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DP97, a DEAD box DNA/RNA helicase, is a target gene-selective co-regulator of the constitutive androstane receptor

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ABSTRACT

The constitutive androstane receptor (CAR) plays a key role in the expression of xenobiotic/steroid and drug metabolizing enzymes and their transporters. In this study, we demonstrated that DP97, a member of the DEAD box DNA/RNA helicase protein family, is a novel CAR-interacting protein. Using HepG2 cells expressing human CAR in the presence of tetracycline, we showed that knockdown of DP97 with small interfering RNAs suppressed tetracycline-inducible mRNA expression of CYP2B6 and UGT1A1 but not CYP3A4. Thus, DP97 was found to be a gene (or promoter)-selective co-activator for hCAR. DP97-mediated CAR transactivation was synergistically enhanced by the co-expression of SRC-1 or PGC1α, therefore it might act as mediator between hCAR and appropriate co-activators.

1. Introduction

The constitutive androstane receptor (CAR), also known as NR1I3, of the nuclear receptor superfamily plays a key role in responses to xenochemical stimuli. CAR enhances the expression of various drug-metabolizing enzymes and transporters, such as CYP2B, CYP3A, CYP2C, glutathione S-transferases, sulfotransferases, UDP-glucuronosyltransferases 1A1 (UGT1A1), Oatp2, Mrp2, and Mrp3 [1–6]. This occurs upon activation, by either direct binding of ligands, such as 6-(4-chlorophenyl)imidazo[2,1-b] [1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl)oxime (CITCO) [7], or signal transduction triggered by activators like phenobarbital [1]. CAR predominantly localizes in the cytoplasm of hepatocytes *in vivo* and in primary cultures, then translocates to the nucleus in response to ligands or activators. Following nuclear translocation, CAR binds to its response elements in the promoter region of the target genes, forming a heterodimer with retinoid X receptor α (RXRα) [8]. The CAR:RXR heterodimer complex then recruits co-activators, such as steroid receptor co-activator 1 (SRC-1) [9], transcriptional intermediary factor 2 (TIF2) [10], and peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PGC1α) [11]. However, CAR translocates spontaneously into the nuclear compartment in immortal cells [12–14]. Because CAR is able to recruit co-activators weakly without ligand binding, it is often referred to as a constitutive androstane receptor [8]. In the presence of agonists, the interaction between CAR and co-activators is tightened. In contrast, chemicals known as "inverse agonists" such as the androstane metabolites androstanol and androstenol [15], and the selective peripheral benzodiazepine antagonist PK11195 inhibit the transactivation potential of ligand-free CAR [16]. Deactivation of CAR by inverse agonists is mediated by dissociation of co-activators followed by association of co-repressors. Furthermore, constitutive transcriptional activity of CAR is affected by the intracellular expression patterns of cofactors. Thus, cofactors are important regulators for the transcriptional activity of CAR.

DP97, a member of the DEAD box DNA/RNA helicase protein family, has been identified as a hormone-dependent interacting protein of estrogen receptors (ERs). DP97 interacts with the AF-2 domain of ERα and act as a co-repressor. In this study, we identified DP97 as a novel CAR-binding protein and showed that it played a role as a gene (or promoter)-selective co-activator for CAR.

2. Material and methods

2.1. Chemicals and plasmid construction

PK11195 and tetracycline were purchased from Sigma Aldrich. The construction of expression plasmids for human (pcDNA-hCAR) and rat CARs (pcDNA-rCAR) has been reported previously [16,17]. The hCAR gene was subcloned into pCMV-3Tag6. The gene encoding the full-length human DP97 was amplified from a cDNA library of human breast cancer MCF-7 cells and inserted in-frame into pcDNA5/TO (Invitrogen) with N-terminal myc tags. The genes

Abbreviations: CAR, constitutive androstane receptor; UGT, UDP-glucuronosyltransferase; SRC-1, steroid receptor co-activator 1; PGC1α, peroxisome proliferator-activated receptor gamma, co-activator 1 alpha.

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encoding the N- and C-terminal truncated fragments of DP97, $DP97_{1-412}$ (amino acids 1–412) and $DP97_{413-865}$ (amino acids 413–865), respectively, were amplified and inserted at appropriate sites in pcDNA5/TO with N-terminal myc tags. To construct the monomer Midoriishi-Cyan (Micy)-tagged hCAR and monomer Kusabira Orange (mKO)-tagged DP97, Micy and mKO coding regions were inserted into the N-terminal region of pcDNA-hCAR or pcDNA-DP97, respectively. The DR4-driven luciferase reporter plasmid (pDR4-Luc) was constructed by inserting three tandem repeats of the DR4 motif into pGL4.24 (Promega).

2.2. Cell culture

The HepTR/hCAR cell line was established from human hepatoma HepG2 cells, in which hCAR was expressed only in the presence of tetracycline (Tet), using the T-REx system (Invitrogen). The human embryonic kidney HEK293, HepG2, HepTR/hCAR and Cos-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; WAKO) containing 10% fetal bovine serum (FBS) and penicillin–streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Co-immunoprecipitation Assay

COS-7 cells were seeded in 60-mm plates and transfected with expression plasmids for myc-DP97, alone or in combination with FLAG-hCAR using the GeneJuice transfection reagent (Novagen). At 48 h post-transfection, cells were harvested after being washed twice with ice-cold phosphate-buffered saline (PBS) and suspended in lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100) containing a protease inhibitor cocktail (Roche). Supernatants were incubated with anti-FLAG M2 agarose (SIGMA) at 4 °C for 2 h. Then, the beads were washed three times with wash buffer (50 mM Tris–HCl, 1 mM EDTA, 650 mM NaCl, and 0.1% Triton X-100) and suspended in sodium dodecyl sulfate (SDS) sample buffer. The co-immunoprecipitated proteins were then separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and detected by western blotting using anti-myc (MBL) and anti-DDDK antibodies (MBL).

2.4. Luciferase reporter analysis

HepG2 cells were transfected using the GeneJuice transfection reagent (Novagen) and the appropriate expression plasmids, with pGL4.74 (hRluc/TK; Promega) used as an internal standard. After overnight incubation, cells were treated with 10 mM PK11195 or solvent (DMSO) for 24 h, before luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The activities of firefly luciferase were normalized against those of Renilla luciferase.

2.5. Knockdown experiments

HepTR/hCAR cells were transfected with DP97 small interfering RNA (siRNA; Invitrogen) or control siRNA (Invitrogen) using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions.

2.6. Quantitative RT-PCR

Total RNA was isolated using ISOGEN II (Nippon Gene Co.) and cDNA was synthesized using a ReverTraAce qPCR RT Kit (TOYOBO). Quantitative RT-PCR (qRT-PCR) was conducted using a THUNDERBIRD™ SYBR qPCR Mix (TOYOBO) in a final volume of 25 μl according to the manufacturer's protocol and using 7500 fast system SDS software (Applied Biosystems). Specific primers were used to target CYP2B6 (5′-AAG CGG ATT TGT CTT GGT GAA-3′ and 5′-TGG AGG ATG GTG GTG AAG AAG-3′), CYP3A4 (5′-CCA AGC TAT GCT CTT CAC CG-3′ and 5′-TCA GGC TCC ACT TAC GGT GC-3′), UGT1A1 (5′-AGT GGA TGG CAG CCA CTG GCT-3′ and 5′-CAG TAA GTG GGA ACA GCC AGA-3′), DP97 (5′-CAT GTG GCT GTG GAA ATG AG-3′ and 5′-AAA TTC CAC CAG CAG TTT GG-3′) and beta-actin (5′-TCC TCC TGA GCG CAA GTA CTC-3′ and 5′-CTG CTT GCT GAT CCA CAT CTG-3′).

2.7. Subcellular localization analysis

HEK293 cells were transfected with Micy-tagged hCAR and mKO-tagged DP97 expression plasmids. At 24 h post-transfection, the intracellular localization of fluorescent protein fused with chimeric proteins were observed using a Zeiss LSM 510 microscope.

3. Results

3.1. DP97 is a novel CAR interacting protein

To identify proteins that regulate the activity of CAR, we carried out co-immunoprecipitation in HepG2 cells stably expressing FLAG-hCAR proteins. DP97 was identified among the proteins co-immunoprecipitated by the anti-FLAG M2 antibody. To confirm physical interaction between hCAR and DP97, we performed co-immunoprecipitation assays in COS-7 cells transfected with FLAG-tagged hCAR (FLAG-hCAR) and myc-tagged DP97 (myc-DP97). Co-immunoprecipitation of hCAR and human DP97 was also observed in simian cells (Fig. 1(A)).

3.2. DP97enhances CAR-mediated transcriptional activation

We analyzed the effect of DP97 on CAR-mediated transcriptional activation using a DR4-luciferase reporter (DR4-luc) assay. Although DP97 is known to repress the transcriptional activity of ERα [18], it significantly enhanced human and rat CAR-mediated transactivation of DR4-luc in a dose-dependent manner (Fig. 1(B)). The C-terminal region of DP97 was reported to harbor the small repression domain and receptor interaction domain, which was sufficient to repress the transcriptional activity of ERα [18]. To localize the critical region for transcriptional activation of hCAR, the N- (amino acids 1–412) and C-terminal (amino acids 413–865) truncated mutants of DP97 were expressed with hCAR. Both mutants did not result in CAR-mediated transcriptional activation of DR4-luc (Fig. 1(C)), suggesting that the entire DP97 is required for transcriptional activation of hCAR.

3.3. Subcellular distribution of hCAR and DP97

The subcellular localization of hCAR and DP97 was investigated by simultaneously transfecting monomer Micy-tagged CAR and mKO-tagged DP97 in HEK293 cells. Micy-CAR and mKO-DP97 were co-localized in the nuclear compartment (Fig. 2). Furthermore, mKO-DP97 was distributed in nuclear speckles together with Micy-CAR.

3.4. Co-expression of DP97 with SRC-1 or PGC1α synergistically activates CAR

Expression of hCAR and DP97 was carried out with or without co-expression of SRC-1 or PGC1α in HEK293 cells. (Fig. 3(A)). SRC-1, PGC1α and DP97 individually enhanced CAR-mediated transactivation. Synergistic activation of CAR was observed when DP97 was co-expressed with either SRC-1 or PGC1α. It is well known that exogenously expressed CAR can interact weakly with

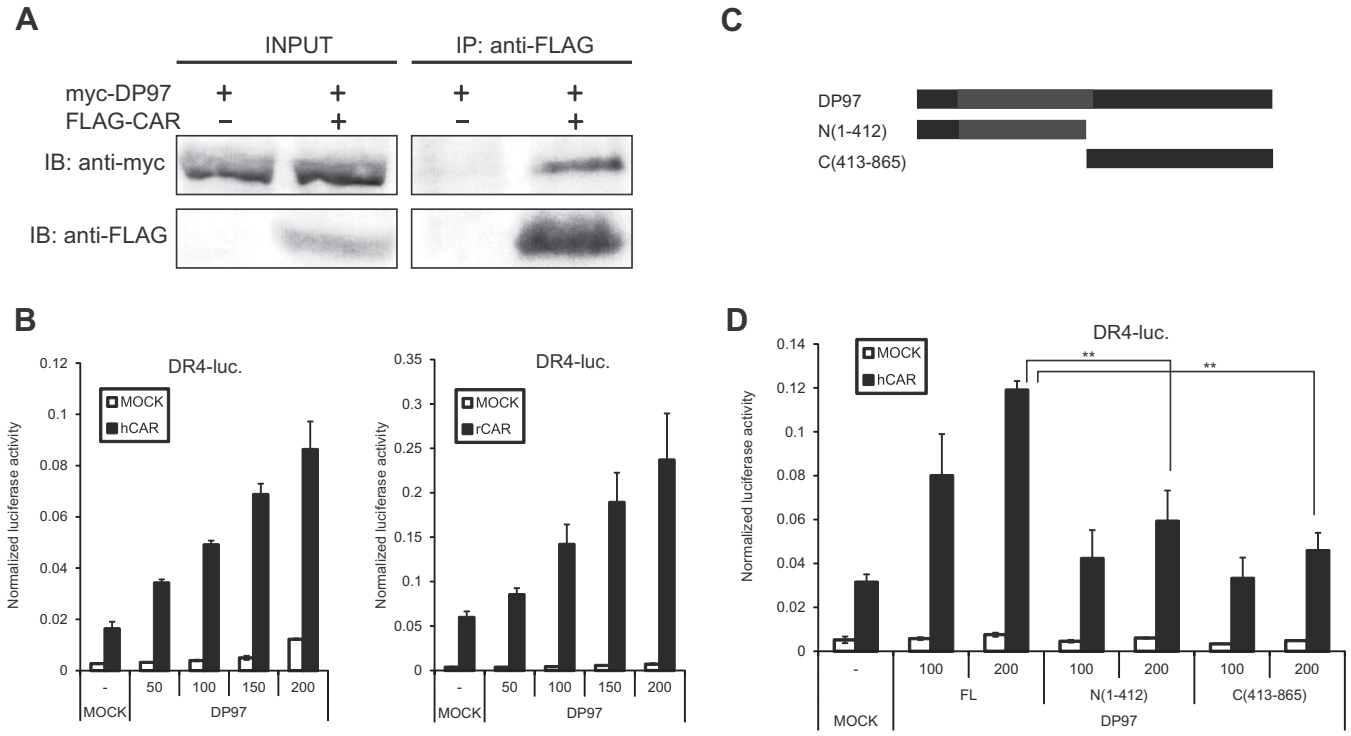

Fig. 1. (A) COS-7 cells were co-transfected with the expression plasmids myc-DP97 and FLAG-CAR or empty vector. Whole cell extracts were then co-immunoprecipitated using anti-FLAG M2 agarose. Co-precipitants were resolved by SDS–PAGE and detected by western blot analysis using anti-myc and anti-FLAG antibodies. (B) HEK293 cells were transfected with the DR4-luciferase reporter (0.1 μg), pGL4.74 (0.01 μg) and expression plasmids (CAR and increasing concentrations of DP97 expression or empty plasmids). (C) Schematic of DP97 deletion mutants. (D) HEK293 cells were transfected with the DR4-luciferase reporter (0.1 μg), pGL4.74 (0.01 μg) and expression plasmids (CAR and deletion mutants of DP97, or empty plasmids). Luciferase activity was measured at 24 h post-transfection. Cells were harvested and luciferase activity measured. Results are presented as Renilla-normalized luciferase activity (mean ± SD). $^{*}P < 0.05$; $^{**}P < 0.01$.

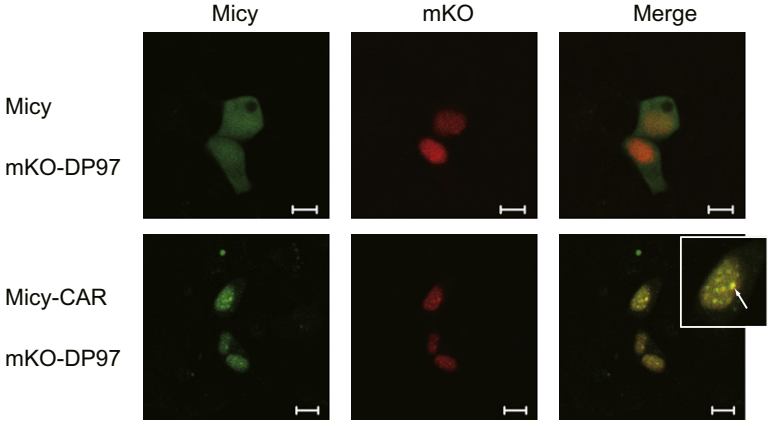

Fig. 2. HEK293 cells were co-transfected with mKO-DP97 and Micy, or Micy-CAR. At 24 h post-transfection, cells were observed using a confocal laser-scanning microscope. Scale bar: 10 μm.

co-activators, such as SRC-1, in the absence of any ligands leading to constitutive transcriptional activation of target genes. This binding is increased by agonists such as TCPOBOP in the case of mCAR, and blocked by inverse agonists when co-activators are replaced with co-repressors. PK11195 repressed constitutive activity of CAR when DP97 was present or absent (Fig. 3(B)).

3.5. Gene-specific regulation of DP97

To investigate the effects of DP97 on endogenous gene regulation by CAR, we established HepG2 cells expressing Tet-dependent hCAR (HepTR/hCAR). Induction of hCAR protein and mRNA expression following treatment with Tet was observed in HepTR/hCAR cells (Fig. 4(A) and (B)). Induction of mRNA for CAR target genes (CYP2B6, CYP3A4 and UGT1A1) was observed after treatment with Tet for 24 h. Knockdown of DP97 with siRNA suppressed Tet-inducible CYP2B6 and UGT1A1 mRNA expression. In contrast, CYP3A4 mRNA induction was not affected by knockdown of DP97 (Fig. 4(C)).

4. Discussion

CAR plays an important role in xenobiotic/drug metabolism. In this study, we identified DP97 as a novel CAR-binding protein

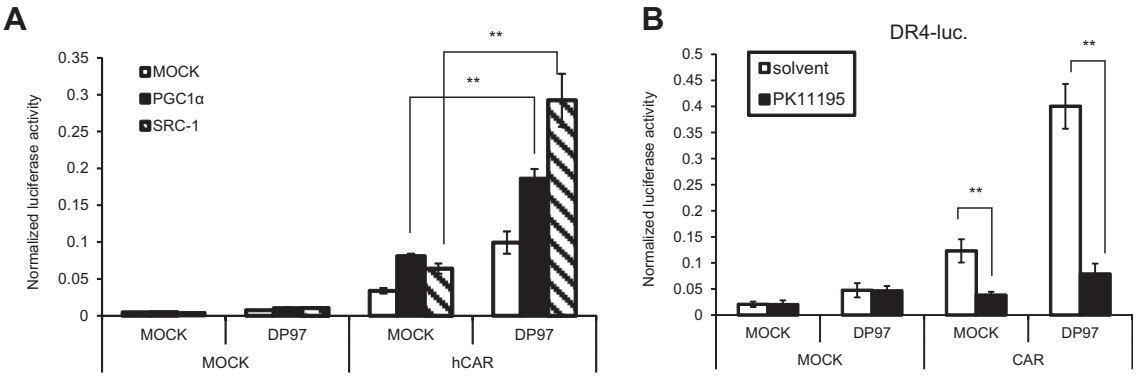

Fig. 3. HEK293 cells were transfected with the DR4-luciferase reporter (0.1 μg), pGL4.74 (0.01 μg) and expression plasmids (CAR, DP97, PGC1α and SRC-1, or empty plasmids). (A) HEK293 cells were transfected with the DR4-luciferase reporter (0.1 μg), pGL4.74 (0.01 μg) and expression plasmids (CAR and DP97, or empty plasmids). After 24 h, cells were treated with PK11195 or DMSO (solvent control). Luciferase activity was measured at 24 h post-transfection (A) or treatment (B). Cells were harvested and luciferase activity measured. Results are presented as Renilla-normalized luciferase activity (mean ± SD). $^{*}P < 0.05$; $^{**}P < 0.01$.

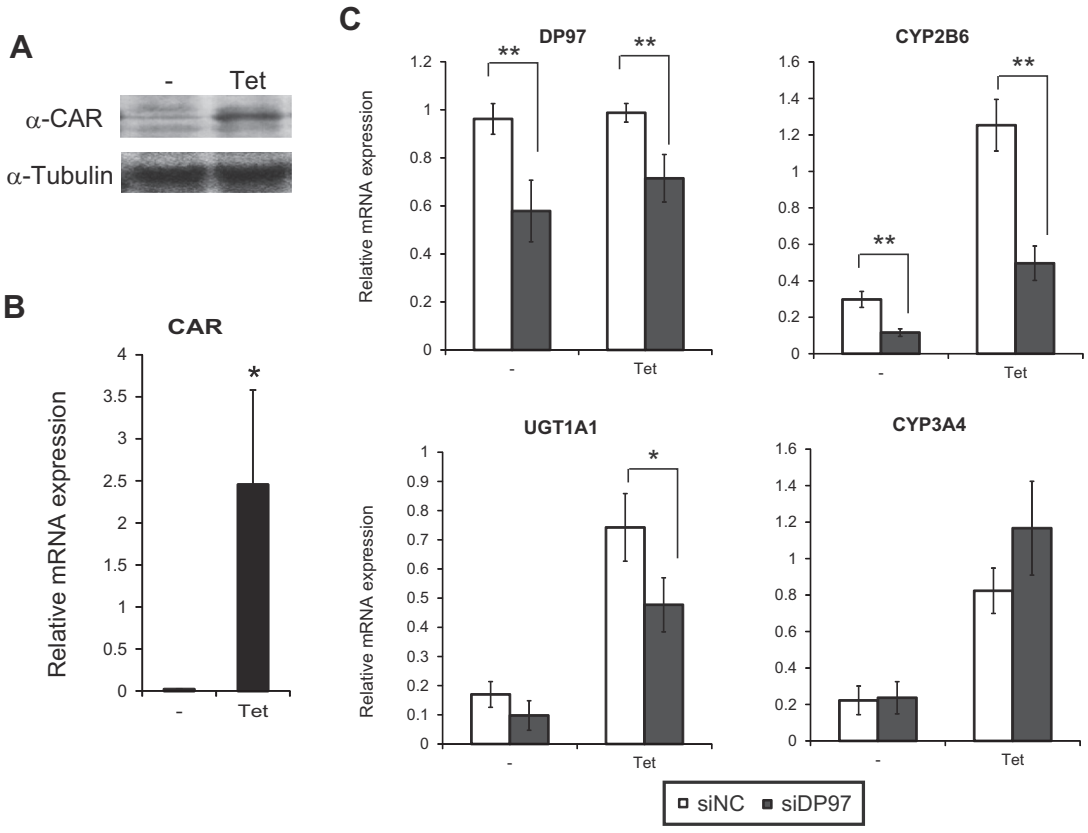

Fig. 4. (A–B) HepTR/hCAR cells were incubated for 24 h with tetracycline (Tet). (A) Whole-cell lysates (20 μg) were resolved by SDS–PAGE, and protein were detected by immunoblotting using anti-CAR and anti-tubulin antibodies. (B) The expression of CAR mRNA was measured by qRT-PCR. The results were normalized against those of β-actin and are expressed as the mean ± S.D. ($n = 3$). (C) HepTR/hCAR cells were treated with DP97 or negative control siRNA. After 48 h, cells were treated with Tet or solvent for an additional 24 h. The expression levels of DP97, CYP2B6, UGT1A1 and CYP3A4 mRNA were measured by qRT-PCR. Results were normalized against those for β-actin and expressed as the mean ± S.D. ($n = 3$).

using co-immunoprecipitation. DP97 has been identified as a hormone-dependent interacting protein of ERs. DP97 can repress transcriptional activity of ERα, ERβ, PR, GR and RARα [18]. DP97 has a repression domain (RD) between amino acids 589–631, therefore its C-terminal region (amino acids 413–865) is sufficient for transcriptional repression of ERα. In this study, we observed that DP97 enhanced the transcriptional activity of CAR. However, enhanced transcriptional activity of CAR was not observed for the C-terminal region of DP97. CAR is regulated by DP97 in a quite different manner from that seen by ERα. It is known that the DEAD box subfamily proteins, p68 and p72, act as co-activators of ERα [19,20]. p72 synergistically enhances ERα transcriptional activity with SRC-1 [21]. Similar to the case of p72-mediated transactivation of ERα, DP97-mediated transactivation of hCAR was synergistically enhanced by the co-expression of SRC-1 or PGC1α. Taken together, DEAD box subfamily proteins might be intermediaries between cofactors and nuclear receptors.

It has been reported that p68 and p72 repress constitutive promoters by interacting with histone deacetylase 1 (HDAC1), a well-known transcriptional repression protein [22]. To clarify the role of

DP97 in CAR-mediated regulation of endogenous genes, we established HepTR/hCAR cells. The expression of mRNA in these cells, specific for CAR target genes, was induced by treatment with Tet. The knockdown of DP97 reduced mRNA expression levels of CYP2B1 and UGT1A1, whereas CAR-mediated up-regulation of CYP3A4 was insensitive to DP97 down-regulation. These results suggest that DP97 acts as a promoter-specific co-regulator of CAR, similar to p68 and p72 [22]. It was recently reported that Hairless acted not only as a co-repressor but also co-activator for the vitamin D receptor in a gene-selective manner [23].

In conclusion, we found that DP97 enhanced the transcriptional activity of hCAR by direct interaction in a gene- or promoter-selective manner. Unlike previous findings concerning the negative roles of DP97 with ERα, ERβ, PR, GR and RARα, we determined that DP97 acts as co-activator of the hCAR gene, selectively enhancing the expression levels of enzymes involved in drug metabolism.

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