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Diverse peroxisome proliferator-activated receptors bind to the peroxisome proliferator-responsive elements of the rat hydratase/dehydrogenase and fatty acyl-CoA oxidase genes but differentially induce expression

(transcriptional induction/cooperative protein/DNA interactions/9-*cis*-retinoic acid receptor/hypolipidemic drugs)

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ABSTRACT **The ability of peroxisome proliferator-activated receptors (PPARs) to induce expression of a reporter gene linked to a peroxisome proliferator-responsive element (PPRE) from either the rat enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene or acyl-CoA oxidase [acyl-CoA:oxygen 2-oxidoreductase, EC 1.3.3.6] gene was examined by transient transfection assays in COS cells. Mouse and rat PPARs, as well as *Xenopus* PPARα (xPPARα) could induce expression of a reporter gene linked to the hydratase/dehydrogenase PPRE in the presence of the peroxisome proliferators ciprofibrate or Wy-14,643, whereas xPPARβ and xPPARγ were ineffective. A similar induction of expression of a reporter gene linked to the acyl-CoA oxidase PPRE was observed with all PPARs except xPPARβ. Extracts from cells transfected with PPAR-encoding genes contained factors that bound to both PPREs. *In vitro* synthesized PPARs could interact weakly with both PPREs; however, binding of each PPAR to both PPREs was significantly increased by the addition of COS cell nuclear extracts, demonstrating that efficient PPAR/DNA binding requires auxiliary cofactors. One cofactor was identified as the 9-*cis*-retinoic acid receptor, RXRα (retinoid X receptor α). Cooperative DNA binding and heteromerization between RXRα and each of the PPARs could be seen with both PPREs. Our results demonstrate that PPAR/PPRE binding and cooperativity with RXRα (and other cofactors) are obligatory but not necessarily sufficient for peroxisome proliferator-dependent transcription induction and that distinct PPREs can selectively mediate induction by particular PPARs.**

Peroxisomes are essential for lipid metabolism (1). Many xenobiotics, including amphipathic carboxylates used as hypolipidemic agents, induce peroxisome proliferation and ultimately hepatocarcinogenesis in rodents (2). These peroxisome proliferators are nongenotoxic carcinogens that apparently act as tumor promoters by modulating the expression of cellular genes involved in growth and differentiation (3, 4).

Administration of peroxisome proliferators leads to the rapid and coordinated transcriptional induction of the nuclear genes encoding the enzymes of the peroxisomal β-oxidation pathway: fatty acyl-CoA oxidase (AOx; acyl-CoA:oxygen 2-oxidoreductase, EC 1.3.3.6]), enoyl-CoA hydratase (EC 4.2.1.17)/3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) (HD), and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) (5, 6). Cis-acting peroxisome proliferator-responsive elements (PPREs) have been identified in the 5′ flanking regions of both the AOx (7, 8) and HD (9) genes. Both PPREs contain direct repeats of the sequence TGACCT, the consensus binding site for several members of the nuclear hormone receptor superfamily. Signal transduction by peroxisome proliferators is apparently mediated through distinct ligand-activated receptors, collectively known as peroxisome proliferator-activated receptors (PPARs), that belong to this family of transcription factors (10–13). Recently, the mouse PPAR (mPPAR) has been shown to bind cooperatively to the AOx PPRE through heteromerization with the 9-*cis*-retinoic acid receptor, RXRα (14).

Here we demonstrate that homologous and heterologous PPARs mediate peroxisome proliferator-dependent transcriptional induction of reporter genes linked to either the AOx or HD PPRE through cooperative protein–DNA interactions between the different PPARs and other cellular factors, including RXRα. However, PPAR–cofactor–DNA interaction is not necessarily sufficient to confer this induction, since we have found that with at least one type of PPAR, induction is differentially accorded by the nature of the PPRE.

MATERIALS AND METHODS

Cells. Rat hepatoma H4IIEC3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) horse serum and 5% (vol/vol) fetal bovine serum. COS-1 cells were maintained in DMEM plus 10% calf serum.

Plasmids and Antibody. pCPSluc contains the minimal promoter from the rat liver carbamoyl-phosphate synthetase (CPS) gene (9). pHD(×3)luc contains three tandem copies of the HD PPRE cloned into pCPSluc. It was constructed by inserting the oligonucleotide 5′-gatCCTCTCCTTTGACCTATTGAACTATTACCTACATTTGA and its complement, 5′-gatcTCAAATGTAGGTAATAGTTCAATAGGTCAAAGGAGAG (nucleotides −2956 to −2919 of the rat HD promoter), into the *Bam*HI site of pCPSluc. pAOx(×2)luc contains two tandem copies of the rat AOx PPRE generated by inserting the oligonucleotide 5′-gatCCTTTCCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCTa and its complement, 5′-gatctAGCAAAAGGGGACCAGGACAAAGGTCACGTTCGGGAAAG (nucleotides −583 to −544 of the rat AOx promoter), into the *Bam*HI site of pCPSluc. Nucleotides

Abbreviations: AOx, fatty acyl-CoA oxidase; CPS, carbamoyl-phosphate synthetase; HD, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; RXR, retinoid X receptor; RXRα, 9-*cis*-retinoic acid receptor; mPPAR, rPPAR, and xPPAR, mouse, rat, and *Xenopus* PPARs.

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designated in lowercase letters were added to provide *Bam*HI–*Bgl* II ends. Plasmids expressing Xenopus PPARs, xPPARα, xPPARβ, and xPPARγ, were kindly provided by W. Wahli (Lausanne, Switzerland). A plasmid expressing mPPAR was kindly provided by S. Green (Macclesfield, U.K.). cDNAs encoding these PPARs were all originally cloned in the expression vector pSG5 (15). Rat PPAR (rPPAR) cloned into pBluescript II SK(+) (Stratagene) was provided by D. Noonan (Ligand Pharmaceuticals, San Diego). The rPPAR cDNA was excised from this plasmid with *Spe* I/*Eco*RV, and the 2.6-kilobase pair fragment was cloned into appropriately modified sites of the expression vector pRc/CMV (Invitrogen, San Diego).

pSKXR3-1 containing a cDNA for human RXRα and a polyclonal antibody to RXRα were provided by R. Evans (Salk Institute, San Diego). The RXRα cDNA was excised from pSKXR3-1 as a 1.8- kilobase pair *Eco*RI fragment and cloned directly into the *Eco*RI site of pSG5 for use in transfections.

Transfections. Transfections of H4IIEC3 cells (10-cm dishes at 50% confluence) were done by the calcium phosphate method followed by a dimethyl sulfoxide shock (9). COS-1 cells were transfected similarly except that cells were incubated for 24 hr before and during transfection in medium without phenol red and containing 5% charcoal-stripped fetal bovine serum. Transfections typically contained 5 μg of a reporter gene construct [pHD(×3)luc or pAOx(×2)luc] and 2 μg of a PPAR expression plasmid. Promoter dosage was normalized for each transfection with pSG5 or pRc/CMV, as appropriate, and the total amount of DNA was maintained at 15 μg with sonicated salmon sperm DNA. Ciprofibrate or Wy-14,643 (each from a ×100 stock in dimethyl sulfoxide) was added to fresh medium to final concentrations of 0.5 mM and 0.1 mM, respectively, at 4 hr and again at 24 hr after transfection (control cells received an equal amount of dimethyl sulfoxide). Cell extracts were prepared 48 hr after transfection. Luciferase activity from equivalent numbers of cells was measured with a luminometer.

***In Vitro* Transcription/Translation.** Transcription of cDNAs for different PPARs and RXRα and subsequent translation in rabbit reticulocyte lysate were performed by using a commercially available kit (Promega). Translations of proteins for use in gel retardation assays were done with unlabeled methionine.

Gel Retardation Analysis. Nuclear extracts were prepared from monolayer cultures of H4IIEC3, COS-1, and COS-1 cells transfected with various PPAR expression plasmids (16, 17). Gel retardation analysis was performed as described (9). All reactions were normalized for protein content. HD PPRE and AOx PPRE double-stranded probes consisting of the oligonucleotides described above were end-labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. Binding reactions were analyzed by electrophoresis at 4°C on prerun 3.5% polyacrylamide gels (30:1 acrylamide/*N,N'*-methylenebisacrylamide weight ratio) with 22 mM Tris base/22 mM boric acid/1 mM EDTA as running buffer. For binding reactions done with *in vitro* synthesized protein, 2 to 4 μl of translation mixture was incubated with labeled probe. Protein concentrations were normalized with unprogrammed rabbit reticulocyte lysate as necessary.

RESULTS

Diverse PPARs Differentially Mediate Peroxisome Proliferator-Dependent Transcriptional Activation via PPREs. Cotransfections of reporter plasmids containing either the HD PPRE or AOx PPRE and expression plasmids encoding various PPARs were carried out with COS cells, which are unresponsive to peroxisome proliferators. Expression of pHD(×3)luc was induced 3- to 5-fold after cotransfection with either xPPARα or mPPAR in the presence of ciprofibrate or Wy-14,643 (Fig. 1*B*). Induction was dependent upon both receptor plasmid and drug, as shown by control transfections done with pSG5. Drug-dependent induction was not observed with genes encoding either xPPARβ or xPPARγ.

mPPAR accorded a 2- to 3-fold drug-independent induction, which was further increased by either drug. This sug-

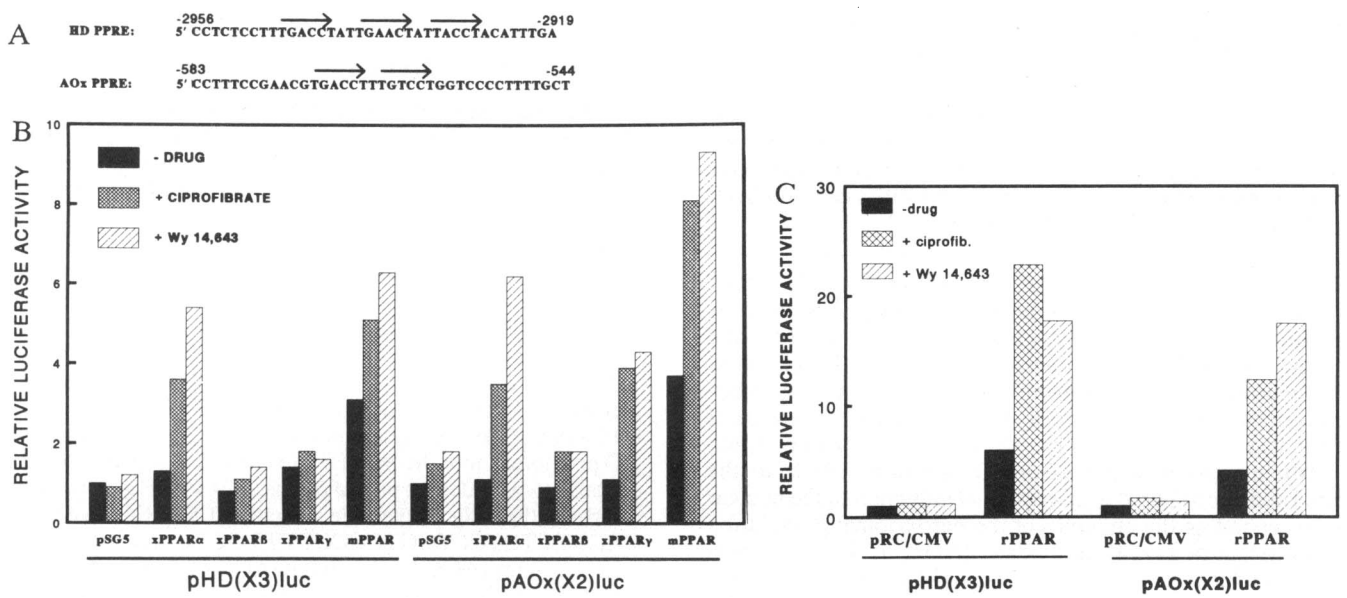

FIG. 1. Activation of a luciferase reporter gene linked to the HD or AOx PPRE by PPARs. (*A*) Sequences of the HD and AOx PPREs. Promoter coordinates are numbered with respect to the transcription start site of each gene. Arrows indicate TGACCT-like motifs. (*B*) Luciferase reporter plasmids pHD(×3)luc and pAOx(×2)luc were cotransfected into COS-1 cells in the presence of control vector pSG5 or pSG5 vectors expressing xPPARα, xPPARβ, xPPARγ, or mPPAR, as indicated. An appropriate amount of pSG5 was included in all transfections to normalize promoter dosage. Transfections were done in the absence or presence of the peroxisome proliferators ciprofibrate and Wy-14,643, as indicated. Luciferase activity was measured from cell lysates corresponding to equal numbers of cells. Values are averaged from at least two independent transfections with duplicate samples and were normalized to the activity of control transfections done with pSG5 in the absence of drug, which was taken as 1. (*C*) Transfections were performed as above except that the luciferase reporter plasmids were transfected with either a pRc/CMV plasmid expressing rPPAR or with the control plasmid pRc/CMV, as indicated.

gests that COS cells may contain endogenous factors that activate mPPAR (and also rPPAR, see below) but that are unable to activate xPPARs.

Drug- and receptor-dependent induction of pAOx(×2)luc expression was observed with xPPARα (3- to 6-fold) and mPPAR (2-fold). Similarly, mPPAR also conferred a 3-fold drug-independent induction, which was further increased by either drug. Interestingly, pAOx(×2)luc expression was also induced by xPPARγ in the presence of ciprofibrate or Wy-14,643, in contrast to the results obtained with pHD(×3)luc. Thus, the ability of xPPARγ to mediate peroxisome proliferator-dependent induction is conditional upon the PPRE used. No drug-dependent induction of expression by the AOx PPRE was observed with xPPARβ. This is in contrast to the results of Dreyer *et al.* (11). In their experiments, the AOx PPRE was placed upstream of the basal thymidine kinase promoter, and transfections were carried out in HeLa cells. The differences in experimental conditions may explain our inability to detect a xPPARβ-dependent induction. Taken together, the results suggest that promoter context or specific cellular coregulators can modulate the induction mediated by particular PPARs.

Both pHD(×3)luc and pAOx(×2)luc responded in a similar fashion to the drugs in transfections performed with rPPAR (Fig. 1*C*). rPPAR was the most effective receptor at mediating peroxisome proliferator signaling (15- to 25-fold induction). There was also a drug-independent but rPPAR-dependent induction of expression observed with both pHD(×3)luc (5-fold) and pAOx(×2)luc (3-fold). This finding further supports the suggestion that COS cells contain specific endogenous PPAR-activating ligands.

PPARs Bind to the HD and AOx PPREs. To determine if the differential response of the HD and AOx PPREs to specific PPARs was due to differences in DNA–protein interactions, gel retardation analyses were performed with nuclear extracts from COS cells transfected with expression plasmids encoding different PPARs (Fig. 2). A protein–DNA complex was observed with both the HD PPRE probe (Fig. 2, lanes i–l) and AOx PPRE probe (Fig. 2, lanes c–f) when using extracts of transfected cells but not of untransfected cells (Fig. 2, lanes b and h). There was a correspondence in the mobilities of the complexes formed between the HD and AOx probes and a particular PPAR, indicating that the same or similar factors bound to both the HD and AOx PPREs. Assays done with extracts from peroxisome proliferator-responsive H4IIEC3 cells generated complexes of similar mobility with both the HD and AOx PPREs (Fig. 2, lanes g and a, respectively).

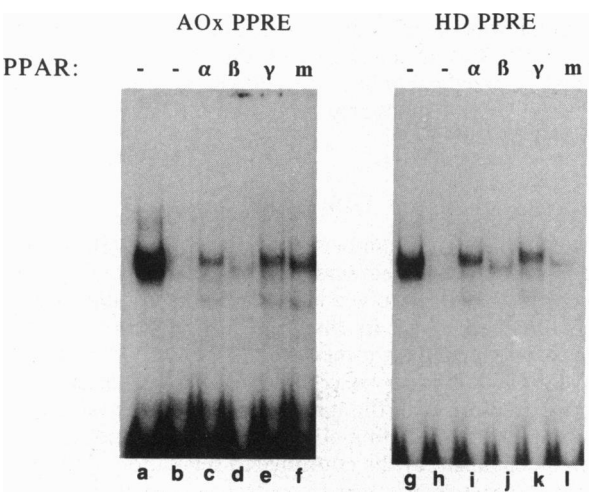

FIG. 2. PPARs expressed *in vivo* bind to the AOx and HD PPREs. Nuclear extracts prepared from COS-1 cells transfected with pSG5 (lanes b and h) or transfected with various PPAR-expressing plasmids (lanes: α, xPPARα; β, xPPARβ; γ, xPPARγ; m, mPPAR) were incubated with labeled AOx PPRE probe (lanes b–f) or HD PPRE probe (lanes h–l) and analyzed by gel retardation. Lanes a and g are reactions carried out with the AOx or HD PPREs, respectively, and extract prepared from H4IIEC3 cells. The PPRE probes used in this and all subsequent binding reactions are described in *Materials and Methods*.

Therefore, the failure of xPPARβ to induce expression via either the AOx or HD PPRE is not due to an inability of this receptor to bind these elements *in vitro* or to the possibility that this particular receptor was unstable and rapidly degraded *in vivo*. Similarly, the differential effects observed with xPPARγ-mediated induction via the AOx PPRE vis-à-vis the HD PPRE cannot be ascribed to differences in the ability of xPPARγ to bind to the HD PPRE as opposed to the AOx PPRE.

A Cellular Cofactor Stimulates PPAR Interaction with PPREs. PPARs belong to the nuclear steroid hormone receptor superfamily. The DNA-binding activity of this class of receptors is stimulated in several cases by cooperative interactions with other cellular factors, including the RXR family of receptors (18–20). Gel retardation analyses were done with *in vitro* translated PPARs to determine if they could interact cooperatively with the HD PPRE in the presence of cellular factors. Fig. 3*A* shows the [^{35}S]methionine-labeled PPARs. Each PPAR bound to the HD PPRE (Fig. 3*B*, lanes c–g), and binding was enhanced by the addition of COS cell nuclear extract (Fig. 3*B*, lanes h–l). Complexes were not generated with unprogrammed rabbit reticulocyte lysate or lysate supplemented with COS cell extract (Fig. 3*B*, lanes a and b, respectively). Similar results were obtained with the AOx PPRE (data not presented).

Cooperative DNA Binding with RXRα. The spacing of the two proximal TGACCT-like repeats in the HD PPRE conforms to that of RXR elements (21, 22). Consistent with this, all PPARs were shown to bind cooperatively to the HD PPRE in the presence of *in vitro* translated RXRα (Fig. 4*A*, compare lanes b–f with lanes i–l). RXRα-dependent stimulation of PPAR binding was most pronounced with xPPARγ, mPPAR, and rPPAR (Fig. 4*A*, compare lanes j–l to lanes d–f) and to a

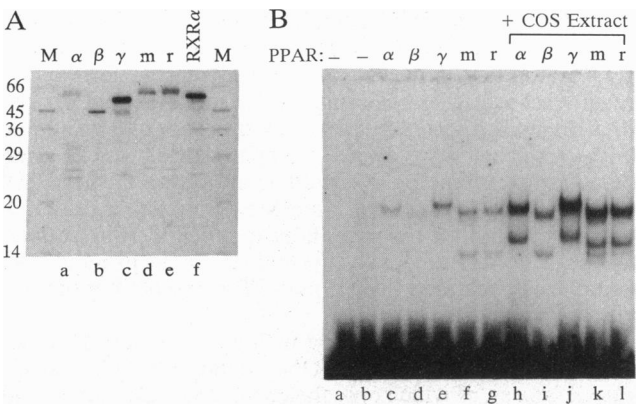

FIG. 3. A cellular factor stimulates PPAR–DNA binding. The cDNAs encoding the various PPARs were transcribed and translated *in vitro*, and the proteins were used for gel retardation assays with the HD PPRE probe. (*A*) SDS/polyacrylamide gel of [^{35}S]methionine-labeled translation products from rabbit reticulocyte lysates programmed with mRNA transcribed *in vitro* from plasmids encoding the various PPARs or RXRα. Lanes: r, rPPAR; M, molecular weight standards (in kDa); others, as in Fig. 2. (*B*) The different PPARs were synthesized *in vitro* as above but with unlabeled methionine. Each translation mixture (2 μl) was incubated with labeled HD PPRE probe in the absence (lanes c–g) or the presence (lanes h–l) of 0.2 μg of nuclear extract from COS-1 cells. Control lanes include probe incubated with 2 μl of unprogrammed reticulocyte lysate (lane a) or with unprogrammed reticulocyte lysate and 0.2 μg of COS extract (lane b). All reactions were normalized as to protein content with bovine serum albumin.

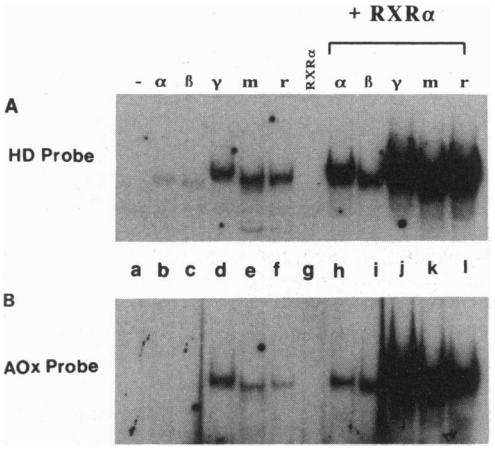

FIG. 4. PPARs bind cooperatively with RXRα to both the HD and AOx PPREs. Unlabeled *in vitro* translated PPARs were incubated with either labeled HD PPRE (*A*) or labeled AOx PPRE (*B*) in the absence (lanes b–f) or in the presence (lanes h–l) of unlabeled *in vitro* translated RXRα. Two microliters of each translation mixture was used. Unprogrammed reticulocyte lysate (2 μl) was added to reactions a–g to normalize for total protein. Lanes: a, unprogrammed reticulocyte lysate incubated with each probe; g, RXRα alone incubated with each probe.

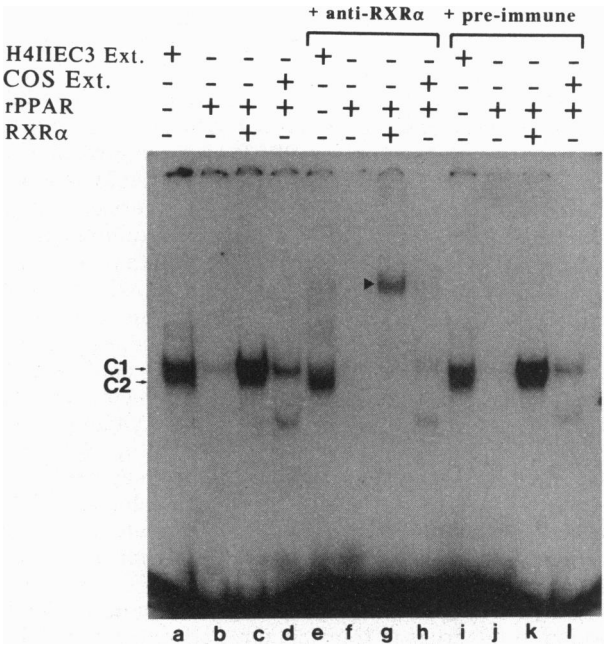

FIG. 5. RXRα is present in protein–DNA complexes formed between H4IIEC3 nuclear extract or *in vitro* translated rPPAR and HD PPRE. Labeled HD PPRE was incubated with extract from H4IIEC3 cells or with *in vitro* translated rPPAR supplemented with RXRα or COS-1 cell extract, as indicated, and analyzed by gel retardation (lanes a–d). Identical reactions were incubated with 1 μl of polyclonal antiserum raised against RXRα (lanes e–h) or with 1 μl of preimmune serum (lanes i–l). C1 and C2 correspond to the two protein–DNA complexes formed between HD PPRE and H4IIEC3 cell extract. The arrowhead corresponds to the supershifted complex observed in reactions carried out in the presence of anti-RXRα serum.

lesser extent with xPPARα and xPPARβ (compare lanes h and i to lanes b and c); RXRα alone had no binding activity (lane g). Similar results were obtained with the AOx PPRE probe (Fig. 4*B*). These results indicate that all the PPARs are capable of interacting cooperatively with RXRα on either the AOx or HD PPRE.

Anti-RXRα antibody decreased the amount of complex formed between *in vitro* translated rPPAR and RXRα and resulted in the concomitant appearance of a supershifted complex, demonstrating the presence of RXRα in this complex (Fig. 5, compare lanes c and g, respectively). Similar results were obtained with *in vitro* translated rPPAR incubated with COS extract (Fig. 5, compare lanes d and h). The amount of protein–DNA complex formed with *in vitro* translated rPPAR alone also decreased in the presence of anti-RXRα antibody; however, a supershifted complex was not readily seen, perhaps because of the small amount of complex originally formed and to the interference of the antibody with complex stability (Fig. 5, compare lanes b and f). These results suggest that one of the cofactors supplied by the COS extract is indeed RXRα. Preimmune serum had no effect on complex stability or electrophoretic mobility (Fig. 5, lanes i–l). RXRα was shown to be present also in the protein–DNA complexes generated by the other PPARs (data not presented).

Incubation of the HD probe with extracts from H4IIEC3 cells generated two protein–DNA complexes (C1 and C2; Fig. 5, lane a), of which only the upper was supershifted with anti-RXRα antibody (lane e). This supershifted complex comigrated with the supershifted complex observed with *in vitro* translated rPPAR and RXRα (Fig. 5, lane g). Therefore, these results suggest that at least one of the complexes formed on the HD PPRE in peroxisome proliferator-responsive H4IIEC3 cells contains RXRα.

xPPARγ Interferes with the Functional Activity of PPARs *in Vivo*. The *in vitro* DNA binding results suggest that the ability of xPPARγ to stimulate drug-dependent transcription from the AOx PPRE but not from the HD PPRE *in vivo* is not due to an intrinsic inability of this receptor to bind to the HD element or to interact cooperatively with cellular factors such as RXRα. To test whether this receptor interferes with signaling by other functional isoforms of PPARs, cells were cotransfected in the presence or absence of ciprofibrate with pHD(×3)luc, a constant amount of rPPAR or xPPARα, and an increasing amount of xPPARγ. Cotransfection of rPPAR and xPPARα with increasing amounts of xPPARγ reduced the luciferase activities mediated by rPPAR and xPPARα in a dose-dependent manner (Fig. 6). xPPARγ had no effect on transfections carried out in the absence of ciprofibrate, indicating that the inhibition was specific and not the result of squelching. Therefore, xPPARγ can interfere with the activity of rPPAR and xPPARα, presumably because xPPARγ is capable of competing *in vivo* with functional receptors for the cognate PPRE binding site.

DISCUSSION

The discovery of a number of related PPARs (10–13) raises the possibility that members of this family of ligand-activated receptors may be involved in distinct and specific regulatory signaling pathways. In this report, we demonstrate that peroxisome proliferator-mediated activation of the HD gene can be elicited by diverse PPARs via direct interaction of these receptors with the upstream PPRE. The AOx gene is activated by PPARs in a similar fashion, illustrating a common mechanism for the coordinated regulation of these, and possibly other, peroxisome proliferator-responsive genes.

Our findings demonstrate that each PPAR, and isoforms of xPPAR, can bind to the AOx and HD PPREs and can do so synergistically through interaction with RXRα. These results are consistent with the structural homologies among the PPARs (10–13) and with the similarities between the AOx and HD PPREs (8, 9). Both PPREs contain two imperfect direct copies of a TGACCT-like motif separated by a single nucleotide (see Fig. 1*A*) and thereby conform to retinoid X response elements (21, 22).

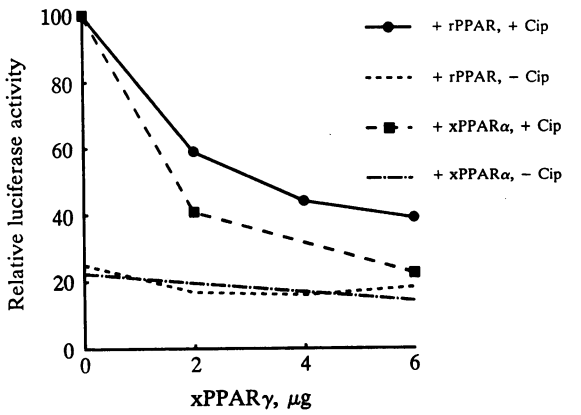

FIG. 6. xPPARγ trans-dominantly inhibits transcription induction mediated by rPPAR and xPPARα. pHD(×3)luc (5 μg) was cotransfected into COS-1 cells with either 2 μg of rPPAR or 2 μg of xPPARα expression plasmid in the absence or the presence of increasing amounts of plasmid expressing xPPARγ, as indicated at the bottom of the figure. Transfections were done in duplicate in the presence (+) or absence (−) of ciprofibrate (Cip). The amount of DNA in each transfection was normalized with pSG5. The values shown are normalized to the activity obtained from the respective reactions carried out in the absence of competitor plasmid, which was taken as 100%.

Interestingly, the expression of the luciferase reporter gene linked to the HD or AOx PPRE was not induced, or was differentially activated, by particular PPARs. For instance, xPPARβ failed to stimulate pHD(×3)luc or pAOx(×2)luc expression in the presence of either ciprofibrate or Wy-14,643. More significantly, xPPARγ stimulated expression of the luciferase reporter linked to the AOx PPRE but not to the HD PPRE. These results show that PPAR–DNA binding or cooperative interactions with cellular factors, including RXRα, are required but not necessarily sufficient to elicit peroxisome proliferator-mediated activation. Importantly, as demonstrated with xPPARγ, activity can depend on the nature of the PPRE. The AOx and HD PPREs are fairly divergent. There are differences both in the sequences of the TGACCT-like repeats as well as in the flanking nucleotides (8, 9). Moreover, the HD PPRE contains a third TGACCT motif two nucleotides farther upstream, which is not present in the AOx PPRE (see Fig. 1*A*). It is likely that some or all of these differences underlie the target gene specificity observed with xPPARγ and perhaps other PPAR isoforms. It is intriguing to speculate that ligand activation or requisite protein–protein interactions (for instance with basal transcription factors or coactivators) may be influenced by differences in receptor–coregulator–DNA interactions or conformation of protein–DNA complexes imparted by different target PPREs.

Finally, the ability of xPPARγ to interfere with the *in vivo* induction of transcription mediated by rPPAR or xPPARα implies that PPAR isoforms may act as both repressors and activators of specific target genes. Our findings reveal a complex interactive network of both positive and negative control pathways underlying the regulation of genes involved in lipid homeostasis and drug detoxification.

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