The orphan nuclear receptor, DAX-1, functions as a potent co-repressor of the constitutive androstane receptor (CAR, NR1I3)

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Abbreviations: NR, nuclear receptor; CAR, constitutive androstane receptor; RXR, retinoid X receptor; DAX-1, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; SHP, small heterodimer protein; DBD, DNA-binding domain; LBD, ligand-binding domain; PBREM, phenobarbital response enhancer module; XREM, xenobiotic response enhancer module; CMV, cytomegalovirus; VP16, virus protein 16; ANDRO, 5α-androstan-3α-ol; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; PB, Phenobarbital; h, human; m, mouse; SRC-1, steroid receptor coactivator 1; bp, base pair(s); DMSO, dimethyl sulfoxide; AF-2, activation function 2; RID, receptor interaction domain.
Abstract

Regulation of gene transcription is controlled in part by nuclear receptors (NR) that function coordinately with coregulator proteins. The human constitutive androstane receptor (CAR; NR1I3) is primarily expressed in liver and regulates the expression of genes involved in xenobiotic metabolism as well as hormone, energy and lipid homeostasis. In this report, DAX-1, a NR family member with corepressor properties, was identified as a potent CAR regulator. Results of transaction and mutational studies demonstrated that both DAX-1’s downstream ‘LXXLL’ and its ‘PCFQVLP’ motifs were critical contributors to DAX-1’s co-repression activities, although two other ‘LXXM/LL’ motifs located nearer the N-terminus had no impact on the CAR functional interaction. Deletion of DAX-1’s carboxy-terminal transcription silencing domain (TSD) restored CAR1 transactivation activity in reporter assays to ~90% of control, demonstrating its critical function in mediating the CAR repression activities. Further, results obtained from mammalian two-hybrid experiments assessing various domain configurations of the respective receptors showed that full length DAX-1 inhibited CAR-SRC1 interaction by ~50%, whereas the same interaction was restored to 90% of control when the DAX-1 TSD was deleted. Direct interaction between CAR and DAX-1 was demonstrated with both alpha-screen and co-immunoprecipitation experiments, and this interaction was enhanced in the presence of the CAR activator, CITCO. Results obtained in primary human hepatocytes further demonstrated DAX-1 inhibition of CAR-mediated CITCO induction of the CYP2B6 target gene. The results of this investigation identify DAX-1 as a novel and potent CAR corepressor and suggest that DAX-1 functions as a coordinate hepatic regulator of CAR’s biological function.
Introduction

Nuclear receptors (NR) are transcription factors that play an essential role in regulation of gene expression and their activity involves a complex interplay of various proteins with diverse functions. NR are comprised several functional regions that dictate their activity. Most NR contain an A/B domain that includes an activator function (AF-1). The C domain contains the DNA binding portion, which is linked to the ligand binding domain E via a hinge region (Domain D). Domain E also contains the interface for NR dimerization, and a transactivation AF-2 function that mediates co-regulator binding (Aranda and Pascual, 2001). Agonist binding induces an AF-2 conformational change, which provides a ‘co-regulator cleft’ for co-activator binding and subsequent activation of transcription. In absence of agonist or the presence of antagonist, the position of AF-2 exposes a co-repressor binding site, causing transcriptional repression (Perissi et al., 1999).

The interaction of co-regulators with NR is accomplished through alpha-helical structures that contain consensus motifs. Generally, NR co-activators contain ‘LXXLL’ sequences (Heery et al., 1997), also known as NR boxes, while NR co-repressors have the consensus motif ‘LXXI/H IXXXI/L’ or CoRNR box (Hu and Lazar, 1999). Numerous co-activators exist and previous studies show that they exhibit distinct NR preferences (Heery et al., 2001; Ding et al., 1998). Further, the specificity of the interaction between NR and these motifs is governed by sequences that flank the N- and C-terminal of the NR box (Chang et al., 1999). The agonist bound may also influence the recruitment of NR coactivators depending on the degree of AF-2 conformational change it induces (Togashi et al., 2005). These findings suggested the intriguing possibility that there are multiple layers that dictate the recruitment of co-regulatory proteins and thus, influence the action NR in a given milieu.

The constitutive androstane receptor (CAR, NR1I3) is an atypical NR in that it does not have an A/B domain and the reference form (CAR1) does not require exogenous ligand for activity (Baes et al., 1994). This constitutive activity is thought to result from a shortened AF-2 domain and hydrogen bonding interactions that stabilize the AF-2 domain in the active conformation (Xu et al., 2004).
Alternative mRNA splicing results in CAR variants, including CAR2 and CAR3 (Auerbach et al., 2003) and unlike the constitutively active CAR1, CAR2 and CAR3 require ligand for activation, and exhibit differences in ligand selectivity (Auerbach et al., 2005; Dekeyser et al., 2009; Dekeyser et al., 2011). CAR and CAR variant transcripts are detectable in many tissues, but are primarily expressed in the liver, where they act as xenosensors, controlling the expression of Phase I and Phase II metabolic enzymes, and Phase III transporter (Timsit and Negishi, 2007). Importantly, CAR is now recognized as regulator of endogenous physiological processes such energy, glucose and lipid homeostasis, and bile acid elimination (Kodama et al., 2004; Konno et al., 2008; Masson et al., 2008; Guo et al., 2003).

With regard to interactions with nuclear co-regulatory proteins, CAR interacts with coactivators in the SRC family, GRIP1, PGC1α, and TIF2 (e.g., Kim et al., 1998; Min et al., 2002; Muangmoonchai et al., 2001; Shiraki et al., 2003. However, only SRC family members and PGC1α appear to enhance CAR transcriptional activity. For example, SRC-1 enhances CAR-mediated induction of CYP2B1 in rat hepatocytes (Muangmoonchai et al., 2001). Further, while all SRC family members enhance mouse CAR activity, SRC-3 seems to be the most important mediator (Chen et al., 2011). The human reference CAR was crystallized the presence of SRC-1 NR box peptides (Xu et al., 2004). With respect to corepressor protein interactions, NCoR and SMRT have been shown to interact with both human and mouse CAR (Dussault et al., 2002; Lempiainen et al., 2005), and NCoR inhibits their transactivation in reporter assays (Lempiainen et al., 2005). SHP (NR0B2) interacts with mouse CAR in pull down and yeast two-hybrid assays (Seol et al., 1996; Park et al., 2004), and represses mouse CAR activity ~50% in transactivation experiments (Bae et al., 2004).

DAX-1 (NR0B1) is an atypical nuclear receptor whose gene (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) was identified as responsible for adrenal hypoplasia congenita (Zanaria et al., 1994). Similar to SHP, DAX-1 is unusual in that it lacks the typical NR DNA binding domain (DBD), and its N-terminal region contains three ‘LXXL/ML’ motifs typically found in NR co-activators. However, DAX-1 appears to function as a transcriptional co-repressor of many NRs, including SF-1 (Ito et al., 1997), ER (Zhang et al., 2000), AR (Holter et al., 2002), Nurr77, (Song et
al., 2004), PPARγ (Kim et al., 2008), LRH-1 (Sablin et al., 2008), GR (Zhou et al., 2008), HNF4α, (Nedumaran et al., 2009), and LXRα (Nedumaran et al., 2010). As well, DAX-1 plays an important role in steroidogenesis, adrenal and reproductive development, and maintenance of stem cell pluripotency (Lalli and Sassone-Corsi, 2003; Jeong and Mangelsdorf, 2009). Recent studies indicated a further role for DAX-1 in hepatic lipogenesis and gluconeogenesis (Nedumaran et al., 2009; Nedumaran et al., 2010).

Since CAR mediates xenobiotic responses and has a role in endogenous homeostatic processes, different co-regulator interactions may direct CAR’s various functional activities. While several CAR co-regulators have been identified, mostly with mouse CAR, the aim of this study was to identify new potential co-regulators of human CAR and CAR splice variants. Screening a library of estrogen receptor interacting peptides (Chang et al., 1999) led to the identification of DAX-1 as a novel and potent repressor of CAR activity.
Materials and Methods

Chemicals and Reagents. General chemicals, dimethylsulfoxide (DMSO, CAS#67-68-5), 6-(4-chlorophenyl: imidazo[2,1-b]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO, CAS#338404-52-7), 5α-androstan-3α-ol (ANDRO, CAS#7657-50-3), anti-mouse IgG-HRP conjugate, anti-FLAG M2 affinity resin, and mouse monoclonal anti-FLAG M2-HRP were purchased from Sigma-Aldrich. Phenobarbital (PB, CAS#50-06-0) was obtained from the Drug Services Division of the University of Washington (Seattle, WA). The human DAX-1 and SHP cDNA clones in the pCMV6-AC expression vector were purchased from Origene (Rockville, MD). Primers were purchased from Integrated DNA technologies (Coralville, IA). Anti-DAX-1 antibody was purchase from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid Constructs and Generation of Mutants. The pM vectors expressing GAL4-DBD-LXXLL peptide motifs were a generous gift of Dr. Donald P. McDonnell (Duke University, Chang et al., 1999). The vectors CMV2-CAR1, CMV2-CAR2, 3.1-RXRα, 2B6-XREM-PBREM luciferase reporter were described previously (Auerbach et al., 2007). The CMV2-CAR3, 3XFLAG-CAR1 and mammalian two-hybrid vectors were also reported previously (Auerbach et al., 2005). Full length DAX-1 was amplified from the pCMV6-DAX-1 expression vector using the primers shown in Table 1 and subcloned into the pM (Gal4-DBD) and pVP16 (AD) mammalian two-hybrid vectors (Clontech, Mountain View, CA). The DAX-1 mutants are represented in figure 1 and were generated using the pCMV6-AC DAX-1 cDNA clone and QuikChange® lightning mutagenesis kit according to the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA). Mutagenesis primers are shown in Table 1 and were designed using the QuikChange® primer design tool. The truncated DAX1-TSD expression plasmid was generated by amplifying bases 1-1325 of the pCMV6-AC-DAX-1 clone using primers shown in Table 1). The truncated DAX-1 product was the inserted into an empty pCMV6-AC vector. This truncated version of DAX-1 was subcloned into the mammalian two-hybrid vectors pM and pVP16. For creation of the pCDH-dCMV-DAX1 construct, the dual promoter pCDH-CMV-MCS-EF1-copGFP (System
Biosciences, Mountain View, CA) was first modified by replacing the EF1-copGFP with CMV-copGFP (excised from pSIH-H1-copGFP; Systems Biosciences), resulting in a dual CMV promoter vector, pCDH-CMV-MCS-CMV-copGFP (pCDH-dCMV). This was done because previous experiments suggested that the EF1 promoter did not function efficiently in primary hepatocytes (unpublished observations). DAX1 was then excised from pCMV6-AC-DAX1 and subcloned into pCDH-dCMV to create a construct in which 2 separate CMV promoters drive DAX1 and copGFP expression.

Transactivation and Mammalian two-hybrid assays. Culture conditions for maintenance of COS-1 cells (ATCC, Manassas, VA) were previously published (Dekeyser et al., 2009). COS-1 cells were used since they are devoid of endogenous CAR expression/activity as demonstrated in previous reports (Auerbach et al., 2005; Auerbach et al., 2007). For transfection and chemical treatments, the same medium was used except dextran/charcoal treated FBS (HyClone, Logan, UT) replaced normal FBS. All transfections and chemical treatments for luciferase reporter and mammalian two-hybrid assays were performed in a 48-well format in triplicate or quadruplicate and repeated at least one time. Transfections for transactivation and mammalian two-hybrid assays were performed as previously described (Dekeyser et al., 2009). All test compounds were diluted in DMSO and levels never exceeded 0.2% (vol/vol). CITCO was used as a positive control for CAR activation (Maglich et al., 2003). Because CAR1 is constitutively active, ANDRO (10µM), a mouse CAR (Forman et al., 1998) and human CAR1 (Auerbach et al., 2007) inverse-agonist, are routinely included in assays to decrease CAR1 activity. This activity is restored in the presence of an agonist, which allows study of agonist-induced CAR1 activity and for relative comparison of CAR1 inhibition. All chemical treatments were for 24 h and luciferase assays were performed as previously described (Auerbach et al., 2007).

Alpha-Screen Assays. Alpha-screen assays were used to assess a direct interaction between DAX-1 and CAR1. AlphaScreen assays (Perkin-Elmer, Waltham, MA) were performed as described for other nuclear receptors (Li et al., 2005). The human CAR1-LBD (residues 103-349) was expressed as a 6xHis fusion
protein from the Novagen expression vector pET24a (EMD Biosciences, San Diego, CA). The protein was purified from a Ni-NTA column followed by a Q-Sepharose column. Human DAX-1 constructs a and b (residues 210-470 and 218-470, respectively) were expressed as GST fusion protein from the expression vector pGEX-4T-1. DAX-1 proteins were directly purified from glutathione agarose column for the assays. The experiments were conducted with approximately 40 nM receptor LBD each in the presence of 5µg/ml donor and acceptor beads in a buffer containing 50 nM MOPS, 50 mM NaF, 50 mM CHAPS, and 0.1 mg/ml bovine serum albumin, all adjusted to a pH of 7.4. Alpha screen assays were performed in duplicate and repeated at least once.

**Immunoprecipitation Assays.** COS-1 cells were seeded at a density of 1.5x10^6 in 60mm dishes. Cells were transfected with 2 µg pCMV6-AC-DAX1 plus 2 µg 3X-FLAG hCAR1 or 2 µg 3X-FLAG empty with Fugene 6 (1 µg DNA:3 µl Fugene6) according to the manufacturer’s directions. The following day, medium was replaced, and ~36 hr after transfection, cells were treated with DMSO, CITCO (3 µM), PB (0.5 mM), or ANDRO (10 µM). After 6 h treatment, the cells were washed with PBS and harvested. Cell pellets were stored at -80°C. Cell pellets were resuspended in 500 µl ice cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TritonX100, 0.5%NP-40 and 1X protease inhibitor cocktail) and sonicated (10 pulses) using a Branson Sonifier 250 (VWR Scientific). The lysates were centrifuged at 14000xg for 20 min at 4°C and supernatants were retained. Protein concentrations were determined by BCA methods. Protein (300 µg) was incubated with 20 µl prewashed and preblocked anti-FLAG M2 resin in a total volume of 500 µl lysis buffer on a rotator overnight at 4°C. The tubes were centrifuged at 7500xg to collect the resin and the supernatant was removed. The resin was then washed 5X with ice-cold wash buffer (lysis buffer with 0.4% TritonX100 and 0.2%NP-40). Protein was eluted with the addition of 20 µl 2X SDS-PAGE sample loading buffer and heating at 95°C for 5 min. The samples were then analyzed by Western blot using BioRad reagents and equipment. The blots were incubated with anti-DAX1 (1:1000 in 2% NFDM, 1X TTBS, 4°C overnight) followed by anti-rabbit HRP (1:5000 in 2% NFDM, 1X TTBS, 2 hr at 24°C) and detection was with Pierce ECL chemiluminescent.
detection kit. Blots were stripped using Pierce Restore reagent and CAR input was detected with anti-FLAG M2 HRP conjugate (1:2000 in 2% NFDM, 1X TTBS, 4°C overnight).

**Culture and Transfection of Human Primary Hepatocytes.** Normal human hepatocytes in a 12 well format were obtained through the Liver Tissue Cell Distribution System, Pittsburgh, PA, funded by NIH Contract #N01-DK-7-0004/HHSN267200700004C. Cell culture conditions were published previously (Goyak et al., 2008). Upon receiving cells, medium was replaced every 24h. For treatments of normal (untransfected) hepatocytes, chemical treatments (DMSO, phenobarbital, 500 μM; CITCO 2 μM; or DEHP 1 μM) were applied 3-5 days after receipt of hepatocytes. After 24h, hepatocytes were washed in 1X phosphate buffered saline (pH 7.4), followed by the addition of 600 μl Trizol reagent (Invitrogen). The Trizol solution was mixed by pipetting and then transferred to 1.5 ml tubes followed by immediate storage at -80°C. For experiments involving transfected primary human hepatocytes, wells were transfected 1-3 days after culture with 2.5 μg pCDH-dCMV-DAX1 or PCDH-dCMV-empty using JetPEI™ hepatocyte transfection reagent (Polyplus Transfection Inc., New York) at a 1:3.2 ratio (μg DNA:μl JetPEI ), according to the manufacturer’s instructions. Approximately 18h after transfection, the medium was removed and replaced with medium containing DMSO or 3 μM CITCO. Treatment continued 24-48h, with fresh treatments prepared every 24h. Following treatment, cells were harvested as described above. RNA was prepared and concentrations and purity were determined using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE). RNA integrity was assessed using a BioRad Experion (Hercules, CA). RNA (2 μg) was used for synthesis of cDNA using the High Capacity Reverse Transcription cDNA kit (Applied Biosystems, Carlsbad, CA) and the remainder was stored at -80°C. The cDNA reaction was performed in a BioRad C1000 Thermocycler using the following conditions: 25°C 10m, 37°C 2h, 85°C 5 m, 4°C forever. Once the reactions had cooled to 4°C, cDNA was stored at -20°C until use.
Real-Time PCR. cDNA was thawed on ice and then diluted 5-fold in nuclease free water to give a concentration of 20 ng/µl. Master mixes were prepared for each target. The volume of components per duplicate reaction was: 15 µl PerfeCTa SYBR Green Supermix, ROX (Quanta Biosciences), 0.6 µl forward primer (final concentration 100 nM), 0.6 µl reverse primer (final concentration 100 nM), 10.8 µl nuclease free water and 3 µl cDNA (final concentration 20 ng/µl). SYBR green primer sets are shown in Table 2. Duplicate aliquots from each mastermix were transferred to a 96 well assay plate. The reactions were run on a BioRad CFX96 Real Time System equipped with a C1000 Thermocycler and CFX Manager Software v. 2. The reaction conditions were as follows: 45°C for 5 min, 95°C for 3 min, 95°C for 15 sec and 60°C for 1 min (40 cycles total). Melt curves were run from 65°C to 95°C with an increment of 0.5°C after each run. Standard curves using serial dilutions of human primary hepatocyte cDNA were run for all targets to determine reaction efficiencies for each primer set. For DAX1, cDNA obtained from hepatocytes transfected with pCDH-CMV-DAX1 was used and for CYP2B6, CDNA from hepatocytes treated with CITCO was used. Efficiencies and standard curve parameters are shown in supplement Table S1. Quantification data were corrected for reaction efficiencies. Expression values were determined using the \( \Delta\Delta CT \) method with GAPDH as the internal reference and expressed as fold-change relative to empty vector transfected or untransfected controls.

Statistical Analysis. All statistical tests were performed using GraphPad Prism Software v. 5.03 (La Jolla, CA). For determining differences in activation of CAR by various treatments and/or interacting proteins, two-way ANOVAs were performed, followed by a Bonferroni test for comparison to controls. A \( P \) value < 0.05 was considered to be significant.
Results

McDonnell Panel Screening Assays. Given the differences in CAR variant sequence and ligand selectivity, we reasoned the CAR variants might have selective co-regulator binding preferences. To investigate this possibility, we screened the McDonnell panel (Chang et al., 1999) of ER-interacting peptides that contain the ‘LXXLL’ NR box motif for interaction with CAR variants in mammalian two-hybrid assays. Although only minor differences in peptide interactions with CAR variants were detected, all CAR variants revealed a relatively strong interaction with a clone designated as D48, containing the sequence SGWENSILYSLSSDLRSDL (See Supplemental Table 2). This clone belongs to the Class III peptides containing NR boxes that are found in RIP140, and also similar to those found in PGC-1, DAX-1 and SHP (Chang et al., 1999). A blast search (Altschul et al., 2005) of the D48 peptide sequence against the human non-redundant protein database revealed sequence homology with the three NR boxes found in human DAX-1 (Figure 1).

Interaction between CAR1 and DAX-1 or SHP. Based on the results of the McDonnell panel peptide screening and the fact that DAX-1 and SHP are related (Ehrlund and Treuter, 2012), we tested DAX-1 and SHP for interaction with CAR1 in reporter assays in COS-1 cells. Both DAX-1 and SHP markedly repressed the ability of CAR1 to activate the 2B6XREM luciferase reporter in a dose dependent manner; however, DAX-1 exhibited much more potent inhibition (~10X) than SHP (Figure 2). These experiments were also conducted for the CAR2 and CAR3 splice variants of CAR1, with similar results obtained (see Supplemental Figure S1). In addition, DAX-1 was potent repressor of pregnane X receptor (PXR) activity in transactivation assays (see Supplemental Figure S2). For CAR1, the activity of DAX-1 was not dependent on the presence or absence of added ligand, i.e., DAX-1 inhibited CAR1 activity in DMSO treated, CITCO (agonist). Interestingly, DAX-1 appeared to decrease CAR1 activity in ANDRO (inverse agonist) treated cells. However, this reduction in activity in ANDRO-treated cells may be due to repression of constitutive activity, since complete inhibition of CAR1 constitutive activity by DAX-1 is
not achieved. These assays were repeated in hepatoma HepG2-C3A cells with analogous results (data not shown). Because of the apparent potent interaction between the CAR variants and DAX-1 in reporter assays, we further investigated the impact of DAX-1 on CAR activity.

**Mutation/Deletion Studies with DAX-1.** To provide further evidence of CAR and DAX-1 functional interaction and to examine the regions of DAX-1 that may contribute to the interaction, mutation analyses were performed. The three DAX-1 LXXLL/M motifs (Figure 1) were mutated individually to produce the LXXAA mutants, M1, M2 and M3. In addition, a fourth reported interacting PCFQVLP motif (Sablin et al., 2008) was mutated to yield PCFQAAP (PCF mutant). Most all the mutants significantly decreased CAR1 activity; however, wild type DAX-1 and the DAX-1 M1 and M2 mutants were the most potent repressors, while the DAX-1 M3 and PCF mutants relieved DAX-1 repression of CAR1 activity to 50-75% of the empty vector control level, depending on the chemical treatment (Figure 3A). Similar results were seen for the CAR2 and CAR3 splice variant receptors (Figure 3A). A truncated version of DAX-1 (DAX1-TSD), where amino acids 442-469 corresponding to the TSD (Figure 1) were deleted, was also constructed. This deletion is equivalent to a naturally-occurring DAX-1 mutant that results in adrenal hypoplasia congenita (Ito et al., 1997). CAR1 activity was restored to 90-100% of control levels with the TSD-deleted DAX-1 construct across the various chemical treatments (Figure 3B). Similarly, deletion of the DAX1-TSD restored CITCO-mediated activation of CAR2 and CAR3 to ~75% of control values, changes that were statistically significant compared with empty vector controls. Experiments with these DAX-1 mutant constructs were also performed with PXR. The results mirrored results seen with the CAR variants (see Supplemental Figure 3).

**Two Hybrid Assays for CAR1 and DAX-1 Interaction.** The interaction between CAR1 and DAX-1 was further assessed in mammalian two-hybrid assays. As SRC1 is known to interact with human CAR (Auerbach et al., 2007), it was used as a positive control. The initial screening suggested no interaction between CAR1 and DAX-1, either in the presence or absence of RXRα (data not shown). However, this
negative result could manifest from the presence of the TSD in DAX-1 that itself likely suppresses transcription of the GAL4 luciferase reporter. Therefore, the DAX1-TSD mutant was also tested in the mammalian two hybrid assay. These assays were performed in two different vector orientations (pm-CAR1 with VP16-test constructs and VP16-CAR with pm-test constructs). An interesting observation derived from the different vector orientations was that CITCO was slightly inhibitory of CAR1 interactions when CAR was in the pm vector, while it increased the interaction with SRC1 in the VP16 vector (Figure 4A). The cause for this difference is unknown, but since the test constructs are expressed as fusion proteins with either the GAL4 DBD (pm vector) or the herpes virus transcription activation domain (VP16 vector), these different configurations may affect the respective conformational changes induced by an agonist, and in turn affect the interaction between the test proteins. Another interesting observation was that pm-CAR1 exhibited a low basal activity (with VP16-empty), while VP16-CAR1 showed no basal activity with pm-empty. This result may extend from the CAR1-DBD fusion protein’s ability to recruit endogenous transcriptional activators.

When pm-CAR1 was tested (Figure 4A, left panel), the DAX1-TSD mutant exhibited higher activity with CAR1 than with the full length DAX-1, although not as robust as the interaction between SRC1 and CAR1. Further, the pm-CAR1:DAX1-TSD activity was lower than that generated by the pmCAR1:VP16-empty vector, suggesting some suppression of CAR1 basal activity. When VP16-CAR1 was assayed with the pm-test constructs (Figure 4A, right panel), an interaction was detected only when SRC1 was present. To help determine whether DAX-1 might compete with SRC1 for binding to CAR1, the effect of DAX-1 on the interaction between CAR1 and SRC1 was assessed. Again, the experiments were performed in both vector orientations. DAX-1 inhibited the interaction between CAR1 and SRC1 by ~ 50%, compared to control. In contrast, when the DAX1-TSD mutant was used, the interaction between CAR1 and SRC1 was inhibited by only 10%, compared to control (Figure 4B).

**Alpha-Screen Assays.** To obtain evidence of a direct interaction between CAR1 and DAX-1, Alpha screen assays were performed. These assays demonstrated direct interaction between CAR1 and two
different DAX1 constructs (Figure 5A). Further, this interaction was significantly enhanced by the addition of the CAR ligand, CITCO, suggesting that DAX-1 preferentially binds CAR1 in the liganded state. It is noteworthy that both of the DAX-1 constructs contained only the PCFQVLP NR box, supporting the conclusion that this sequence is important for interaction with the CAR proteins.

Co-Immunoprecipitation of DAX-1 with CAR1. Additional evidence of a direct interaction between CAR1 and DAX-1 were obtained with immunoprecipitation experiments conducted using lysates from COS-1 cells that were cotransfected with 3XFLAG-CAR1 and pCMV6-DAX1, and treated with model CAR direct and indirect activators, as well as the inverse agonist, ANDRO. Anti-FLAG beads were used for immunoprecipitation and the precipitated proteins were subjected to Western blot analysis with anti-DAX1 antibody. As shown in Figure 5B and C, the highest levels of DAX-1 were detected from cells that were treated with CITCO, with lower levels detected in the other treatment groups. These results agree with the alpha screen results suggesting that DAX-1 preferentially binds with CAR1 in the liganded state.

Overexpression of DAX-1 in Primary Human Hepatocytes. CITCO induces CYP2B6 in human liver via activation of CAR (Maglich et al., 2003). In order to assess the impact of DAX-1 on CAR-mediated CYP2B6 induction ex vivo, primary human hepatocytes from 5 individual donors were transfected with DAX-1, treated with CITCO, and then assessed for CYP2B6 mRNA expression. The donor characteristics are shown in Table 3. Based on visualization of GFP, the transfection efficiency was estimated as ranging from ~5-20% in the different hepatocyte donor preparations (data not shown). However, it should be noted that the pCDHdCMV vector used in the transfections contains dual promoters, with GFP driven by a separate promoter than DAX-1 and thus the relative efficiencies of expression of these protein may differ. In this respect, the Ct values obtained in for DAX-1 expression clearly demonstrated DAX-1 overexpression in the cultured cells. RT-PCR analyses of the respective hepatocyte cDNAs showed that DAX-1 expression attenuated CAR-mediated induction of CYP2B6 by
CITCO in all donors, with the extent of CYP2B6 inhibition ranging from 18% to 92% across individual hepatocyte cultures.

**Effect of CAR Activation on DAX-1 Expression in Primary Human Hepatocytes.** In order to assess whether CAR activation induces hepatic DAX-1 expression, untransfected primary human hepatocytes were treated with prototypical CAR (phenobarbital, CITCO) or CAR2 (DEHP) activators for 24h and assessed quantitatively for levels of DAX-1 mRNA expression. Four separate hepatocyte donors were tested but in no case was DAX-1 induction detected by these treatments (data not shown).
Discussion

Screening a panel of peptides containing different ‘LXXLL’ motifs (or NR boxes) led to the identification of DAX-1 as a potential CAR-interacting protein and repressor of CAR transcriptional activity. DAX-1 has been most clearly characterized for its role in human development, as mutations in the gene are associated with both X-linked congenital adrenal hypoplasia and hypogonadotropic hypogonadism (Zanaria et al., 1994; Tabarin et al., 2000). More recently, a role for DAX-1 as a regulator of liver physiology has emerged, with its reported activity as a co-repressor of hepatocyte nuclear factor 4α (HNF4α) and liver X receptor (LXR) resulting in negative regulation of gluconeogenic pathways (Nedumaran et al., 2009) and lipogenesis, respectively (Nedumaran et al., 2010). Although DAX-1 exhibited the highest degree of homology with the detected CAR-interacting peptide, we also examined SHP, as they belong to the same NR sub-family (Ehrlund and Treuter, 2012) and like CAR, SHP is expressed in the liver and was previously demonstrated to interact with mouse CAR, (Park et al., 2004), (Bae et al., 2004). Further, DAX-1 and SHP both lack a traditional NR DNA binding domain (DBD), contain LXXLL NR interaction motifs otherwise found in nuclear co-activators, and act as transcriptional co-repressors of ligand-activated NRs (Ehrlund and Treuter, 2012). Despite these similarities, our results demonstrate that DAX-1 is a much more potent repressor of human CAR1 activity than is SHP.

Similar to SHP (Lee et al., 2000), DAX-1 likely functions through two different protein interaction mechanisms for repression of NR activity. The first is based on the 4 NR boxes in the DAX-1 sequence. The three NR boxes localized in its N-terminal region are analogous to the ‘LXXLL’ motifs commonly found in NR co-activators. A fourth sequence, ‘PCFQVLP’ is thought to mimic the LXXLL motifs and forms the interface for interaction with the AF-2 domain of LRH-1 (Sablin et al., 2008). Interestingly, SHP also contains a similar “PSFCHLP’ sequence, although no functional relevance has been demonstrated (Ehrlund and Treuter, 2012). These 4 DAX-1 NR boxes allow for competition with co-activators. A second mechanism mediating DAX-1 repression lies with a C-terminal transcription silencing domain (TSD). The TSD allows for recruitment of transcriptional corepressors such as NCoR.
We further tested CAR1 and its splice variants, CAR2 and CAR3, for differences in their interaction with DAX-1. For CAR1, CAR2 and CAR3, mutation of the first 2 NR boxes (M1 and M2, Figure 1) had no effect on DAX-1 repression of CAR activity, whereas mutation of the 3rd NR box and the atypical PCFVQLP NR box (M3 and PCF, Figure 1), inhibited DAX-1’s repression of CAR activity by ~50-75%. The results also suggested that the 3rd NR box interacted more strongly with CAR2 and CAR3 than with wild type CAR1. These differences may stem from conformational changes in the CAR variant proteins resulting from amino acid insertions in their sequences (Auerbach et al., 2003). Nevertheless, the third ‘LXXLL’ and the PCFQVLP NR boxes of DAX-1 were most critical in mediating the functional interaction with CAR. Interestingly, deletion of DAX-1’s TSD restored CAR1 activity to control levels, whereas the comparative activities of CAR2 and CAR3 were only restored to about 65% of control. Therefore, these results suggest that: 1) the third ‘LXXLL’ and the PCFQVLP NR boxes of DAX-1 are the principal effectors of co-activator competition and interaction with CAR, and 2) DAX-1 competition with co-activators may be more for CAR2 and CAR3 than CAR1.

Initial mammalian two hybrid studies failed to detect a direct interaction between CAR1 and DAX-1. Explanations for this result may implicate another “bridging” protein required for the interaction and/or that the intrinsic repressor effects from the TSD of DAX-1 are blocking activation of the GAL4 reporter in these assays. The latter possibility appears likely as the basal activity generated with pm-CAR1 and VP16 empty was repressed in the pm-CAR1:VP16-DAX-1 assay (Figure 4A, left panel). To address whether TSD was masking detection of the CAR1-DAX-1 interaction, a DAX-1-TSD construct was used in the mammalian two-hybrid assays. The basal activity of pm-CAR1 was partially restored with VP16-DAX-1-TSD. We further tested DAX-1 and DAX-1-TSD in a mammalian two-hybrid competition assay with CAR1 and SRC1. While intact DAX-1 inhibited the CAR1-SRC1 interaction 50-75% depending on the vector orientation, DAX-1-TSD was not as efficient. In sum, these results support the concept that competition with SRC1 and the intrinsic transcription repressor domain are both
important mechanisms in DAX-1 repression of CAR transcriptional activity. However, the results did not clarify whether a direct interaction existed between CAR1 and DAX-1.

In these respects, both alpha screen and co-immunoprecipitation experiments and clearly indicated a direct interaction between these proteins. Further, in both assays the CAR1/DAX-1 direct interaction was enhanced by the presence of the ligand, CITCO, consistent with known functions of DAX-1 acting as a repressor of ligand-activated receptors. Typically, corepressors bind to NR in the absence of ligand (Perissi et al., 1999). Although CAR1 is ‘constitutively active’ in vitro, binding of the inverse agonist ANDRO favors the recruitment of the co-repressor SMRT (Dussault et al., 2002). Thus, while SMRT or other corepressors help to regulate activity of ‘unactivated’ CAR, DAX-1 serves to repress the activity of CAR1, as well as the ligand-activated variants of the receptor, CAR2 and CAR3. In this manner, DAX-1 may provide another level through which CAR activity is fine tuned.

In vivo, CAR is a hepatic ‘xenosensor’ that upon chemical activation translocates to the nucleus where it facilitates the transcription of genes encoding xenobiotic metabolism and transport (Timsit and Negishi, 2007). CYP2B6 is a prototypical phase I gene activated by CAR in human liver (Honkakoski et al., 2003). The current studies demonstrated that DAX-1 can mediate CAR transcriptional activity in primary human hepatocytes, with overexpression of DAX-1 resulting in decreased CAR-mediated induction of CYP2B6 in CITCO treated cells. Given the interindividual differences noted in inducer responsiveness among humans (Dekeyser et al., 2009), the degree of CYP2B6 induction in CITCO treated cultures was variable among donors, as was the extent of DAX-1 repression. In general, greater levels of CYP2B6 repression were observed in donors that were most responsive to CITCO induction. Differential xenobiotic responsiveness among humans likely results from a complex interplay of genetics, previous chemical exposures, and perhaps differences in the expression profiles of the xenobiotic receptors that mediate these responses. As CAR activation in the liver mediates induction of xenobiotic metabolic enzymes and transporters, DAX-1 repression may contribute to the etiology of adverse drug reactions in select individuals.
While the physiological implications of DAX-1 repression of hepatic CAR activity are currently unclear, DAX-1 appears to negatively regulate hepatic gluconeogenesis and lipogenesis in mice (Nedumaran et al., 2009; Nedumaran et al., 2010). These reports demonstrated that hepatic DAX-1 expression is modulated by insulin and nutritional status and that DAX-1 repressed the transcriptional activity of hepatocyte nuclear factor 4α (HNF4α), a transcription factor known to positively regulate gluconeogenic enzymes (Nedumaran et al., 2009). Further, adenoviral mediated overexpression of DAX-1 in mice fed a high fat diet significantly reduced fasting blood-glucose levels. In another study, DAX-1 was shown to repress hepatic LXRα transcriptional activity resulting in decreased in expression of SREBP-1c, a transcription factor that mediates expression of lipogenic enzymes, as well as decreased liver triglyceride levels (Nedumaran et al., 2010). Interestingly, CAR activation decreases lipogenesis in mouse models fed a high fat diet and different, but perhaps interrelated, mechanisms have been proposed for this effect. CAR activation induces expression of Insig-1, which in turn, represses the activation of SREBP-1c, resulting in decreased expression of lipogenic enzymes (Roth et al., 2008). Another study outlined an indirect mechanism whereby CAR-mediated induction of SULT2B1b results in inactivation of LXRα oxysterol agonists, in turn causing decreased activation of SREBP-1c by LXRα (Dong et al., 2009). Further, CAR activation has also been shown to suppress expression of gluconeogenic enzymes in mouse models, resulting in improved antidiabetic effects (Dong et al., 2009). Given these observations, DAX-1 antagonism of CAR activity in the liver would be predicted to have opposing effects compared with DAX-1 antagonism of hepatic HNF4α and LXRα. Thus, it is conceivable that DAX-1 binds to CAR or HNF4α and LXRα preferentially, based on nutritional status and perhaps, the ligand bound to the receptor. Future studies are required to sort out the complex NR-mediated regulatory mechanisms controlling hepatic lipogenesis and gluconeogenesis.

Given that DAX-1 and SHP share structural and functional similarities (Ehrlund and Treuter, 2012) and that hepatic SHP expression is induced by bile acid-activated farnesoid X receptor, ultimately down-regulating bile acid and fatty acid synthesis (Lee et al., 2007), we investigated if CAR activation
might induce DAX-1 expression in primary human hepatocytes. After 24h treatment with CAR agonists, no induction of DAX-1 expression was observed (data not shown); however, it is intriguing to speculate that DAX-1 may be induced by a downstream metabolic product to mediate CAR activity.

Another tissue in which DAX-1 mediated repression of CAR activity may be important is the adrenal gland. DAX-1 is highly expressed in the adrenal gland and mutations in DAX-1 result in adrenal hypoplasia congenita (for review see Lalli and Sassone-Corsi, 2003; Niakan and McCabe, 2005). Relatively high levels of CAR1 and CAR variants are also detected in the adrenal gland (Savkur et al., 2003; Arnold et al., 2004; Lamba et al., 2004), although a physiologic role in adrenal function has not been identified. However, a glucocorticoid response element (GRE) has been identified in the CAR promoter sequence and studies in human hepatocytes demonstrated that activated GR induces CAR expression (Pascussi et al., 2000; Pascussi et al., 2003). Further, DAX-1 is known to repress ligand activated GR transactivation (Zhou et al., 2008). Thus it seems likely that interactions of DAX-1, CAR and GR in the adrenal gland may influence glucocorticoid homeostasis; however, additional studies are necessary to explore these interactions.

This study demonstrates for the first time that DAX-1 functions as a co-repressor for activated human CAR and its splice variants, CAR2 and CAR3. These effects are likely mediated through competition with coactivators, such as SRC1, and via the intrinsic TSD of DAX-1, which is responsible for recruitment of co-repressors. Although the physiological implications of the CAR-DAX-1 interaction are yet to be determined, DAX-1 mediated repression of CAR transcriptional activity represents an additional level through which CAR activity may be precisely regulated in liver hepatocytes.
Acknowledgments. The authors wish to thank Denise Coslo for technical assistance with the primary human hepatocytes.
Authorship Contributions.

Participated in research design: Laurenzana, Chen, and Omiecinski

Conducted experiments: Laurenzana, Chen, Kannuswamy, Sell, and Li

Contributed new reagents or analytical tools: Li and Strom

Wrote or contributed to the writing of the manuscript: Laurenzana and Omiecinski
References


Constitutive Androstane Receptor (CAR) and Requires Steroid Co-Activator 1 (SRC-1) and the Transcription Factor Sp1. *Biochem J* **355**:71-78.


Footnotes

This study was supported by a grant from the National Institutes of Health National Institute of General Medicine [Grant GM066411].
Figure Legends

Figure 1. DAX-1 protein sequence showing the locations of three ‘LXXL/ML’ NR boxes (bold and shaded in gray), the PCFQVL NR interacting sequence (double underlined), and the transcription silencing domain (TSD, single underlined).

Figure 2. Effect of DAX-1 or SHP on CAR1 Activity. Results shown here represent single transfection experiments, with all treatments in quadruplicate. COS-1 cells were transfected with the CMV2-CAR1 and 3.1-RXRα expression vectors, the 2B6-XREM-PBREM reporter, the pRL-CMV vector for normalization of transfection efficiency and varying amounts of PCMV6-DAX1 (Panel A) or pCMV6-SHP (Panel B). All treatments were for 24h and the data are represented as normalized luciferase values. Each data point represents the mean (+/- S.D.). Asterisks indicate significant difference from respective control (no DAX-1 or SHP) within a chemical treatment group (p<0.01).

Figure 3. Effect of DAX-1 mutations on CAR variant activity. Results shown here represent single transfection experiments, with all treatments in quadruplicate. COS-1 cells were transfected with the 3.1-RXRα expression vector, the 2B6-XREM-PBREM reporter, the pRL-CMV vector for normalization of transfection efficiency and varying amounts of CMV2-CAR1, -CAR2 or CAR3 and PCMV6-empty, -DAX1 WT, -DAX1 M1, -DAX1 M2, -DAX1 M3, or -DAX1 PCF (Panel A) or PCMV6-DAX1-TSD (Panel B). All treatments were for 24h and data are represented as normalized luciferase values and each data point represents the mean (+/- S.D.). Asterisks indicate significant difference from respective control (no DAX1) within a chemical treatment group (p<0.05).

Figure 4. Panel A. Mammalian two-hybrid assays to assess CAR1 interaction with DAX-1. COS- cells were cotransfected with pmGAL4-CAR1-LBD and VP16-empty (negative control), VP16-SRC1 (positive control) VP16-DAX1 or VP16-DAX1 TSD, 3.1 RXRα-LBD, pFR-luciferase reporter and pRL-CMV vector for transfection efficiency normalization (left side). On the right half of the graph, CAR1 was in the VP16 vector and the test constructs were in the pmGAL4 vector. Panel B. Mammalian two
hybrid assay to assess the effect of DAX-1 on CAR1-SRC1 interaction. COS-1 cells were cotransfected with pmGAL4-CAR1 and VP16-SRC1 along with either VP16-empty, -DAX1, or -DAX1-TSD. The 3.1 RXRα-LBD, pFR-luciferase reporter and pRL-CMV vectors were included in all transfections. On the right half of the graph, VP16-CAR1-LBD and pmGAL4-SRC1 were tested in the presence of pmGAL4-empty, -DAX1, or –DAX1-TSD. Both panels are representative single transfection experiments, with all treatments in quadruplicate and each data point represents the mean (+/- S.D.). Asterisks indicate that each treatment was significantly different from its respective empty vector control (p<0.05).

Figure 5. Direct interaction between CAR1 and DAX-1. Alpha screen assay for CAR1 interaction with DAX-1 (Panel A). Purified CAR1-LBB and DAX-1 GST (~40 nM each) were incubated in the presence of 5µg/ml donor and acceptor beads. The DAX-1a construct contains aa 210-470 and DAX-1b contains aa 218-470. Bars represent the mean (+/- S.D.) of 2 separate experiments, each performed in duplicate. Representative co-immunoprecipitation of CAR1 and DAX1 (Panel B). COS-1 cells were cotransfected with pCMV6-DAX1 andp3XFLAG CAR or 3XFLAG empty. After ~40h, the cells were treated with DMSO, CITCO ANDRO or PB for 5-6h and then harvested. Cell lysates were immunoprecipitated with anti-FLAG beads and precipitated protein was subjected to Western blot with anti-DAX1 antibody, followed by anti-FLAG antibody. Panel C shows the densitometry analysis of the Western blot data.

Figure 6. Effect of DAX-1 on CAR-mediated induction of CYP2B6 by CITCO in primary human hepatocytes. Human primary hepatocytes were transfected with DAX-1, empty vector and then treated with CITCO or DMSO. RT-PCR was performed on cDNA samples isolated from the cells to quantify DAX-1, CYP2B6 and GAPDH (as an internal reference). Values were expressed as fold induction relative to untransfected or empty vector transfected DMSO treated cells and represent the average of two replicate samples. Percentages indicate the decrease in CYP2B6 induction relative to untransfected or empty vector transfected CITCO treated.
Table 1. Primers used for creation of DAX-1 constructs

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This article has not been copyedited and formatted. The final version may differ from this version.
### Table 2. SYBR Green PCR Primer Sets

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Supplemental Material for:

Title: The orphan nuclear receptor, DAX-1, functions as a potent co-repressor of the constitutive androstane receptor (CAR, NR1I3)

Authors: Elizabeth M. Laurenzana, Tao Chen, Malavika Kannuswamy, Brian E. Sell, Stephen C. Strom, Yong Li, and Curtis J. Omiecinski

Journal: Molecular Pharmacology

Table S1. RT-PCR Standard Curve Target Data

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Table S2. Interaction of McDonnell\textsuperscript{1} Panel peptides with human CAR variants

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\textsuperscript{2} Peptides were classified into three different classes based on sequences flanking the conserved LXXLL motif. The highlights showed conserved sequences in the classes and the ‘LXXLL’ motifs found in all of the peptides.
Supplemental Figure S1. Effect of DAX-1 and SHP on CAR2 and CAR3 activity. Results shown here represent single transfection experiments, with all treatments in quadruplicate. COS-1 cells were transfected with the CMV2-CAR2 or CMV2-CAR3 and 3.1-RXRα expression vectors, the 2B6-XREM-PBREM reporter, the pRL-CMV vector for normalization of transfection efficiency and varying amounts of PCMV6-DAX1 (Panel A) or pCMV6-SHP (Panel B). All treatments were for 24h and the data are represented as normalized luciferase values. Each data point represents the mean (+/- S.D.).
Supplemental Figure S2. Effect of DAX-1 and SHP on pregnane X receptor (PXR) activity. Results shown here represent single transfection experiments, with all treatments in quadruplicate. COS-1 cells were transfected with the CMV2-PXR and 3.1-RXRα expression vectors, the 3A4-XREM-PBREM reporter, the pRL-CMV vector for normalization of transfection efficiency and varying amounts of PCMV6-DAX1. All treatments (TO901317 is a PXR agonist; Mitro N, Vargas L, Romeo R, Koder A, Saez E. T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. FEBS Lett. 2007;581:1721-1726) were for 24h and the data are represented as normalized luciferase values. Each data point represents the mean (+/− S.D.).
**Supplemental Figure S3.** Effect of DAX-1 mutations on pregnane X receptor (PXR) activity. Results shown here represent single transfection experiments, with all treatments in quadruplicate. COS-1 cells were transfected with the 3.1-RXRα expression vector, the 3A4-XREM-PBREM reporter, the pRL-CMV vector for normalization of transfection efficiency and varying amounts of CMV2-CAR1, -CAR2 or CAR3 and PCMV6-empty, -DAX1 WT, -DAX1 M1, -DAX1 M2, -DAX1 M3, or -DAX1 PCF (Panel A) or PCMV6-DAX1-TSD (Panel B). All treatments (TO901317 is a PXR agonist; Mitro N, Vargas L, Romeo R, Koder A, Saez E. T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. FEBS Lett. 2007;581:1721-1726) were for 24 h and data are represented as normalized luciferase values and each data point represents the mean (+/- S.D.). Asterisks indicate that each treatment was significantly different from its respective empty vector control (p<0.05).