR binding sites of the *CYP2C9* promoter. Our results indicate that NCOA6 is responsible for the synergistic activation of *CYP2C9* by HNF4 α and PXR and NCOA6 differentially regulates *CYP2C9* and *CYP3A4* gene expression though both the genes are regulated by the same nuclear receptors.

Keywords

NCOA6; Co activators; PXR; HNF4 α ; *CYP2C9*; Synergistic transactivation

1. Introduction

Cytochrome P450 enzymes constitute a super family of hemoproteins found throughout the animal kingdom [1]. *CYP2C9*, the major member of the *CYP2C* subfamily in human liver, metabolizes more than 16% of clinically used drugs, and forms physiologically active metabolites from the endogenous substrate arachidonic acid [2,3]. Studies in primary hepatocytes have shown that, *CYP2C9* mRNA and protein to be up regulated by numerous drugs such as rifampicin, hyperforin (the active ingredient in St. John's Wort), phenobarbital and taxol [4–8]. Furthermore, clinical studies have confirmed that the clearance of *CYP2C9* substrates is increased *in vivo* after the administration of drugs [9–12].

Studies have shown that the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) both bind to the same responsive elements (RE's) at –2998 (DR5) and

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Conflicts of Interest

The authors declare that they have no conflict of interest.

–1839 (DR4) to mediate the transcriptional activation of the *CYP2C9* gene by various drugs [13–15]; however, the proximal site at –1839 appears to be the more important site. PXR belongs to the NR1I2 nuclear family of receptors [16,17], which are activated by a wide range of structurally unrelated compounds [18–21]. PXR preferentially binds many xenobiotic ligands such as rifampicin, taxol, and hyperforin [22,23]. Ligand dependent PXR activation up regulates many of the phase I and II, drug metabolism genes and I and genes involved in transport and clearance pathways including bile acid homeostasis [24]. There is considerable cross talk between PXR and CAR for similar sets of responsive elements in the DNA of various promoters [23,25,26]. Hetero-dimerization of PXR and CAR with RXR (retinoid X receptor) facilitates binding DR-3, DR-4 or DR-5 sites in the promoter region of various genes [18,27–31].

CYP2C9 and *CYP3A4*, like many other *CYP* genes, are preferentially expressed in the liver and developmentally regulated by hepatic enriched transcriptional factors such as HNF4 α (hepatic nuclear factor 4 α) [32–34]. HNF4 α activates the transcription of target genes through recognition of a direct repeat (DR1) motif and recruitment of the chromatin remodeling system [35,36]. Three proximal DR-1 sites have been reported in the *CYP2C9* gene which bind HNF4 α and increase transcription in response to HNF4 α [34]. Studies in our laboratory have reported cross talk between the CAR and HNF4 α binding sites in the *CYP2C9* promoter [34]. HNF4 α and CAR/or PXR both synergistically activate the *CYP2C9* promoter in HepG2 cells.

Our present study addresses the mechanism of the cross talk between PXR and HNF4 α . GST pull-downs show that NCOA6 interacts with PXR and HNF4 α in the presence and absence of the PXR ligand rifampicin. Mapping the interacting domains, we have shown that the first LXXLL motif is essential for PXR interaction, while both the first and second LXXLL motifs were involved in the interaction with HNF4 α . We have confirmed the interaction *in vivo* with mammalian two-hybrid assay. Ectopic expression of NCOA6 modestly arguments the activation of the *CYP2C9* and *CYP3A4* promoters by PXR and HNF4 α individually and the synergistic activation by the combination of these two receptors. However, silencing endogenous NCOA6 abolished the synergistic activation of the *CYP2C9* but not that of the *CYP3A4* promoter. Similarly, NCOA6 differentially regulated the increase in *CYP2C9* and *CYP3A4* mRNA after over expression of PXR and HNF4 α individually or in combination. Finally, chromatin immunoprecipitation (ChIP) analysis showed that PXR and HNF4 α were recruited to their respective binding sites on the *CYP2C9* promoter and NCOA6 bound to both sites. These studies are consistent with the hypothesis that NCOA6 forms a bridge between the PXR and HNF4 α binding sites in the *CYP2C9* promoter providing the platform for the recruitment of other co activators to facilitate the cross talk between these distal sites.

2. Materials and methods

2.1. Cell culture, transient transfections and ligands

HepG2 cells were maintained in the Eagle's minimal essential medium supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C under 5% CO₂. All transient transfections were carried out as described in Lipofectamine 2000 protocol (Invitrogen, CA). Briefly, 0.2 μ g of *CYP2C9* or *CYP3A4* luciferase constructs, 0.1 μ g of each receptor construct(s), and 0.2 μ g of co activator construct with 0.02 μ g of pRL-TK vector as internal control, pcDNA 3.1 as the empty vector to make the total amount of DNA transfected to 0.8 μ g were combined in 50 μ L OPTI-MEM and mixed with transfection reagent as suggested. Twenty-four hours later, medium was replaced, and drugs were added at the appropriate concentrations (0.1% of DMSO or 10 μ M rifampicin). Ligands were incubated with the HepG2 cells for 24 h and assayed for promoter activity using Promega's dual luciferase

assay kit (Promega, Madison, WI). Firefly luciferase readings were normalized with renilla readings to calculate promoter activity.

2.2. Plasmids and adenovirus mediated over expression and RNA interference

PXR, HNF4 α , NCOA6, Med1, PGC-1 α and SRC-1 were cloned in pcDNA3.1 by PCR amplification. GST-PXR and GST-HNF4 α were cloned in pGEX-4T1. NCOA6 domains, NCOA6 I (1–353 aa), NCOA6 II (338–673 aa), NCOA6 III (648–998 aa containing 1st LXXLL motif at 851 aa), NCOA6 IV (986–1327 aa), NCOA6 V (1292–1641 aa coding for 2nd LXXLL motif at 1491 aa) and NCOA6 VI (1625–2065aa) were cloned in pGEX-4T1 in-frame of GST and pACT for expression in two hybrid assay. For transient transfection assay of *CYP2C9* promoter expression, *CYP2C9*-1.9Kb/pGL3 construct has been previously described (Chen et al 2005). *CYP3A4* promoter construct was a kind gift (Dr. Stephen Kliewer, Southwestern University Texas Medical School, Dallas, TX). Full-length PXR and HNF4 α were cloned into pShuttle-CMV vector of AdEasy™ XL Adenoviral Vector system (Stratagene, CA) at the *EcoRV* site. *PmeI* linearized shuttle vectors were electroporated into BJ5183-AD1 cells containing the Ad Easy vector backbone for homologous recombination. Positive recombinant Adeno plasmids were selected on kanamycin and confirmed with restriction digestion. The recombinant plasmids were digested with *Pac I* and transfected into AD-293 cells for replication and packaging into virus particles. Virus particles were purified on continuous cesium chloride, dialyzed and stored in Tris buffered sucrose. pShuttle-CMV-lacZ supplied as control was used for making Ad-lacZ virus. shRNA were designed to silence the NCOA6 mRNA coding sequence were described earlier [37]. Double stranded shRNA oligos were designed for pRNAT-H1.1/Adeno (SD-1219) with H1 promoter and cGFP as the marker. The final NC-III shRNA target sequence was used to design the scrambled shRNA 5'-CACTGAAGTATACCAAGAGCA-3'. Adenoviruses expressing each shRNA were prepared as described before. HepG2 cells were routinely infected with 2.5×10^9 virus particles (VP).

2.3. Expression and purification of recombinant proteins, GST-Pull Down assay

Full-length recombinant proteins of PXR and HNF4 α were expressed as GST fusion proteins in *E.coli BL21 (DE3)*. Briefly, sequenced verified pGEX4T-PXR/HNF4 α constructs were transformed in *E.coli BL21* and initially screened for protein expression. Liquid cultures grown up to A₆₀₀ of 0.4 were induced with 1 mM IPTG at room temperature for 3h and the bacteria were collected. The cells were lysed by three freeze thaw cycles and suspended in TEDG (Tris 50mM pH 7.4, 2 mM EDTA, 2 mM DTT and 10% glycerol) buffer containing 0.42 M NaCl, 1% Triton X -100 and complete protease inhibitors. The lysate was centrifuged at 100,000 $\times$ g for 45 min and the supernatant was used for immobilizing GST fusion proteins on GSH-sepharose matrix. Immobilized proteins were washed 3–4 times with NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride) and analyzed by SDS-PAGE for purity and quantification of the fusion proteins. GST pull-down assays were performed by incubating 5 μ L of ³⁵S methionine labeled proteins in a 500 μ L of NETN buffer containing 1mg/ml fatty acid free BSA in the presence and absence of the PXR ligand rifampicin. The bound proteins were washed 3X with NETN buffer and were heat denatured in SDS sample buffer. These proteins were separated on 4–20% gradient gels and after fixing and amplifying the signal; the gels were dried and autoradiographed.

2.4. Mammalian two-hybrid assay

To map the interaction domains between NCOA6 and HNF4 α , or NCOA6 and PXR, the ligand-binding domain of HNF4 α and PXR were fused to the GAL4 DBD in a pBind vector individually. NCOA6 domains from the previous GST fusion constructs were transferred to pACT vector individually. The promoter luciferase reporter 9XGAL4 UAS was employed to

assay the interaction in HepG2 cells along with the pBind construct and pACT constructs as shown. Promega's Dual-Glo System was used to measure the luciferase activity.

2.5. qPCR

Total RNA was extracted using RNeasy mini prep system (QIAGEN, Valencia, CA) following the manufacturer's procedure. RT-PCR analysis was performed in two steps by initial reaction with Superscript II (Invitrogen) reverse transcriptase. PCR with Taqman® Universal PCR Master Mix (Applied Biosystems) was then performed with gene-specific primers using relative quantification methods. For the RT reaction, 200 ng of total RNA was combined with 2 µl (40 units) of RNase inhibitor (PerkinElmer Life and Analytical Sciences, Boston, MA), 1× First Strand Buffer (Invitrogen), 10 mM dithiothreitol, 0.5 mM dATP, dTTP, dGTP, and dCTP, and 1 µl (200 units) of Superscript II to a total volume of 20 µl, incubated at 42°C for 50 min and then inactivated at 70°C for 15 min, and stored at –20°C or immediately used in PCR analysis. PCR was performed on an Applied Biosystems 7900HT using relative quantification $2^{-\Delta\Delta CT}$ method with taqman probes for CYP2C9 (Cat# HS00426397_m1, lot# 465955), with TBP as the internal control (4333769F, lot# 0706051).

2.6. Chromatin immunoprecipitations

Five plates (15 cm) of 90% confluent HepG2 cells were infected with 2.5×10^{11} VP for 48 h each with adenovirus expressing, LacZ, PXR, HNF4α, PXR-HNF4α and PXR-HNF4α with shRNA for NCOA6 individually. A similar set of HepG2 cells were treated with 10 µM rifampicin. After 48h, the cells were cross-linked with 1% formaldehyde directly in the media, and cross-linking was stopped by the addition of 0.125 M glycine. The cells were washed in ice cold PBS and lysed in hypotonic solution (5mM Pipes). The nuclei were harvested after 30 min, washed and solubilized in Buffer A containing 50mM Tris-Cl, pH 8.0, 1% SDS, 5 mM EDTA, 5 mM EGTA, 0.5 mM PMSF and complete protease inhibitor mix (Roche Molecular Biochemicals). The homogenate was sonicated 6–7 times on ice at 40% setting (Branson sonicator) to shear the chromosomal DNA to an average length of <500 bp. The insoluble material was removed by high-speed centrifugation and chromatin extracts were stored at –80°C. The soluble chromatin was diluted 5 fold with Buffer B containing 20 mM Tris-Cl pH 8.0, 1% Triton X-100, 1.2 mM EDTA and 150 mM NaCl. The chromatin was diluted and precleared by incubating for 3 h with 5 µL of pre-immune serum coupled to protein-A agarose beads saturated with BSA (1 mg/mL) and salmon sperm DNA (0.4 mg/mL). Precleared chromatin was incubated with 2 µg of individual antibodies overnight, the complexes were precipitated with 50 µL of 50% protein A agarose beads. The complexes were washed 2X with 1 mL buffer C (50 mM Tris-Cl pH 8.0, 2 mM EDTA, 0.2% sarkosyl), and 4X with 1 mL buffer D (100 mM Tris-Cl pH 9.0, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid) sequentially. Immune complexes were eluted from the protein A beads by vortexing in 150 µL of Buffer E (50mM sodium bicarbonate, 1% SDS) for 15 min twice [38]. The eluants were pooled and adding 5 M NaCl to a final concentration of 200 mM reversed the protein-DNA cross-linking. RNA was removed with the addition of 10 µg of DNase free RnaseA followed by incubation at 65°C for 4 h. Genomic DNA from the immune complexes was EtOH precipitated and the proteins in the complex were digested with 15 µg of Proteinase K. Finally, the DNA binding proteins bound cognate cis acting elements were purified with phenol-chloroform-isoamyl alcohol and further on qiagen columns. Similarly purified DNA fragments from the chromatin extracts (input) were used as control for PCR reactions. The primers used for amplification of the human CYP2C9 promoter are 5'-TAAAGACAGCAACCGAGC-3' and 5'-TACAATGATTCAGGATTTTCG-3' spanning for the proximal CYP2C9 PXR response element and 5'-ATATACAAGGCATAGAATATGGCC-3' and 5'-GACCAATCACCTAGGTCCAC-3' spanning the CYP2C9 HNF4α response elements.

3. Results

3.1. NCOA6 interacts with PXR

We have previously shown that NCOA6 interacts with CAR and HNF4 α and silencing NCOA6 interferes with the synergistic activation of the *CYP2C9* promoter by HNF4 α and CAR [37]. In this paper, we tested if NCOA6 would also interact with PXR? A partial fragment of NCOA6 coding for the first LXXLL motif was expressed as a fusion protein of GST, which was immobilized on GSH sepharose beads. Full-length hPXR and HNF4 α were labeled with ³⁵S methionine and allowed to interact with the immobilized GST-NCOA6 fragment. Both PXR and HNF4 α interacted strongly with the first LXXLL motif of NCOA6, and the PXR ligand rifampicin had no significant effect on these interactions (Fig. 1a and b). Reciprocally, we have immobilized full length human PXR (hPXR) and hHNF4 α expressed as GST fusion proteins, allowed them to interact with full length NCOA6 and known co activators such as Med1, GRIP-1, SRC-1 and PGC-1 α in the presence and absence of rifampicin. NCOA6 interacts with PXR robustly, and the PXR ligand rifampicin has little effect on this interaction. Med1, the anchoring protein of the mediator complex, GRIP-1, SRC-1 are part of the CBP bound HAT containing enzyme complex and PGC-1 α is a co activator implicated in energy regulation. These established co activators are known to interact with most nuclear receptors and were tested for their ability to interact with PXR and HNF4 α (Fig. 1c). Although rifampicin is not a ligand for HNF4 α , the presence of rifampicin appeared to enhance the binding of co activators to HNF4 α suggesting that rifampicin might alter fatty acid binding to the HNF4 α ligand-binding pocket.

3.2. Mapping of the interaction domains and two-hybrid assay

To define the interacting domain(s) of NCOA6, GST-NCOA6 domains were allowed to interact with ³⁵S-methionine labeled PXR. GST and GST-NCOA6 fusion proteins are shown schematically in Fig. 2a. GST-NCOA6-III domain (648–998aa contains the first LXXLL at 851aa) interacted significantly with PXR (Fig. 2b) in a ligand independent manner. No other NCOA6 domain was found to bind PXR suggesting that only the first LXXLL motif was involved in the interaction. Previously, the first LXXLL motif of NCOA6 was shown to interact with PPAR nuclear receptors (α and γ) TR α and ER α [39]. Taken together, these data suggest that there is only one functional interaction domain. When the interacting domains of NCOA6 and HNF4 α were similarly mapped with ³⁵S-methionine-HNF4 α [37], the GST-NCOA6-III domain strongly retained the radio labeled HNF4 α , but there was also a moderate interaction between the GST-NCOA6-V domain (containing the second LXXLL motif) and HNF4 α , suggesting a possible additional role for this LXXLL motif. This second LXXLL motif of NCOA6 was shown to be involved in the interaction with LXR (another liver specific receptor) in regulating lipogenesis and cholesterol/bile acid homeostasis in the liver and interaction with ER α with no significant physiological function [40].

3.3. Mammalian two-hybrid assay

To assess the strength and specificity of the interaction between NCOA6 and the nuclear receptors PXR and HNF4 α , we employed a mammalian two-hybrid assay. The six partial fragments shown in Fig. 2a spanning the entire coding region, were cloned in pACT vector to express each as a fusion protein downstream of the VP-16 activating domain. The ligand binding domain of HNF4 α and PXR were cloned individually in pBind vector to express each as a fusion protein of the GAL4 DNA binding protein. All the constructs were individually tested for activation with the promoter construct pSG9. An interaction assay with the HNF4 α ligand binding domain and the NCOA6 fragments showed that the first LXXLL motif was essential for the interaction and there was a small interaction with the second LXXLL motif too (Fig. 3a). On co expression of the ligand-binding domain of PXR

with the activation domain of VP-16 fused to NCOA6 fragments, again the domain III that codes for the first LXXLL motif interacted with the ligand-binding domain of PXR, which could be further, boosted by the presence of rifampicin (Fig. 3b). There was small but significant interaction with the second LXXLL motif (domain V). Thus, although the second LXXLL motif showed a small interaction with PXR *in vivo* and with HNF4 α *in vitro*, it did not show significant enhancement of ligand dependent transcription activation in the two-hybrid assay for the PXR.

3.4. NCOA6 differentially regulates CYP2C9 and CYP3A4 expression

PXR transactivates the *CYP2C9* and *CYP3A4* promoters and the activation is enhanced by the presence of 10 μ M rifampicin as the ligand. Expression of NCOA6 with PXR has no significant effect on PXR mediated transactivation of either the *CYP2C9* or *CYP3A4* promoters in the presence of rifampicin (Fig. 4). Ectopic HNF4 α also activated *CYP2C9* promoter activity but had no effect on *CYP3A4* promoter activation. When NCOA6 was ectopically co expressed with HNF4 α there was a modest enhancement of activity of *CYP2C9* and *CYP3A4* promoter activity, which was augmented further in the presence of rifampicin (Fig. 4). But when PXR and HNF4 α were co expressed with the *CYP2C9* or *CYP3A4* reporter constructs, there was a synergistic increase in *CYP2C9* and *CYP3A4* promoter activity specifically in the presence of rifampicin (Fig. 4). In the absence of PXR ligand rifampicin, the synergistic activation is not seen, suggesting that the ligand is required for the PXR activation, which could assist in correct refolding of the AF2 domain on PXR facilitating the recruitment of co activators, which in turn bind to HNF4 α . Addition of ectopic NCOA6 boosted this activation several fold for both the promoters (*CYP3A4* and *CYP2C9*) only upon the synergistic activation. In conclusion, there is a synergistic activation of *CYP2C9* and *CYP3A4* promoter expression by the nuclear receptors PXR and HNF4 α particularly in the presence of the PXR ligand, rifampicin. The synergism was moderately augmented by NCOA6 consistent with a possible role for the co activator in the regulation of *CYP2C9* and *CYP3A4* by PXR and HNF4 α .

3.5. NCOA6 is critical to bridge between PXR and HNF4 α

We have conclusively demonstrated that NCOA6 is a bonafide co activator for HNF4 α [37]. To test its transcriptional role in the activation of *CYP2C9* by PXR and HNF4 α , we expressed small interfering RNA (shRNA) directed against NCOA6 in HepG2 cells using an adenoviral system to silence NCOA6 expression. Among five targets, NC-III and NC IV reduced the expression of NCOA6 mRNA levels by 90% and 75% respectively as quantified by qPCR [37]. To test whether silencing of NCOA6 affects the ligand dependent synergistic activation of the *CYP2C9* promoter by PXR and HNF4 α , the *CYP2C9*-luc promoter construct and PXR, HNF4 α or PXR-HNF4 α were transiently transfected into HepG2 cells, and 24 h later the cells were infected with adenovirus expressing scrambled or shRNA for NCOA6(NC-III). The synergistic activation of the *CYP2C9* promoter by PXR-HNF4 α could be dramatically repressed by the expression of shRNA for NCOA6 in the presence of a ligand but not in absence of ligand rifampicin (Fig. 5a). However, silencing NCOA6 did not have any effect on the synergistic activation of the *CYP3A4* promoter (Fig. 5b).

3.6. Down regulation of NCOA6 does not affect constitutive levels of CYP2C9 mRNA or its induction by PXR or HNF4 α but does block the synergistic induction in vivo

HepG2 cells express CYP2C enzymes poorly, possibly due to low expression of factors such as CA, PXR, PGC-1 and/or SRC-1. We examined the effect of silencing of NCOA6 on CYP2C9 mRNA in HepG2 cells. The shRNA for NCOA6, down regulated the constitutive levels of CYP2C9 mRNA by 75% compared to the scrambled shRNA controls. (Fig. 6a) In contrast, ectopic over expression of PXR or HNF4 α induced substantial amounts of CYP2C9 mRNA levels, but NCOA6 shRNA failed to represses this induction, suggesting

that NCOA6 may not play a direct role in the PXR or HNF4 α mediated up regulation of the transcription of *CYP2C9* (Fig 6b and 6c). Silencing NCOA6 with shRNA decreases the NCOA6 protein levels as seen in the western blot compared to lacZ or scrambled virus. However RNA Pol II as a control does not change its expression when NCOA6 protein levels were diminished (Fig. 6d), suggesting that shNCOA6 is specifically acting towards down regulating the expression of NCOA6 alone. On the other hand, we have shown above that the down regulation of NCOA6 levels inhibits the synergistic activation of *CYP2C9* promoter activity by the ectopic expression of PXR and HNF4 α (Fig 5 a). To translate these data to gene expression, we infected AdPXR and AdHNF4 α individually in HepG2 cells and simultaneously down regulated NCOA6 levels with shRNA for NCOA6. Expression of PXR and HNF4 α produces a synergistic 50-fold induction of *CYP2C9* mRNA in HepG2 cells in the presence of rifampicin compared to a less than 5-fold induction by HNF4 α or PXR in the absence of rifampicin. The synergistic induction by ectopic expression of HNF4 α and PXR on *CYP2C9* mRNA in HepG2 cells was essentially abolished by down regulating NCOA6 with adenoviral shRNA (Fig. 7a). In contrast there was no synergistic induction of *CYP3A4* mRNA by ectopic expression of PXR and HNF4 α and silencing NCOA6 had no effect on its induction (Fig. 7b). Taken together, the transactivation and mRNA induction results strongly indicate that NCOA6 may act as a bridging partner between PXR and HNF4 α resulting in a synergistic up regulation of *CYP2C9* gene expression but not for *CYP3A4* gene expression.

3.7. Chromatin immunoprecipitation assay

ChIP assays demonstrated that PXR is recruited strongly to the PXR binding site on the promoter region of *CYP2C9* in chromatin extracts prepared from HepG2 cells after ectopic expression of PXR and moderately after ectopic expression of PXR-HNF4 α with or without infection with shRNA for NCOA6. As expected, HNF4 α was recruited to its binding sites on the promoter in all the chromatin extracts from cells over expressing HNF4 α , PXR-HNF4 α and shRNA for NCOA6 did not affect this binding. A specific antibody to NCOA6 was able to immunoprecipitate the PXR and the HNF4 α binding sites of *CYP2C9* in chromatin extracts from cells ectopically expressing PXR and PXR-HNF4 α . Silencing NCOA6 prevented immunoprecipitation of both the PXR and HNF4 α binding sites of the *CYP2C9* promoter by antibody to NCOA6 (Fig. 8). Thus ChIP assays show that PXR and HNF4 α can bind to their respective binding sites on the *CYP2C9* promoter *in vivo* and NCOA6 binds to both sites supporting the hypothesis that the NCOA6 may be necessary for formation of a bridge between the HNF4 α and PXR sites and form the basis for the recruitment of other co activators involved in the massive induction of *CYP2C9* gene expression by ectopic over expression of both PXR and HNF4 α .

4. Discussion

Hepatic drug metabolism is one of the mechanisms whereby organisms protect themselves from exposure to environmental chemicals and either activates or deactivates [34,37] therapeutic drugs. *CYP2C9* is an important drug-metabolizing enzyme which metabolizes ~16% of all clinical drugs as well as some endogenous compounds. *CYP2C9* is up regulated by drugs and other xenobiotics primarily via two xenobiotic sensing receptors, CAR (NR1I3) and PXR (NR1I2). PXR is one of the most important xenobiotic sensing receptors since it is promiscuous, interacting with a wide array of ligands including clinical drugs such as rifampicin, paclitaxol, and dietary supplements such as St. John's Wort. PXR regulates the response of a variety of CYP enzymes, drug transporters, and phase II drug metabolizing enzymes in response to environmental stimuli. Many of the CYP enzymes and phase II enzymes are found in high concentrations in liver, where HNF4 α , a hepatic enriched factor, regulates both their constitutive expression and enhances their response to the xenobiotic sensing receptors CAR and PXR [41–43]. In fact, for a number of these enzymes such as

CYP2C9 and CYP3A4, the receptors HNF4 α and CAR have been reported to synergistically activate their promoter activity in cell lines, and studies in primary hepatocytes [34,44] and HNF4 α knockout mice have confirmed that HNF4 α plays a central role in regulating the response of drug metabolizing enzymes to PXR and CAR [41].

It has become apparent that cross talk with other transcription factors, coactivators and growth signals is necessary to explain the interplay between different receptors [45,46]. In these and recent studies [37], we performed yeast two-hybrid screens to identify receptors/ and or co activators which might explain the interplay between these receptors. One of the co activators identified in a yeast two-hybrid screen using HNF4 α as bait was the co activator NCOA6 which was subsequently shown to interact with the receptor CAR using protein-protein interaction techniques principally through the first LXXLL motif with some interaction through the second LXXLL motif [37]. The present study shows that the xenoreceptor PXR also interacts with NCOA6 through the first LXXLL motif. Mammalian two hybrid assays further confirm that NCOA6 interacts with PXR and HNF4 α principally through its first LXXLL motif although a smaller interaction is seen with the second LXXLL motif.

Exogenous NCOA6 has little effect on *CYP2C9* or *CYP3A4* promoter activity in the presence or absence of PXR, but increases HNF4 α activation and the synergistic activation by PXR and HNF4 α particularly in the presence of the PXR ligand rifampicin. Silencing NCOA6 decreased the synergistic activation of the *CYP2C9* promoter by PXR and HNF4 α , but surprisingly did not affect that of the *CYP3A4* promoter. Parallel effects were seen on *CYP2C9* and *CYP3A4* mRNA. shNCOA6 preferentially affected the synergistic increase in *CYP2C9* mRNA by HNF4 α and PXR but had no effect on *CYP3A4* mRNA induction.

There are many examples of crosstalk between CAR or PXR and HNF4 α . Cross-talk between PXR and HNF4 α has been reported for the human sulfotransferase SULT2A1 whereby this enzyme is up regulated by HNF4 α and down regulated by PXR [47]. Rifampicin induction of *CYP3A4* has been proposed to involve cross talk with HNF4 α and PXR via various co activators such as PGC-1 α , SRC-1, and the small heterodimer partner (SHP) may compete with HNF4 α and SRC-1 for PXR [48]. The observed differential regulation of *CYP2C9* and *CYP3A4* could be due the fact that at the promoter level, the constructs of *CYP2C9* and *CYP3A4* are of similar length and the distance between the PXR binding site and the HNF4 α binding site is about 1.6 kbp, which could be a possible explanation for the synergistic activation of the promoter constructs. But at the chromatin level, though *CYP2C9* is about 1.6 kbp, the distance between the binding sites is about 8 kbp for *CYP3A4*. This long distance between the binding sites could prevent the proper loop formation required for the synergistic activation. Our data with mRNA and silencing of NCOA6 strongly supports this hypothesis. Negishi and coworkers [45] reported that the signal molecule EGR1 (early growth factor) binds to the proximal promoter of *CYP2B6* and coordinates interaction with a nearby HNF4 α site which cross-talks with a CAR binding at a distal enhancer site causing synergistic induction by drugs. A later paper from the same group provided evidence that EGR1 is involved in a loop formation between the distal enhancer and the proximal sites [46]. Similarly, our studies are consistent with the hypothesis that NCOA6 may assist in loop formation between the distal PXR sites and the proximal HNF4 α sites of the *CYP2C9* promoter as depicted in the graphical abstract.

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Abbreviations

PXR	pregnane X receptor
CAR	constitutive activate/androstane receptor
HNF4α	hepatocyte nuclear factor 4 α
RE	response element
NCOA6	Nuclear receptor coactivator 6 (PRIP/RAP250/ASC-2)
PBP	peroxisome proliferator-activated receptor binding protein (TRAP220/MED-1/DRIP205)
CBP	cAMP response element-binding protein binding protein
PCR	polymerase chain reaction
SRC-1	steroid receptor coactivator-1
PGC-1α	peroxisome proliferator-activated receptor coactivator-1 α
PIMT	PRIP-interacting protein with methyltransferase domain
GRIP-1	glucocorticoid receptor interacting protein
ChIP	chromatin immunoprecipitation
qPCR	quantitative polymerase chain reaction
CREB	cAMP response element-binding protein
aa	amino acid
shRNA	small hairpinRNA
bp	base pair(s)
TEGN	Tris, EDTA, glycerol, and Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
VP	virus particles
Ad	adenovirus
MS/MS	tandem massspectrometry
NIEHS	National Institute of Environmental Health Sciences

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Fig. 1. NCOA6 interacts with PXR and HNF4α

The first LXXLL motif of NCOA6 interacts with HNF4α and PXR. Immobilized GST-NCOA6 (656–900 aa) was allowed to interact with labeled HNF4α (a) and hPXR (b) in the presence and absence of 10 μM rifampicin. The proteins were denatured in SDS-sample buffer and separated on 4–20% SDS-PAGE gel. (c) GST-PXR and HNF4α were immobilized on GSH-sepharose beads. NCOA6, PBP, GRIP-1, SRC-1 and PGC-1α were translated *in vitro* in the presence of ³⁵S methionine using Promega's *in vitro* tr/trl system. Full length radio labeled proteins were allowed to interact with GST, GST-PXR, and GST-HNF4α (±10 μM rifampicin or DMSO as the control). Bound proteins were heat denatured, separated by electrophoresis on a 4–20% gradient gels, fixed, signal was amplified and dried for autoradiography. 1/10th of the tr/trl mixes were loaded as input.

Fig.2. NCOA6 interacts with PXR specifically through the LXXLL domain

(a). Schematic representation of NCOA6 domains generated as GST fusion proteins. (b). PXR interacts specifically through the 1st LXXLL domain. Truncated GST-NCOA6 domains were allowed to interact with full-length ³⁵S labeled PXR in the presence and absence of 10 μ M rifampicin. The bound proteins were washed extensively and denatured and electrophoretically separated on a 4–20% gradient PAGE. The gels was stained and fixed. The signal was amplified by enhancer and dried for autoradiography. 1/10th of the tr/trl mixes were loaded as input.

(a)

Fig. 3. Mammalian two-hybrid assay for In vivo interaction

HepG2 cells were transfected with 9XGal4 luciferase promoter and Gal4-HNF4! ligand binding domain (pBind fusion) and VP-16 NCOA6 domains (pACT fusion) as shown in (a) individually or in combination. Luciferase assays confirms the interaction is through the LXXLL motif in domain III and to a minor extent with domain V of NCOA6. Values represent means $\pm$ SE of triplicate analyses (*, significantly greater than control at $p < 0.001$). (b). GAL4-PXR ligand binding domain and VP-16 NCOA6 domains along with the GAL4 promoter constructs were transfected to HepG2 cells and after 24 h, the cells were treated with 10 μ M rifampicin for 24. The luciferase assays confirmed the PXR-HNF4! α

interaction is through the LXXLL domain. Data represent means $\pm$ SE, n=3, * statistically significant increase between groups indicated by bars, $p<0.05$.

Fig. 4. Nuclear Receptor Co activator 6 (NCOA6) promotes the synergistic activation of CYP2C9 and CYP3A4

HepG2 cells were transfected with promoter constructs of either CYP2C9 (a) or CYP3A4 (b) individually along with nuclear receptors PXR and HNF4A α . NCOA6 has no effect on either of the promoters in the absence of nuclear receptors PXR or the presence of nuclear receptors. Synergistic activation of CYP2C9 or CYP3A4 promoters by co expression of PXR and HNF4A α simultaneously is enhanced by the presence of NCOA6 and PXR ligand rifampicin. All transfections were performed in triplicates. Values are means of triplicates (n=3). * Statistically significant increase in CYP2C9 transactivation, p<0.05.

Fig. 5. NCOA6 differentially regulates the activation of CYP2C9 and CYP3A4

HepG2 cells were transfected with CYP2C9 promoter construct (a) and CYP3A4 promoter construct (b). shNCOA6 has no effect on the promoter expression with both the reporter constructs. Synergistic activation of CYP2C9 or CYP3A4 with PXR and HNF4 α was profound in the presence of PXR ligand rifampicin. Silencing NCOA6 to the abolished the synergistic activation of CYP2C9 (a) but did not abolish the CYP3A4 (b) activation. All transfections were performed in triplicate. Values are means of triplicates (n=3). (*, Statistically significant increase in CYP2C9 transactivation, $p<0.05$. #, CYP2C9 promoter activity is significantly decreased when NCOA6 is silenced, $p<0.05$).

Fig. 6. NCOA6 does not play a direct role in nuclear receptor mediated constitutive regulation of CYP2C9 mRNA

HepG2 cells were infected with Adenovirus LacZ, scrambled and shNCOA6 individually. shNCOA6 down regulates constitutive CYP2C9 mRNA (a) while scrambled has no effect. Ectopic expression of PXR (b) and HNF4 α (c) induces CYP2C9 mRNA but simultaneous reduction of NCOA6 levels with shNCOA6 did not effect the nuclear receptor mediated induction of CYP2C9 mRNA. Data represent means $\pm$ SE of fold differences in mRNA compared to LacZ, n=3, #, significantly decreased expression of NCOA6 by shNCOA6, p<0.05. (d) shNCOA6 specifically decreases the NCOA6 protein levels in the nuclear extracts from HepG2 cells as detected on immuno blotting, lacZ, scrambled and shNCOA6 infected HepG2 cells, as a control RNA Pol II was detected at normal levels.

Fig. 7. CYP2C9 and CYP3A4 are differentially regulated by NCOA6

HepG2 cells were infected with scrambled, PXR or HNF4α individually or in combination as shown and treated with 10μM rifampicin for 24h. Induction of CYP2C9 (a) and CYP3A4 (b)mRNA were quantified by qPCR. CYP2C9 (a) was induced by the co expression of PXR and HNF4α and on PXR ligand rifampicin treatment. This synergistic ligand activated CYP2C9 induction was abolished by silencing NCOA6. On the other hand, CYP3A4 (b) though induced by rifampicin activated PXR did not synergize with HNF4α. Silencing NCOA6 did not have any effect on the mRNA levels. Data represent means $\pm$ SE, n=3, #, significant decrease in CYP2C9 induction by silencing NCOA6, $p < 0.05$.

Fig.8. Recruitment Analysis of the nuclear receptors PXR, HNF4α and NCOA6 to the CYP2C9 promoter by Chromatin Immunoprecipitation analysis

ChIP analysis for PXR-HNF4α mediated recruitment of nuclear receptor cofactors to the *CYP2C9* gene promoter. Chromatin extracts isolated from HepG2 cells infected individually with adenovirus expressing, LacZ, PXR, HNF4α, PXR-HNF4α and PXR-HNF4α with shNCOA6 (NC-III) in the presence and absence of PXR ligand rifampicin were pre-cleared and immunoprecipitated with antibodies for PXR, HNF4α and NCOA6. PCR was used to analyze the *CYP2C9* promoter at the PXR binding sites (a) and HNF4α binding sites (b). Expression of PXR and HNF4α increased their recruitment to their respective sites. Immunoprecipitation with NCOA6, showed its association with both PXR and HNF4α binding sites on *CYP2C9* promoter. Silencing of NCOA6 essentially abolishes the recruitment of NCOA6 to the PXR and HNF4α binding sites.