

Activation of Orphan Nuclear Constitutive Androstane Receptor Requires Subnuclear Targeting by Peroxisome Proliferator-activated Receptor γ Coactivator-1 α

A POSSIBLE LINK BETWEEN XENOBIOTIC RESPONSE AND NUTRITIONAL STATE*

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In contrast to the classical nuclear receptors, the constitutive androstane receptor (CAR) is transcriptionally active in the absence of ligand. In the course of searching for the mediator of CAR activation, we found that ligand-independent activation of CAR was achieved in cooperation with the peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α). PGC-1 β , a PGC-1 α homologue, also activated CAR to less of an extent than PGC-1 α . Coexpression of the ligand-binding domain of a heterodimerization partner, retinoid X receptor α , enhanced the PGC-1 α -mediated activation of CAR, although it had a weak effect on the basal activity of CAR in the absence of PGC-1 α . Both the N-terminal region, with the LXXLL motif, and the C-terminal region, with a serine/arginine-rich domain (RS domain), in PGC-1 α were required for full activation of CAR. Pull-down experiments using recombinant proteins revealed that CAR directly interacted with both the LXXLL motif and the RS domain. Furthermore, we demonstrated that the RS domain of PGC-1 α was required for CAR localization at nuclear speckles. These results indicate that PGC-1 α mediates the ligand-independent activation of CAR by means of subnuclear targeting through the RS domain of PGC-1 α .

Nuclear receptors comprise a large family of ligand-regulated transcription factors that mediate various physiological responses (1). Nuclear receptors are composed of highly conserved structural domains, including a DNA-binding domain (DBD)¹ and a ligand-binding domain (LBD). In response to ligand binding, the LBDs of nuclear receptors undergo a structural rearrangement, resulting in the recruitment of transcriptional coactivators, such as CBP, cAMP-response element-bind-

ing protein-binding protein (CBP)/p300, p300/CBP-associated factor (P/CAF), steroid receptor coactivator 1, peroxisome proliferator-activated receptor gamma (PPAR γ) coactivator-1 α (PGC-1 α), and the thyroid hormone receptor-associated proteins/vitamin D receptor-interacting proteins/activator-recruited cofactor (TRAP/DRIP/ARC) complex, which lead to either histone acetylation or recruitment of the RNA polymerase II complex (2).

The constitutive androstane receptor (CAR, also named NR1I3 or NR1I4) (3) and the pregnane X receptor (PXR, also named NR1I2) (3), which are both expressed abundantly in the liver and intestine, play critical roles in the xenobiotic response, the metabolic responses of the body against toxic molecules and drugs (4). The activation of these nuclear receptors induces the expression of several target genes, including cytochromes P450. Recently, CAR gene knock-out mice exposed to acetaminophen were spared from the toxic effects of the drug (5). However, natural ligands to these receptors are unknown, and thus they are included in the orphan nuclear receptors. Unlike classical nuclear receptors, such as steroid hormone receptors, CAR shows constitutive transcriptional activity in the absence of ligand when expressed in cell lines and in yeast (6, 7). The constitutive activity of mouse CAR is inhibited by sex steroids, 5 α -androstan-3 α -ol (androstanol) and 5 α -androst-16-en-3 α -ol (androstenol) (7). Yet, it is still unclear whether or not these steroids are true natural ligands for CAR, because a superphysiological concentration was required to inhibit the CAR activity. Thus, the elucidation of the mechanism of CAR activation is a current issue.

During a search for a coactivator coupled with CAR, we found that the transcriptional coactivator PGC-1 α mediated the ligand-independent activation of CAR. PGC-1 α was originally identified as a PPAR γ -binding protein in a cell line derived from brown adipose tissue (8) and subsequently was shown to couple with several nuclear receptors (9–15). Unlike other transcriptional coactivators, PGC-1 α has the distinctive structural features of a serine/arginine-rich domain (RS domain) and an RNA recognition motif (RRM) at the C-terminal end, which are both observed in many splicing factors (16). We demonstrate here that the RS domain of PGC-1 α plays an important role for CAR activation by interacting with CAR and targeting CAR to nuclear speckles. The significance of the interaction of CAR with PGC-1 α , which is considered to be a key modulator in glucose homeostasis, is also discussed.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmids pCMX-FLAG-mPGC1 α , pCMX-FLAG-mPGC1 β , pCMX-GAL4-hERR3 (amino acids 173–436), pCMX-GAL4-hPPAR γ 2 (amino acids 204–506), pCMX-GAL4-hPXR (amino acids

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¹ The abbreviations used are: DBD, DNA-binding domain; LBD, ligand-binding domain; CBP, cAMP-response element-binding protein-binding protein; P/CAF, p300/CBP-associated factor; PPAR γ , peroxisome proliferator-activated receptor γ ; PGC-1 α , PPAR γ coactivator-1 α ; CAR, constitutive androstane receptor; PXR, pregnane X receptor; androstanol, 5 α -androstan-3 α -ol; androstenol, 5 α -androst-16-en-3 α -ol; RS domain, serine/arginine-rich domain; RRM, RNA recognition motif; GST, glutathione S-transferase; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; ERR3, estrogen-related receptor 3; ER α , estrogen receptor α ; RXR α , retinoid X receptor α ; HNF4, hepatocyte nuclear factor 4; PBS, phosphate-buffered saline.

110–434), pCMX-GAL4-hRXR α (amino acids 222–426), pCMX-GAL4-hER α (amino acids 251–595), pCMX-GAL4 DBD, and UASx4-tk-Luc were kindly provided by Dr. A. Kakizuka (Kyoto University). The plasmids pOZ-Tip60 and pOZ-P/CAF were obtained from Dr. T. Ikeda (Hiroshima University). The cDNA for human CAR LBD (amino acids 77–348) was obtained by PCR from a human liver cDNA library (Origene Technologies, Inc.) using the primers CARLs (5'-TAGATGCT-GAATTC AGG AAA GAC ATG ATA C; underlining indicates the introduced EcoRI site) and CARLa (5'-GCGGATCC TCA GCT GCA GAT CTC CTG GAG C; underlining indicates the introduced BamHI site). The PCR fragment was cloned into pBluescript KS(–), and the sequence was confirmed. The EcoRI-BamHI fragment was excised and ligated to EcoRI-BamHI-digested pCMX-GAL4 DBD.

The pCGN-TFIIB plasmid was constructed by inserting an *Nhe*I-BamHI fragment of TFIIB, excised from phiIB (obtained from Dr. D. Reinberg, Harvard University), into *Xba*I-BamHI-digested pCGN (obtained from Dr. K. Simon). The *Hind*III-*Kpn*I fragment containing a FLAG peptide sequence was excised from pCMX-FLAG-mPGC1 β and inserted into *Hind*III-*Kpn*I-digested pCMX-GAL4-RXR α , giving rise to pCMX-FLAG-RXR α .

The pCMX-FLAG-mPGC1 α plasmid was digested with *Nhe*I and *Xba*I, and the large fragment was recircularized by ligation, giving rise to the PGC-1-Nhe deletion mutant. The large fragment was blunted by Klenow DNA polymerase and was circularized by self-ligation, giving rise to the PGC-1-Nhe/Xba mutant. The pCMX-FLAG-mPGC1 α plasmid was digested with *Xba*I, blunted by Klenow DNA polymerase, and circularized by self-ligation, giving rise to the PGC-1-Xba mutant. The pCMX-FLAG-mPGC1 α plasmid was digested with *Eco*RI, and the large fragment was circularized by self-ligation, giving rise to the PGC-1-Eco mutant. The *Eco*RI-*Mfe*I fragment containing the RS domain of PGC-1 α was inserted into the *Eco*RI-digested PGC-1-Eco mutant, giving rise to the PGC-1-Mfe mutant.

The *Eco*RI-*Not*I fragment of hCAR LBD was cloned in-frame with the glutathione S-transferase (GST)-encoding sequence of pGEX6P-1 (Amersham Biosciences). Deletion mutants of GST-fused PGC-1 α were constructed in a similar manner as described above. For the S tag fusion protein, PGC-1 α was digested with the indicated restriction enzymes and cloned into pET30 (Novagen).

For the enhanced green fluorescent protein (EGFP) fusion protein, the *Eco*RI-BamHI fragment of hCAR LBD was first cloned into pBlue-script KS(–), and then the *Sal*I-BamHI fragment was excised and cloned into pEGFP-C1 (Clontech).

Cell Culture and Transient Transfections—COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone Laboratories). One day before transfection, the cells were plated in 24-well plates at a density of 6×10^4 /well. The cells were transiently transfected with 1 μ g of the DNA mixture by LipofectAMINE2000 (Invitrogen), according to the manufacturer's recommendations. Typically, we used 200 ng of nuclear receptor plasmid, 200 ng of coactivator plasmid, 400 ng of reporter construct, 200 ng of pEYFP-C1 as an internal control, and pcDNA3 as a normalization plasmid. After 6 h of transfection, the medium was changed to phenol red-free Dulbecco's modified Eagle's medium (Invitrogen) containing 10% charcoal/dextran-treated fetal calf serum (Sigma), and the ligands were added for 24 h when necessary.

Luciferase Assay—One day after transfection, the enhanced yellow fluorescent protein (EYFP) fluorescence was measured with a microplate fluorescence reader, model FL500 (Bio-Tek Instruments) to examine the transfection efficiency. Then the cells were washed once with PBS and lysed with 150 μ l of PicaGene lysis buffer (Toyo Ink Co., Ltd.). After the cell debris was removed by centrifugation, the luciferase activity was measured with PicaGene, using a luminometer, model TD-20/20 (Promega). Luciferase activities were normalized to the EYFP fluorescence value and are referred to as relative light units. All of the experiments were repeated at least three times and yielded similar results.

Pull-down Assay—GST fusion proteins were expressed in DH5 α cells and were purified using glutathione-Sepharose 4B (Amersham Biosciences). If necessary, the GST tag was removed by PreScission protease (Amersham Biosciences). His-tagged proteins were expressed in the BL21(DE3) strain (Novagen) and were purified using a Ni²⁺-bound metal chelating column (Amersham Biosciences). Ten μ g of GST-fused and S-tagged PGC-1 α proteins were each bound to glutathione-Sepharose 4B and S protein-agarose (Novagen), respectively. After the unbound proteins were removed by washing, 20 μ g of GST-free CAR proteins were added to the beads, which were incubated at 4 °C for 2 h. The beads were extensively washed with 20 mM Tris-Cl, pH 7.4, containing 150 mM NaCl, 0.1 mM EDTA, 0.1% Nonidet P-40, and 10%

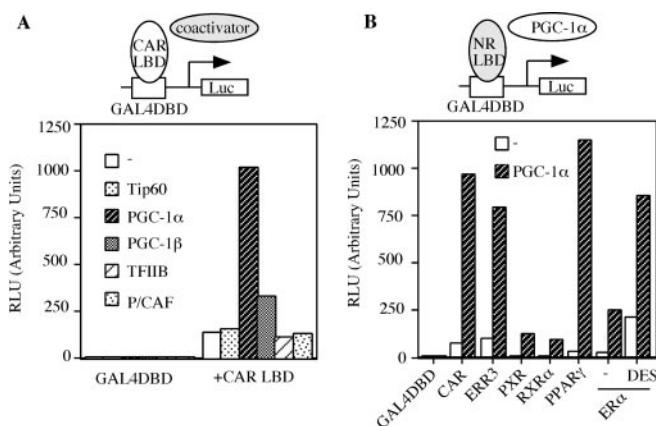


FIG. 1. Coactivation of CAR by PGC-1 α . *A*, selectivity of various coactivators in CAR activation. COS-7 cells were transiently transfected with vectors expressing the CAR LBD fused to the GAL4 DNA-binding domain (GAL4DBD) and the indicated coactivators together with a reporter construct. Transcriptional activation was analyzed by the luciferase activity, normalized to EYFP fluorescence as an internal control. The GAL4 DBD-fused CAR showed constitutive activity as compared with the GAL4 DBD alone. Cotransfection of PGC-1 α enhanced the CAR activity, although PGC-1 α alone did not activate transcription. PGC-1 β also showed slight enhancement of CAR activity. *B*, coactivation of nuclear receptors by PGC-1 α . The cells were transiently transfected with vectors expressing the LBDs of the indicated nuclear receptors fused to the GAL4 DBD, with or without PGC-1 α . In the absence of ligand, coexpression of PGC-1 α greatly enhanced the CAR, ERR3, and PPAR γ activities. PGC-1 α slightly increased the basal activities of PXR and RXR α . Activation of ER α by PGC-1 α was enhanced by the addition of the ER α agonist diethylstilbestrol (DES, 10 μ M).

glycerol. The bound CAR proteins were eluted with SDS-PAGE sample buffer and were detected by Western blotting using an anti-CAR polyclonal antibody (Santa Cruz) with the ECL Western blotting detection reagent (Amersham Biosciences).

Purification of Recombinant CAR-PGC-1 α Complex Coexpressed in Bacteria—The His-tagged NeoI-MfeI fragment of PGC-1 α and the GST-fused CAR LBD were coexpressed in BL21 (DE3) cells under ampicillin and kanamycin selection. The cells were grown in LB medium at 37 °C until an A_{600} of 0.8 and were induced for 18 h with 0.05 mM isopropyl-1-thio- β -D-galactopyranoside at 18 °C. The cells were harvested by centrifugation and frozen until use.

The cell pellets (1 liter of culture) were resuspended in PBS and sonicated on ice. After the debris was removed by centrifugation (9000 \times g at 4 °C for 20 min), the soluble extract was loaded onto glutathione-Sepharose 4B. The column was washed with PBS, and the bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0. The eluate was dialyzed against loading buffer (20 mM Hepes, pH 7.4, 250 mM NaCl, and 25 mM imidazole) and was loaded onto a Ni²⁺-bound metal chelating column. The column was washed with the loading buffer and a wash buffer (20 mM Hepes, pH 7.4, containing 150 mM NaCl). The proteins were eluted by the wash buffer containing 50 mM EDTA, pH 7.4, and were directly transferred to a GSTrap column (Amersham Biosciences). The column was further washed with 20 mM Tris-Cl, pH 7.4, containing 150 mM NaCl and was equilibrated with PreScission cleavage buffer (50 mM Tris-Cl, pH 7.4, containing 150 mM NaCl, 10 mM EDTA, and 1 mM dithiothreitol). Ten units of PreScission protease (Amersham Biosciences) were then injected into the column by a syringe. The cleavage was carried out for 2 days at 4 °C, and the purified recombinant CAR-PGC1 complex was eluted with 20 mM Tris-Cl, pH 7.4, containing 150 mM NaCl.

Histochemistry—COS-7 cells were seeded onto 35-mm dishes and were transfected with vectors expressing EGFP-fused CAR, FLAG-tagged RXR α , or FLAG-tagged PGC-1 α . After 24 h, the cells were fixed with 3% formaldehyde for 15 min at room temperature. The cells were washed twice with PBS, permeabilized with 0.2% Nonidet P-40, and then incubated for 1 h in 3% bovine serum albumin in PBS to block nonspecific binding. FLAG-tagged proteins were visualized with anti-FLAG M2 antibody (Cosmo Bio Co. Ltd.) and rhodamine-conjugated anti-mouse IgG (Cosmo Bio Co. Ltd.). EGFP and rhodamine fluorescence were observed with a laser scanning microscope, model LSM510 (Zeiss).

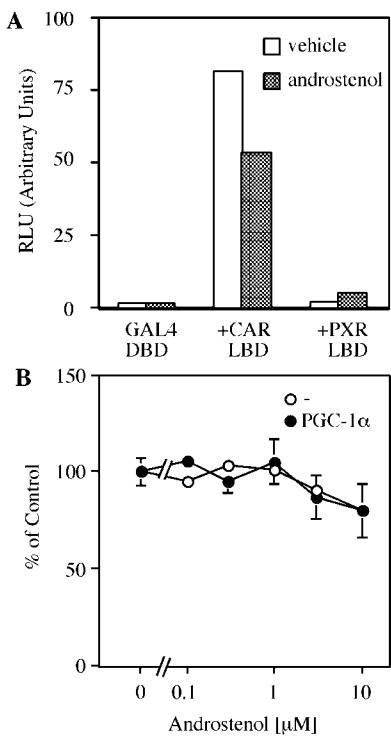


FIG. 2. Effects of putative CAR ligands on the activity. *A*, effects of putative ligands on CAR or PXR activity. COS-7 cells were transiently transfected with the expression plasmids for the GAL4 DBD, the GAL4 DBD-fused CAR, or the GAL4 DBD-fused PXR and the reporter construct. The cells were treated with 10 μ M of androstenol for 24 h. Androstenol slightly inhibited the basal CAR activity. In contrast, androstenol slightly stimulated PXR activity. *B*, effects of androstenol on the PGC-1 α -dependent CAR activity. The cells were transfected with the GAL4 DBD-fused CAR with (closed circles) or without PGC-1 α (open circles). The cells were treated with androstenol at the indicated concentrations for 24 h. The vertical axis indicates the activity relative to that in the absence of androstenol (100%) in each case.

RESULTS

Functional Coupling of PGC-1 α with CAR—To find the mediator of CAR activation, we examined the coupling of various nuclear receptor coactivators with CAR (Fig. 1A). The CAR LBD was fused with the GAL4 DBD and was recruited to the promoter region with the GAL4 binding element, to drive the expression of the luciferase reporter gene. After transfection of the respective plasmids into COS-7 cells, transcriptional activation was measured by the luciferase activity. Without any exogenous coactivator, CAR showed the constitutive activity (white bar). Cotransfection of various coactivators with CAR revealed that PGC-1 α strongly enhanced the CAR activity. PGC-1 β also stimulated CAR activity to a lower extent than PGC-1 α . The other coactivators, such as Tip60, TFIIB, and P/CAF, did not stimulate the CAR activity. Next, we compared the magnitude of CAR activation by PGC-1 α with that of other nuclear receptors (Fig. 1B). PGC-1 α also enhanced the ERR3 and PPAR γ activities in the absence of their ligands, as well as the CAR activity. A similar level of transcriptional enhancement by PGC-1 α was observed with agonist-stimulated ER α . On the other hand, PGC-1 α showed weak activation of PXR, although PXR has a sequence similar to that of CAR, and both receptors function in xenobiotic responses (4, 17). PGC-1 α evoked weak activation of the retinoid X receptor α (RXR α) in the absence of its ligand, 9-cis-retinoic acid.

Effects of Putative Ligands on PGC-1 α -mediated CAR Activation—The sex steroids androstanol and androstenol reportedly inhibit CAR activity (7, 17). In fact, we observed that androstenol (10 μ M) partially inhibited the basal activity of

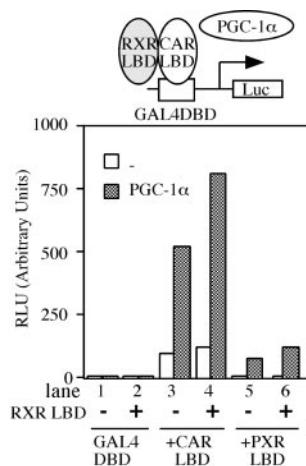


FIG. 3. Enhancement of PGC-1 α -stimulated CAR activity by heterodimerization with RXR α . Although RXR α fused to the GAL4 DBD was slightly activated by PGC-1 α (Fig. 1B), the RXR α LBD in the absence of the GAL4 DBD did not couple with PGC-1 α (*lanes 1* and *2*). When the GAL4 DBD-fused CAR was expressed with or without the RXR α LBD in the absence of PGC-1 α , a slight enhancement of the basal activity of CAR was observed (*lanes 3* and *4*, white bars). In the presence of PGC-1 α , the RXR α LBD enhanced the CAR activity (*lanes 3* and *4*, shaded bars). The RXR α LBD had no effect on the basal activity of PXR (*lanes 5* and *6*, white bars) and weakly activated the PGC-1 α -mediated activation of PXR (*lanes 5* and *6*, shaded bars).

CAR (Fig. 2A). In contrast, androstenol (10 μ M) weakly activated PXR, which is consistent with the previous report (17). Next, we examined the effect of this putative ligand on the PGC-1 α -mediated CAR activation and observed that androstenol moderately inhibited but did not abolish the CAR activation by the coexpression of PGC-1 α (Fig. 2B). We also investigated the effect of a synthetic ligand, the antimycotic clotrimazole and observed that clotrimazole slightly inhibited but did not abolish the PGC-1 α -mediated activation of CAR as well (data not shown).

Effects of Heterodimerization with RXR α on PGC-1 α -mediated CAR Activation—RXR α is known to heterodimerize with CAR to enhance the constitutive activity of CAR (7, 18) and its androstanol sensitivity (7, 19). We therefore investigated the effect of heterodimerization with RXR α on the PGC-1 α -mediated activation of CAR (Fig. 3). Because RXR α itself was slightly activated by PGC-1 α , as shown in Fig. 1, we used the construct of RXR α LBD that was not fused with GAL4 DBD, to avoid the direct recruitment of PGC-1 α by RXR α to the promoter. In fact, PGC-1 α did not couple with the RXR α LBD without the GAL4 DBD (*lane 2*). Coexpression of the RXR α LBD had a weak effect on the basal activity of CAR but enhanced the PGC-1 α -mediated CAR activation (*lanes 3* and *4*). The RXR α LBD had no effect on the PXR basal activity and a very weak effect on the PXR activity when coexpressed with PGC-1 α (*lanes 5* and *6*). Although RXR α enhanced the PGC-1 α -mediated activation of CAR, the coexpression of the RXR α LBD did not alter the required domains of PGC-1 α for CAR activation (Fig. 4 and data not shown). Then we further characterized the PGC-1 α -mediated CAR activation without RXR α .

Functional Domains within PGC-1 α Required for CAR Activation—PGC-1 α has a putative nuclear receptor interaction region containing the LXXLL motif, which is conserved in several nuclear receptor coactivators, the RS domain, and the RRM, the latter two of which are observed in many non-snRNP splicing factors (16). To define the functional domains of PGC-1 α required for CAR activation, we made a series of deletion mutants of PGC-1 α (Fig. 4A) and investigated their ability to activate CAR using the luciferase assay (Fig. 4B, panel *a*). Removal of the LXXLL motif (PGC-1-Nhe/Xba) de-

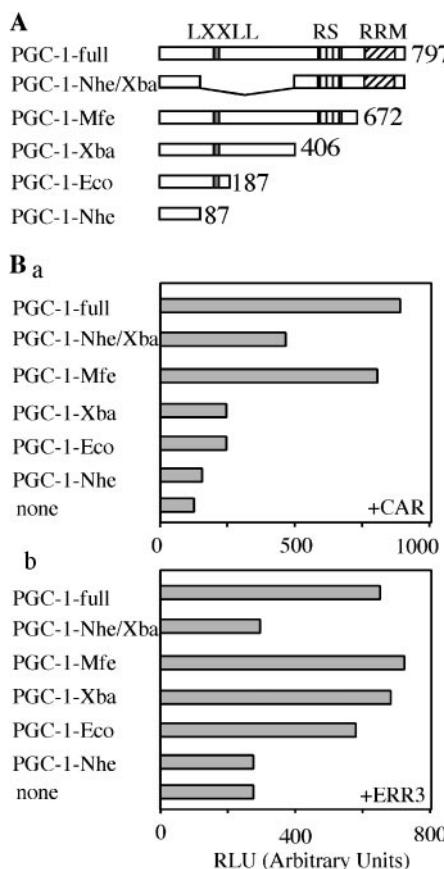


FIG. 4. PGC-1 α domains required for CAR activation. *A*, schematic representation of PGC-1 α domain structures and deletion mutants used for cotransfection experiments. The putative nuclear receptor interaction domain containing the LXXLL motif is indicated by a gray box, the RS domain is a striped box, and the RRM is a hatched box. The numbers at the ends of the constructs show the amino acid positions where the stop codon was introduced. *B*, PGC-1 α deletion mutants were cotransfected with the GAL4 DBD-fused CAR (*panel a*) and ERR3 (*panel b*). The PGC-1-Nhe/Xba mutant lacking the LXXLL motif activated CAR but not ERR3. The PGC-1-Mfe mutant lacking RRM activated both CAR and ERR3. The CAR activation was dramatically reduced in the PGC-1-Xba and PGC-1-Eco mutants lacking the RS domain and RRM, although these mutants activated ERR3 to the same extent as the full length. The PGC-1-Nhe mutant activated neither CAR nor ERR3.

creased but did not abolish the ability of PGC-1 α to activate CAR. The deletion of RRM (PGC-1-Mfe) did not affect the CAR activation, whereas the deletion of both the RRM and RS domains (PGC-1-Xba and PGC-1-Eco) further reduced the ability. A deletion mutant lacking all three regions (PGC-1-Nhe) completely lost the ability to activate CAR. These results indicated that both the LXXLL motif and the RS domain in PGC-1 α are required for the full activation of CAR.

Next we investigated whether the requirement of these domains for the enhancement was specific to CAR. We took advantage of ERR3, which was activated by cotransfection of PGC-1 α (Fig. 1*B*) and investigated the required domains of PGC-1 α for the activation of ERR3 (Fig. 4*B*, *panel b*). Removal of the LXXLL motif (PGC-1-Nhe/Xba) completely abolished the ability of PGC-1 to activate ERR3, but the deletion of the RRM and RS domains (PGC-1-Xba and PGC-1-Eco) did not alter the ERR3 activation. A deletion mutant lacking all three regions (PGC-1-Nhe) completely lost the ability to activate ERR3. These results indicated that the LXXLL motif, but not the RS domain, is sufficient for coupling with ERR3. The difference in the regions of PGC-1 α necessary for CAR and ERR3 activation suggested that the requirement of the RS domain for the acti-

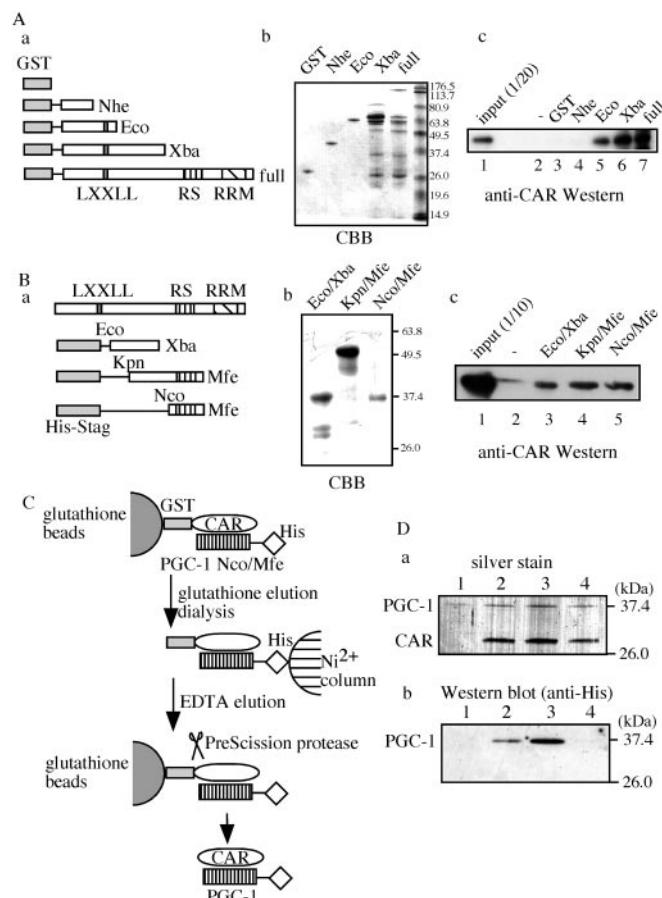


FIG. 5. Multiple PGC-1 α domains interact with the CAR LBD. *A*, Pull-down experiments using a series of PGC-1 α mutants with C-terminal deletions. *Panel a*, the GST-fused PGC-1 α deletion mutants are schematically shown. The restriction enzyme shown at the right indicates the position where the stop codon was introduced. The domains and the motifs indicated below the full-length PGC-1 α were described in the legend to Fig. 4*A*. *Panel b*, PGC-1 α deletion mutants, immobilized on glutathione-Sepharose beads, were resolved by SDS-PAGE and stained by CBB. *Panel c*, the CAR LBD was incubated with GST or GST-fused PGC-1 α deletion mutants. After the beads were washed, the bound proteins were separated by SDS-PAGE. The CAR LBD interaction with PGC-1 α deletion mutants was detected by Western blotting using an anti-CAR antibody. *B*, pull-down experiments using PGC-1 α fragments lacking the LXXLL motif. *Panel a*, S-tagged PGC-1 α fragments are shown. *Panel b*, proteins immobilized on S-protein beads were separated and stained by CBB. *Panel c*, the CAR LBD interaction with the PGC-1 α fragments was detected by Western blotting. *C*, schematic representation of the procedure to obtain the CAR-PGC-1 α complex by serial affinity purification. The His-tagged NcoI-MfeI fragment of PGC-1 α containing the RS domain was coexpressed and copurified with the GST-fused CAR LBD. *D*, recombinant CAR LBD-PGC-1 α RS domain complex. The number above each lane indicates the fraction eluted by PreScission cleavage. *Panel a*, the complex was separated by SDS-PAGE and was visualized by silver staining. *Panel b*, the PGC-1 α fragment was detected by Western blotting using an anti-His antibody.

vation is dependent on the nuclear receptor with which it is coupled.

The Regions within PGC-1 α That Interact with CAR LBD—We asked whether PGC-1 α would physically interact with the CAR LBD and which domains were required for the interaction. Deletion mutants of GST-fused PGC-1 α were expressed and purified from *Escherichia coli* (Fig. 5*A*, *panels a* and *b*). The CAR LBD was purified as a GST-fused protein, and then the GST portion was removed by PreScission protease digestion. The CAR LBD was mixed with GST-PGC-1 α or its mutant proteins immobilized onto glutathione-Sepharose beads, and the bound CAR LBD was dissolved in sample buffer

and visualized by Western blotting using an anti-CAR antibody (Fig. 5A, *panel c*). The CAR LBD was able to bind to PGC-1-Eco (*lane 5*), PGC-1-Xba (*lane 6*), and PGC-1-full (*lane 7*) but not to PGC-1-Nhe (*lane 4*). The difference in the CAR binding ability between PGC-1-Eco and PGC-1-Nhe indicated that the region containing the LXXLL motif was required for the association with the CAR LBD. A tighter interaction was observed in PGC-1-Xba (*lane 6*) and PGC-1-full (*lane 7*) than in PGC-1-Eco (*lane 5*). Although PGC-1-Xba and PGC-1-full were contaminated with many lower bands (Fig. 5A, *panel b*), the CAR interaction was not correlated with those lower bands. These results suggest that PGC-1 α interacts with the CAR LBD through the C-terminal region in addition to the LXXLL motif.

We produced additional deletion mutants lacking the LXXLL motif, as His- and S-tagged (His-Stag) proteins, to locate other regions of PGC-1 α interacting with the CAR LBD, besides the LXXLL motif region (Fig. 5B, *panels a* and *b*). The CAR LBD was mixed with the Stag-PGC-1 α proteins immobilized onto S-protein beads, and the bound CAR LBD was pulled down and visualized by Western blotting using an anti-CAR antibody (Fig. 5B, *panel c*). The CAR LBD was able to bind to these His-Stag PGC-1 α proteins (Fig. 5B, *panel c*), indicating that multiple regions of PGC-1 α directly interact with CAR. It was notable that the NcoI-MfeI fragment, containing the RS domain, directly bound to CAR (*lane 5*), which possibly reflects the fact that the RS domain had a crucial effect on CAR activation (Fig. 4B, *panel a*).

Purification of the Recombinant CAR LBD-PGC-1 α RS Domain Complex—To prove that the CAR LBD directly interacts with the RS domain of PGC-1 α , we coexpressed the GST-fused CAR LBD and the His-tagged RS domain in *E. coli* and purified the complex of these two proteins. Serial affinity purification allowed us to isolate the CAR LBD-PGC-1 α RS domain complex (Fig. 5C). The complex was eluted from glutathione-Sepharose beads by cleaving off the GST tag by PreScission protease, which ensured the direct binding of PGC-1 α to the CAR LBD rather than nonspecific binding to the column (Fig. 5C). Aliquots of the eluate were subjected to SDS-PAGE and were analyzed by silver staining (Fig. 5D, *panel a*). The protein appeared as a 29-kDa band, corresponding to the CAR LBD. The coeluted 37-kDa protein was identified as a PGC-1 α fragment by Western blotting using an anti-His antibody (Fig. 5D, *panel b*).

PGC-1 α -dependent Arrangement of CAR Subnuclear Localization—In the course of the experiment investigating the colocalization of CAR with PGC-1 α in cells, we found that CAR moved to nuclear speckles when PGC-1 α was coexpressed. We used the EGFP-fused CAR LBD to determine the localization of CAR (Fig. 6A, *center panel*), which showed a distribution similar to that of the GAL4 DBD-fused CAR, as determined by immunofluorescence (Fig. 6A, *left panel*). Coexpression of RXR α did not affect the distribution of CAR (Fig. 6B). When PGC-1 α was coexpressed, CAR colocalized at nuclear speckles with PGC-1 α (Fig. 6C, *panel a*). In all of the 50 total cells counted, colocalization in nuclear speckles was observed (100%). A significant amount of CAR was still present in the cytoplasm even when PGC-1 α was coexpressed, suggesting that the subnuclear distribution, but not the cytoplasm-nuclear shuttling of CAR, is important for the activity. PGC-1 α expressed without CAR localized at nuclear speckles; however, a diffusely distributed signal was observed in the entire nucleus (Fig. 6C, *panel a*, *PGC-1 alone*). Coexpression of CAR with PGC-1 α seemed to weaken the diffuse distribution of PGC-1 α in the nucleus and to intensify the signal in nuclear speckles, suggesting that the binding of CAR and PGC-1 α might cause a mutual effect on each other.

We used the deletion mutants of PGC-1 α to investigate which domain of PGC-1 α was responsible for the subnuclear targeting of CAR. The PGC-1-Nhe/Xba mutant lacking the LXXLL motif was able to shift the localization of CAR to the nuclear speckles (Fig. 6C, *panel b*). Of 44 total cells counted, 30 cells showed colocalization of CAR and PGC-1 α in nuclear speckles (68%). The PGC-1-Mfe mutant lacking RRM was not able to localize in nuclear speckles by itself (Fig. 6C, *panel c*, *PGC-1 alone*). This observation is consistent with the previous report (20). Interestingly, the localization of PGC-1 α in nuclear speckles was observed even in the absence of RRM but only when CAR was coexpressed (Fig. 6C, *panel c*). Colocalization of CAR and PGC-1 α was observed in 32 cells of the 38 total cells counted (84%). Using the PGC-1-Xba mutant lacking both the RS domain and RRM, neither CAR nor PGC-1 localized in the nuclear speckles (Fig. 6C, *panel d*). The PGC-1-Eco mutant no longer showed exclusive nuclear localization, because of the absence of a nuclear localization signal (Fig. 6C, *panel e*, and *D*). A comparison between the activity and the localization suggested that the colocalization in nuclear speckles may be a limited process for PGC-1 α -mediated CAR activation.

DISCUSSION

In this study, we report that the transcriptional coactivator PGC-1 α mediates the ligand-independent activation of the nuclear receptor CAR. Our results showed that the N-terminal region containing the LXXLL motif and the C-terminal region containing the RS domain participate in the interaction and the activation with CAR. Both regions were necessary for full activation. In addition, the RS domain was required for colocalization of PGC-1 α in nuclear speckles with CAR. These data indicated that the ligand-independent activation of CAR by PGC-1 α is achieved by subnuclear targeting through the RS domain of PGC-1 α .

CAR shows constitutive activity in the absence of exogenous ligand when transfected into cell lines, such as HepG2 and CV-1 cells; however, it is reportedly inactive in primary cultured hepatocytes because of its cytoplasmic localization (21). This study gave us the idea that an additional factor may be required for the constitutive activity of CAR. In the course of searching for a coactivator coupling with CAR, we found that PGC-1 α greatly enhanced the CAR activity in the absence of ligand. This activation was specific to PGC-1s, because several other coactivators we examined did not show the enhancement. This link of CAR with PGC-1 α surprised us, because the liver is not the major organ of PGC-1 α expression (8). However, important experimental data have recently described how PGC-1 α expression in the liver is dynamically regulated by the nutritional state of the body; PGC-1 α is induced by starvation or fasting and plays a critical role in gluconeogenesis (12, 22).

Recently, the crystal structures of several orphan nuclear receptors, including ultraspiracle, hepatocyte nuclear factors (HNF4 α and HNF4 γ), retinoic acid-related orphan receptor β , ERR3, and PXR, have been solved (23–28). Notably, the crystals of the ultraspiracle, HNF4 α , HNF4 γ , and retinoic acid-related orphan receptor β LBDs prepared from *E. coli* contained lipid ligands, presumably derived from cell lipid constituents, in the hydrophobic pocket. According to these studies, one possible difference between the cell lines and the primary hepatocytes was that CAR might be activated by an unknown endogenous ligand produced by the cell lines. Alternatively, a natural ligand, which exists in the primary hepatocytes but not in the established cell lines, may repress the CAR activity, and its repression might be modulated by the interaction with PGC-1 α . The CAR reverse agonist, androstenol, did not completely inhibit the PGC-1 α -mediated CAR activation, although the human CAR, which we used, is less sensitive to

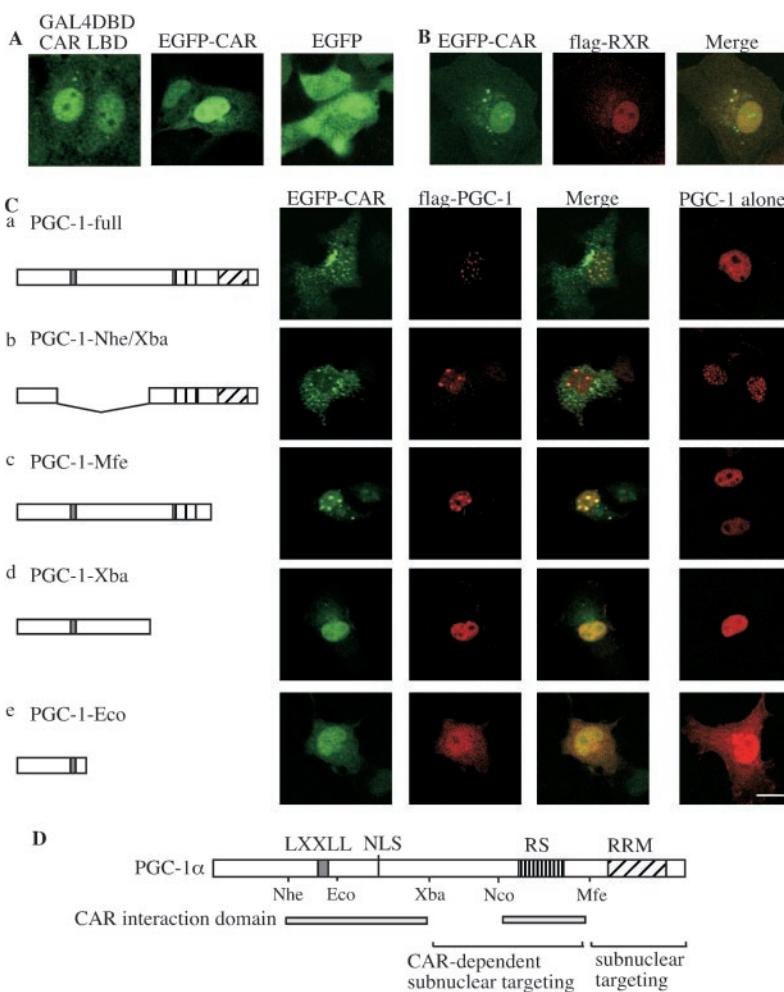


FIG. 6. PGC-1 domains required for subnuclear targeting of CAR. *A*, subcellular localization of CAR, detected by either immunofluorescence or EGFP fusion. COS-7 cells were transfected with the GAL4 DBD-fused CAR LBD or the EGFP-fused CAR LBD and were fixed after 24 h. The GAL4 DBD-fused CAR was detected by indirect immunofluorescence using an anti-CAR antibody and an fluorescein isothiocyanate-conjugated secondary antibody (*GAL4DBD-CAR LBD*). CAR was distributed throughout the cells with a slight accumulation in nucleus. The EGFP-fused CAR (*EGFP-CAR*) showed a pattern of distribution similar to that detected by immunofluorescence. EGFP alone did not show any nuclear accumulation but was distributed diffusely (*EGFP*). *B*, the effect of coexpression of the RXR α LBD on the subcellular localization of CAR. RXR α was detected by indirect immunofluorescence, using an anti-FLAG antibody and a rhodamine-conjugated secondary antibody (*FLAG-RXR*). The pattern of CAR localization was not changed by RXR α (*EGFP-CAR*). *C*, the effect of coexpression of PGC-1 α on the subcellular localization of CAR. CAR was visualized with an EGFP fusion (*EGFP-CAR*). PGC-1 α was visualized with indirect immunofluorescence using an anti-FLAG antibody and a rhodamine-conjugated secondary antibody (*FLAG-PGC-1*). The colocalization of CAR and PGC-1 α was investigated by confocal microscopy (*Merge*). The localization of PGC-1 α without CAR is shown on the *left* (*PGC-1 alone*). *Panel a*, nuclear CAR moved to the nuclear speckles when PGC-1 α was coexpressed. PGC-1 α by itself was located at the nuclear speckles. *Panel b*, the PGC-1-Nhe/Xba mutant lacking the LXXLL motif was located at the nuclear speckle and shifted the location of CAR to the nuclear speckles when coexpressed. *Panel c*, the PGC-1-Mfe mutant lacking RRM did not reside at the nuclear speckle but was distributed throughout the nucleus. However, the coexpression of CAR with the PGC-1-Mfe mutant induced the colocalization of CAR and the PGC-1 α mutant at the nuclear speckles. *Panel d*, the PGC-1-Xba mutant lacking the RS domain and RRM resides at the nucleus without any nuclear speckles, and coexpression of CAR and the PGC-1-Xba mutant did not change their respective locations. *Panel e*, the PGC-1-Eco mutant was distributed throughout the cells, and the coexpression of CAR and the PGC-1-Eco mutant did not change their respective locations. *Scale bar*, 20 μ m. *D*, schematic representation of the functional PGC-1 α domains. CAR interacts with PGC-1 α through multiple domains, including the LXXLL motif in the N terminus and the RS domain in the C terminus. PGC-1 α by itself localizes at the nucleus by its nuclear localization signal (NLS) and at the nuclear speckles by RRM in the C terminus. The interaction with CAR induces the CAR-dependent targeting of PGC-1 α to the nuclear speckles with CAR.

androstanol than the mouse CAR (17). In addition, the effect of a synthetic antagonist of ERR3, diethylstilbestrol, on PGC-1 α -mediated ERR3 activation was small, and furthermore, the recombinant ERR3-PGC-1 α complex was so stable that it could not be dissociated by diethylstilbestrol stimulation (data not shown). Thus, the binding of CAR to PGC-1 α is rather stable, and the complex might also be resistant to androstanol. The answer to this issue awaits the development of strong and specific agonists and antagonists, which are expected for both the clinical and experimental fields.

Because CAR forms a heterodimer with RXR α (7, 18, 19), we assessed the effect of the heterodimerization between CAR and RXR α on the PGC-1 α coactivation. RXR α itself slightly coupled

with PGC-1 α in the absence of ligand, but the enhancement of PGC-1 α -mediated CAR activation by the RXR α LBD was not additive but synergistic, suggesting that the CAR-RXR α heterodimer may stabilize PGC-1 α binding to CAR rather than CAR and RXR α binding to PGC-1 α independently. Using deletion mutants of PGC-1 α , we observed that the coexpression of the RXR α LBD did not change the domains of PGC-1 α required for CAR activation (data not shown), supporting the idea that RXR α stabilizes the binding. These results suggest that the heterodimerization with RXR α does not explain the constitutive activity of CAR. This idea does not contradict the *in vivo* observation that RXR α remains at the nucleus, even when CAR is inactive in the cytosol in hepatocytes (21).

PGC-1 α reportedly couples with several other nuclear receptors in ligand-dependent and ligand-independent manners (8–15). PGC-1 α interacts with these nuclear receptors through the LXXLL motif in its N-terminal region. In this study, we observed that CAR also directly interacted with the region containing the LXXLL motif. In addition, we found that another region containing RS domain in PGC-1 α also interacted with CAR by pull-down experiments and probed the complex formation using purified proteins. The C-terminal fragment containing the RS domain and RRM reportedly interacts with an ER α hinge region in a ligand-independent manner (10). Taken together, the RS domain in PGC-1 α may provide a novel interface to nuclear receptors. The interaction between PGC-1 α and nuclear receptors through multiple domains may confer the specificity of the coactivation.

The RS domain as well as RRM in PGC-1 α is shared with SR proteins, which are essential splicing factors that participate in mRNA processing through their RS domains and RRMs (20, 29). PGC-1 α diffusely localized at the nucleus and concentrated in the nuclear speckles, where splicing factors are colocalized (20). Nuclear speckles of splicing factors are considered to be a site for storage and assembly of splicing factors (30). Nuclear speckles are highly dynamic structures. Upon gene activation, the splicing factors, visualized by GFP fusions, are released from the nuclear speckles to transcriptionally active sites (31). In the absence of RRM, PGC-1 α did not reside in the nuclear speckles by itself; however, when CAR was coexpressed, the PGC-1 α moved to nuclear speckles together with CAR, suggesting that the coexpression of CAR and PGC-1 α has a mutual effect on their localization (Fig. 6D). This targeting activity was totally dependent on the RS domain and was observed even in the absence of the LXXLL motif in PGC-1 α . The RS domains in the splicing factors are reportedly required for both targeting to and dissociation from nuclear speckles (32–34). These results strongly suggest that CAR binds to PGC-1 α through the RS domain and rearranges the domain to acquire the ability to move to the nuclear speckles for transcriptional activation or for altering mRNA processing. It is interesting that the CAR-mediated transcriptional activation of cytochrome P450 by xenobiotics in hepatocytes is abolished by the serine/threonine-specific phosphatase inhibitor, okadaic acid (21), which is also known to prevent the movement of splicing factors into nuclear speckles (35). The involvement of the serine/threonine-specific phosphatases in regulating PGC-1 α -mediated CAR activation will be investigated in the future.

Although the physiological relevance of PGC-1 α -mediated CAR activation will be needed for further experiments, our data strongly suggest that PGC-1 α regulates the activity of CAR in hepatocytes. Microarray data have suggested that CAR regulates several enzymes catalyzing fatty acid or carbohydrate metabolism (36). Thus, it would be intriguing to investigate the CAR activity in the liver under different nutritional conditions and the PGC-1 α levels after exposure to xenobiotics, which would provide clues to the relationships between these important pathways, drug catalysis and metabolic conditions.

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Coactivator-1 α : A POSSIBLE LINK BETWEEN XENOBIOTIC RESPONSE AND
NUTRITIONAL STATE**

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