Nuclear receptor corepressor-dependent repression of peroxisome-proliferator-activated receptor $\delta$-mediated transactivation

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The nuclear receptor corepressor (NCoR) was isolated as a peroxisome-proliferator-activated receptor (PPAR) $\delta$ interacting protein using the yeast two-hybrid system. NCoR interacted strongly with the ligand-binding domain of PPAR$\delta$, whereas interactions with the ligand-binding domains of PPAR$\gamma$ and PPAR$\alpha$ were significantly weaker. PPAR–NCoR interactions were antagonized by ligands in the two-hybrid system, but were ligand-insensitive in vitro pull-down assays. Interaction between PPAR$\delta$ and NCoR was unaffected by coexpression of retinoid X receptor (RXR) $\alpha$. The PPAR$\delta$–RXR$\alpha$ heterodimer bound to an acyl-CoA oxidase (ACO)-type peroxisome-proliferator response element recruited a glutathione S-transferase–NCoR fusion protein in a ligand-independent manner. Contrasting with most other nuclear receptors, PPAR$\delta$ was found to interact equally well with interaction domains I and II of NCoR. In transient transfection experiments, NCoR and the related silencing mediator for retinoid and thyroid hormone receptor (SMRT) were shown to exert a marked dose-dependent repression of ligand-induced PPAR$\delta$-mediated transactivation; in addition, transactivation induced by the cAMP-elevating agent forskolin was efficiently reduced to basal levels by NCoR as well as SMRT coexpression. Our results suggest that the transactivation potential of liganded PPAR$\delta$ can be fine-tuned by interaction with NCoR and SMRT in a manner determined by the expression levels of corepressors and coactivators.

Key words: cAMP, SMRT, transcriptional repression.

INTRODUCTION

Peroxisome-proliferator-activated receptor $\delta$ (PPAR$\delta$) nuclear receptor type 1, class 2 (NR1C2) is a member of the PPAR subfamily of nuclear receptors many of which have been shown to be critical in the control of cellular growth, differentiation and homeostasis (reviewed in [1]). The PPAR subfamily comprises in addition to PPAR$\delta$ also PPAR$\alpha$ (NR1C1) and PPAR$\gamma$ (NR1C3). Numerous studies have clearly established roles for PPAR$\alpha$ and PPAR$\gamma$ in the regulation of cellular growth, differentiation and lipid homeostasis [2], whereas the biological significance of PPAR$\delta$ has remained elusive. However, recent studies have identified the role of PPAR$\delta$ in cholesterol metabolism [3], adipocyte differentiation [4,5], neuronal function [6], epidermal differentiation [7,8], colon cancer [9] and uterine implantation [10].

Transactivation by PPAR$\delta$ is mediated by RXR (retinoid X receptor)–PPAR$\delta$ heterodimers bound to peroxisome-proliferator-response elements (PPREs) in the promoter region of target genes. The PPREs are generally of the direct repeat with 1-bp spacing (DR1) type, which is also the target of a number of related nuclear receptors. Relatively little is known about the molecular mechanisms controlling PPAR$\delta$-mediated transactivation. The transcriptional activity of nuclear receptors depends on and is regulated by a complex interplay of coactivators and corepressors. Coactivator-dependent transactivation by nuclear receptors is facilitated when the nuclear receptor ligand-binding domain (LBD) adopts a conformation that allows the C-terminal helix of activator function 2 (AF2) to pack tightly against the body of the LBD, forming a surface that accommodates a short helical structure present in the receptor interaction domains of most coactivators [11]. The crystal structures of PPAR$\delta$ and other nuclear receptors have revealed that this conformation of the LBD is the prevalent one upon ligand binding [12,13].

A large number of coactivators have been identified and shown to interact with nuclear receptors in a ligand-dependent manner (reviewed in [14]). In the unliganded state, several nuclear receptors have been found to interact with the nuclear receptor corepressors (NCoRs) [15] and silencing mediator for retinoid and thyroid hormone receptor (SMRT) [16]. These corepressors are evidently required for the active repressing function of unliganded thyroid hormone receptor (TR) and retinoic acid receptor (RAR) [17] and also of Rev-Erb [18], chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF) [19] and DAX1 [20]. Moreover, NCoR and SMRT interact with antagonist-bound steroid receptors and appear essential for full antagonist activity [21]. Although the unliganded PPAR$\alpha$...
and PPARγ are not transcriptional repressors they have been shown to associate with NCoR and SMRT [22–24].

Substructures of the LBD that are also involved in coactivator recruitment seem to play a role in the interactions of corepressors with nuclear receptors [25–28]. The interaction domains of the corepressors contain extended helical structures, which are predicted to interact with the part of the LBD that has been shown to accommodate the short helical structure present in the coactivators [26,27]. The ligand-induced folding of the AF2 helix probably precludes binding of these extended helical structures; however, additional structures flanking the helices also appear to partake in the binding process, which may involve slightly different structures of different receptor LBDs [28]. NCoR also partake in the binding process, which may involve slightly different structures of different receptor LBDs [28]. NCoR also probably precludes binding of these extended helical structures; however, additional structures flanking the helices also appear to partake in the binding process, which may involve slightly different structures of different receptor LBDs [28].

Moreover, both NCoR and SMRT repressed PPARα transactivation, induced either by ligand or by a cAMP-elevating agent. Interestingly, we find that the ligand dependency of this interaction is conditional, as it is absent when assayed in vitro, but prominent in vivo. Moreover, both NCoR and SMRT repressed PPARδ-mediated transactivation, induced either by ligand or by a CAMP-elevating agent, suggest a high degree of flexibility in the regulation of the transcriptional potential of PPARδ.

EXPERIMENTAL

Plasmids

Expression vectors for the yeast two-hybrid system pGBT9 (pG9), pGDAD10 (pG10) and pGDAD242 (pG4) were obtained from Clontech. pG9NCoR (residues 1454–2453) was generated by cloning the EcoRI fragment containing the partial cDNA of human NCoR (isolated in the yeast two-hybrid library screening) from pG10NCoR (residues 1454–2453), into the EcoRI site of pGBT9. pG10mNCoR (residues 1944–2453) and pG10mNCoR (residues 1454–2453) was subcloned from pGEX–NCoR (residues 2239–2453) into pM (Clontech). All PCR-based constructions were verified by nucleotide sequencing.

Yeast two-hybrid library screening

A human leukaemia (Jurkat) MATCHMAKER library (Clontech) was screened with pG9–mPPARδ (HLBD) as bait in a mating-based two-hybrid screening as described by Bendixen and co-workers [36].

Yeast two-hybrid assay

The yeast strain SFY526 (Clontech) was transformed with vectors expressing GAL4(DBD) fusion as bait and a GAL4(AD) (activator domain) fusion as the activator. Clones were selected on selection medium plates containing 2% (w/v) glucose. Filter assays were performed according to the Clontech MATCHMAKER manual. To quantify β-galactosidase activities, selected clones were grown in selection minimal medium with 2% (w/v) glucose. At $D_{600}$ 0.5, activators or vehicle were added. After 5 h the cultures were harvested, the cells were washed in Z-buffer and cell density was measured spectrophotometrically. The β-galactosidase activity was measured either as described in [37] or by submitting the cells to one freeze–thaw cycle and lysing in 20 mM DTE, 0.055% (w/v) SDS, 1% (v/v) Triton X-100 in Z-buffer in microtitre plates. Subsequently, the β-galactosidase activity was measured in an automatic platereader (iEMS with automatic injection of o-nitro-phenyl-galactoside) using single kinetic mode. Each assay was performed twice on 4–8 individual clones of each type. In addition, the ability of yeast transformants to grow in the presence of 3-amino-1,2,4-triazole, aminotriazol (Sigma), a competitive inhibitor of the HIS3 gene product, was used to detect functional interactions between PPARs and NCoR. For Western analysis of protein expression, anti-Gal4(DBD) (Clontech) and anti-TATA-binding protein (TBP) (Santa Cruz, sc-273) antibodies were used.

Dynabead DR1 pull out

High-salt yeast extracts were prepared as described previously [32]. A total of 40 µl of yeast extract was preincubated on ice for 15 min in a 176 µl reaction containing 60 mM KCl, 20 mM Tris/HCl, pH 7.5, 10% (v/v) glycerol, 2 mM DTE, 2 mM MgCl2, 80 µg/ml sonicated herring sperm DNA and protease inhibitors, with or without 10 µM cyclic prostaglandin I2 (cPGLI) or 1 µM BRL49653 as indicated. A biotinylated oligonucleotide containing the DR1 element of the rACO promoter [5′-biotin-C6-(tgcaacctgggagccgtatgcttcttaggctgctccctgc-3′] annealed to complementary non-biotinylated oligonucleotide was bound.
to streptavidin-coated paramagnetic beads (Dynabeads, M280, Dynal®) following the procedure recommended by Dynal®. After preincubation, the reaction was precleared for non-specific Dynabead/DNA binding by incubation with Dynabeads (with non-DR1-oligo bound) for 10 min at room temperature. The beads for preclearing were then removed by magnetic force and 0.2 mg of probe beads was added to the reaction. Binding was allowed for 10 min at room temperature at 22 rpm end-over. Bacterially produced glutathione S-transferase (GST)–NCoR (residues 2239–2453) or GST, approx. 0.65 μg, was added to the reactions and binding was allowed for another 10 min at room temperature. The beads were pulled out by magnetic force, washed four times in 60 mM NaCl, 20 mM Tris/HCl, pH 7.5, 10% (v/v) glycerol, 2 mM MgCl₂, 2 mM DTE, protease inhibitors and bound proteins were eluted by boiling in 30 μl SDS/PAGE loading buffer. The proteins were resolved by electrophoresis, transferred to Immobilon P membrane and visualized by immunodetection using ECL®. The primary antibodies used were α-GST (Pharmacia), α-RRXr (D-20) (Santa Cruz Biotechnology), α-PPARγ and α-PPARγ2. The last two were kindly provided by Poul Grimaldi and Mitchell Lazar, respectively. Stripping was done by boiling in 20 mM Tris/HCl, pH 7.5, 0.2% (w/v) SDS.

Peptide mapping

Bacterially produced GST–NCoR (residues 2239–2453) was subjected to SDS/PAGE on a 10%, (w/v) acrylamide gel. Bands were visualized with Coomassie-Blue staining, excised, in situ digested [38] and analysed by matrix-assisted laser-desorption/ ionization mass spectrometry (MALDI–MS) analysis. In order to determine precisely the truncation point (±1 residue), the GST–NCoR was also directly submitted to the same analysis.

In vitro transcription and translation

Full-length rPPARγ, full-length mPPARγ, full-length mPPARγ, full-length RRXr and full-length hTR/β cDNAs were transcribed and translated using the TnT reticulocyte lysate kit (Promega) in the presence of [35S]Methionine.

GST pull down

GST fusion proteins were expressed in Escherichia coli BL21(pGROESL) by induction with 0.1 mM isopropyl β-D-thiogalactoside at 30 °C. Proteins were isolated by cell lysis with a French press and purified according to the Pharmacia manual. In vitro translated 10 μl of rPPARγ, mPPARγ, mPPARγ and RRXr were incubated in buffer A, [20 mM Tris/HCl, pH 8.5, 100 mM NaCl, 0.1% (v/v) NP-40, 10% (v/v) glycerol, protease inhibitors and 10 mg/ml BSA (essential fatty-acid free)] with 100 μM Wy14643, 25 μM 2-bromopalmitate, 10 μM BRL49653, 10 μM cPGI₂, and 1 μM of thyroid hormone (T3) respectively for 15 min on ice. GST–NCoR coupled to glutathione beads was added and the interaction was allowed to proceed for 2 h at 4 °C. The beads were then washed three times in buffer A with ligand where appropriate and finally in 10 mM Tris/HCl, pH 8.5. The bound proteins were eluted by boiling in SDS/PAGE sample buffer and resolved by electrophoresis on a 10%, (w/v) SDS gel. [35S]Methionine-labelled proteins were visualized by autoradiography.

Transient transfections

NIH-3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% (v/v) calf serum (Sigma). The cells were cultured in 21 × 5 cm² plates and transfected in triplicate at 50% confluence, employing the DC-Chol lipofection procedure [39] in DMEM without calf serum. Cells were transfected with luciferase reporter vector, β-galactosidase expression vector for normalization, receptor expression vectors, NCoR expression vector and SMRT expression vector. pBlueScriptKS+ was used to equalize the total quantity of transfected DNA. Six hours after transfection, the liposomes were removed and the medium was changed to DMEM containing 10% (v/v) resin-charcoal-stripped calf serum and 1 μM BRL49653, 100 μM Wy14643 (TIC+), 25 μM 2-bromopalmitate, 5 μM cPGI₂ (Sigma), 0.5 μM L165041 (Merck Research Laboratories, NJ, U.S.A.), 10 μM forskolin or solvent (DMSO). Cells were harvested after 48 h and lysed in Galacto-Light (TROPiX) lysis buffer. Luciferase and β-galactosidase activity were measured in triplicate in microtiter plates in a Berthold MicroLumat LB96P luminometer. The luciferase values were normalized to β-galactosidase activity.

RESULTS

PPARγ interacts strongly with NCoR

A partial cDNA encoding the C-terminal 1000 amino acids (residues 1454–2453) of the human NCoR was isolated in a yeast two-hybrid screening of a human leukaemia (Jurkat) cDNA library using the mouse PPARγ HLBD as bait. Further analysis using the yeast two-hybrid system showed that the isolated NCoR fragment interacted exclusively with the LBD (H1–H12) as defined by the crystal structure of the PPARγ LBD [12] of PPARγ (Figure 1A). Inclusion of further N-terminal domains of PPARγ appeared to neither enhance nor reduce the interaction with the NCoR fragment. In agreement with previous reports [22,30], we found that the NCoR fragment also interacted well with the PPARγ and PPARγ HLBDs in the two-hybrid system. The relative interaction strength of the three PPAR subtypes with NCoR (residues 1454–2453) was measured in a liquid culture yeast two-hybrid assay. As shown in Figure 1(B), the strength of interaction was strongly PPAR subtype-dependent, with PPARγ exhibiting an almost 4-fold stronger interaction with NCoR than PPARζ. Equal levels of expression of the three GAL4(DBD)–PPAR(HLBD) fusion proteins were confirmed by Western blotting using immunodetection of yeast TBP for normalization of protein load (Figure 1C).

Ligand dependence of the PPARγ–NCoR interaction

The interaction of NCoR with nuclear receptors is generally characterized by a strong ligand dependency. Interactions between PPARγ and NCoR, and between PPARγ and NCoR have previously been shown to exhibit some degree of ligand dependency in yeast and mammalian two-hybrid assay, respectively [22–24]. When cPGI₂, which binds to all PPAR subtypes, was added to the liquid cultures in the yeast two-hybrid assay, the PPARγ–NCoR (residues 1454–2453) interaction was strongly reduced, whereas the PPARγ–NCoR and PPARγ–NCoR interactions were only modestly reduced (Figure 2A). In a mammalian two-hybrid system, a pronounced ligand-dependent reduction of the interaction with NCoR was observed for all three PPAR subtypes (Figure 2B).

The NCoR receptor interaction domain (ID) is composed of two discrete nuclear receptor IDs [25] and one N-terminally located interaction-enhancing domain [25,40]. Yeast two-hybrid analysis showed that PPARγ interacted in a ligand-dependent fashion with both the C- and N-terminal autonomous interaction domains (named ID-I and ID-II, respectively) (Figure 2C).
Interaction between PPARδ and ID-II appeared weak. However, the strength of the interactions of the different NCoR fragments with PPARδ cannot be determined in this assay because the three fragments are expressed at very different levels as assessed by Western blotting (results not shown).

Using GST–NCoR fusions and in vitro translated nuclear receptors for pull-down analyses, we found that TRβ as previously reported interacted strongly with the N-terminal NCoR ID-II fragment, whereas interaction with the C-terminal ID-I fragment was weak [25]. Interaction was significantly decreased by the addition of T3 (Figure 2D). On the other hand, PPARδ interacted equally well with the C- and N-terminal NCoR ID-I and ID-II fragments. Similar results were obtained with PPARα and PPARγ (results not shown). In the pull-down assays, PPARδ–NCoR interactions were unaffected by the addition of ligand (Figures 2D and 3). Addition of a 2-fold excess of in vitro translated RXRα did not affect the NCoR–PPAR interactions in vitro (Figure 3). The same conclusion was reached using a yeast two-hybrid assay, where coexpression of RXR did not affect the interaction between NCoR and PPARδ (results not shown). Both PPAR and RXR were observed in the pull-down mixtures, although the RXR signals were very weak compared with the PPAR signals. Whether RXR was pulled down by direct interaction with NCoR or as part of a PPAR–RXR heterodimer, or whether NCoR binding to PPARδ impedes interaction between PPARδ and RXR cannot be concluded from this assay. Yet, as shown below, a preformed PPARδ–RXR heterodimer is able to recruit NCoR. In a control experiment (see Figure 3), the addition of T3 significantly reduced or completely abolished TRβ interaction with NCoR, confirming the functionality of the assay [15]. In agreement with previous reports, the TR signal was much stronger than the RXR signal in pull-down analyses [30].

Binding of nuclear receptors to cognate DNA response elements has been shown to enhance heterodimerization and influence the recruitment of cofactors [41,42]. Thus the formation of a ternary complex consisting of RAR, RXR and an RAR response element enhanced recruitment of NCoR in the absence of ligands, but also enhanced ligand-dependent recruitment of the steroid receptor coactivator 1 [42]. It was reported that DNA binding of PPARc prevented interaction with NCoR when assayed in electrophoretic mobility assays [30]. To investigate whether the PPARδ–NCoR interaction persisted in a DNA-bound context and, if so, how ligands would affect this interaction, we tested the PPARδ–RXRα–NCoR complex formation on an oligonucleotide containing the ACO DR1. A biotinylated DNA probe containing the ACO DR1 was used to pull down RXRα and PPARδ from high-salt extracts of yeast expressing these receptors (Figure 4A). When bacterially produced GST–NCoR (residues 2239–2453) was added to the reaction it was pulled down with the DR1/RXRα–
PPARδ complex bound to DR1. Interestingly, and in keeping with previously published functional assays [21], we were also able to detect interaction between NCoR and a DR1/RXRα–PPARγ complex (Figure 4B). Small amounts of GST–NCoR were also pulled down using extracts of yeast expressing RXRα alone, consistent with previous reports showing weak interaction between RXRα and the NCoR ID-I [22]. Addition of 10 μM cPGI2 did not diminish binding of NCoR to the DNA-bound PPARα–RXRα heterodimer. Similarly, addition of 10 μM cPGI2 or 1 μM BRL49653 did not decrease NCoR binding to the DNA-bound PPARγ–RXRα heterodimer (Figure 4). Other ligands including the RXR ligand 9-cis retinoic acid were tested with similar results (results not shown).

During purification from E. coli extracts, GST–NCoR was partially degraded. It appeared that RXRα predominantly pulled down the full-length fragment, whereas the PPAR–RXR complexes displayed no particular preference for the full-length fragment and even tended to pull down slightly more of a truncated form. To determine the degree of truncation, a sample of the GST–NCoR fusion protein was submitted to SDS/PAGE. Bands corresponding to the two largest peptide forms were excised and submitted to tryptic in-gel digestion followed by mass-spectrometric peptide mapping. The largest peptide was identified as full-length GST–NCoR (residues 2239–2453). The truncated peptide predominantly pulled down by PPARs was found to contain GST–NCoR (residues 2239–2377), thus lacking a part of the NCoR C-terminal which included the 38 amino-acid region previously shown to impair NCoR receptor-interacting domain (RID) interaction with TR/β [15].

In conclusion, DNA binding of the PPARδ–RXRα heterodimer did not prevent interaction with NCoR (residues 2239–2453). PPARδ–NCoR interaction was ligand-dependent when assayed in GST-pull-down assays. In vitro translated PPARα or TRβ was pulled down with GST–NCoR (residues 1944–2453) in the absence or presence of ligand. Values on the y-axis denote the relative [35S]PPARδ signal strength (arbitrary units) obtained using QMS phosphorimager software. The data shown are representative of at least four independent experiments.

NCoR is a potent repressor of PPARα-mediated transactivation

To investigate the functional significance of the observed interactions between NCoR and PPARα–RXR we expressed the receptors in NIH 3T3 cells and assayed the effect of coexpression of NCoR on the activity of a PPARETK-Luc reporter construct (Figure 5A). NCoR coexpression led to a dose-dependent reduction of ligand-induced PPARα–RXRα-mediated transactivation. Confirming previously published results [22–24],

Figure 2 NCoR–PPAR interaction is ligand-dependent in vivo but not in vitro, and involves the entire NCoR RID

(A) Top panel: schematic presentation of the genomically integrated 3 × (UASGal)–lacZ reporter construct, containing three copies of the UASGal in front of the Gal1 minimal promoter. Bottom panel: yeast two-hybrid analysis of the interactions between NCoR (residues 2239–2453) and the PPAR LBDs with or without ligand (5 μM cPGI2). Interaction in the absence of ligand was set to 100. (B) Top panel: schematic presentation of the pTK-4 × UASGal-luc construct, containing three copies of the ACO PPRE inserted in front of a minimal thymidine kinase (TK) promoter. Bottom panel: mammalian two-hybrid analysis of the interactions between GAL(DBD)NCoR (residues 2239–2453) and the PPAR HLBDs fused to GAL(AD) with or without ligand (100 μM WY14643, 1 μM BRL49653 or 0.5 μM L165041). Interaction in the absence of ligand was set to 100. (C) Yeast two-hybrid analysis showing that GAL(DBD)PPARδ interacts with both the N- and C-terminal parts of the NCoR RID fused to GAL(AD) in liquid culture β-galactosidase assays. Addition of 25 μM 2-bromopalmitate (BrPal) reduced the strength of the interaction with either part of the RID. The values on the abscissa represent β-galactosidase activity above the background level. (D) The NCoR–PPARδ interaction is ligand-independent when assayed in GST-pull-down assays. In vitro translated PPARδ or TRβ was pulled down with GST–NCoR (residues 1944–2453) in the absence or presence of ligand. Values on the y-axis denote the relative [35S]PPARδ signal strength (arbitrary units) obtained using QMS phosphorimager software. The data shown are representative of at least four independent experiments.
NCoR coexpression also led to repression of the ligand-induced transcriptional activity of PPARα and PPARγ (Figure 5A), although repression of PPARα was significantly weaker than that observed for PPARγ and PPARδ. Using GAL4(DBD)–PPAR(HLBD) fusions and the GAL UAS Luc reporter, we observed significant, but less NCoR-mediated repression supporting the finding that NCoR interacted with LBDs of the PPARs. The PPARs are generally characterized as transcriptional activators without the repression potential of the related receptors TR, RAR and Rev-Erb. Accordingly, coexpression of NCoR did
Figure 5 NCoR and SMRT repress PPARδ-mediated transactivation in vivo

NIH-3T3 cells were transfected with a pTK-3 × PPRE-luc (A) or pTK-4 × UAS,β-galuc (B) reporter construct and with expression vectors for RXRα, NCoR and either full-length PPARs (A) or GAL4–PPAR(HLBD) fusions (B). Transfected cells were incubated with or without the PPAR ligands: L165041 (0.5 μM), WY14643 (100 μM), BRL49653 (1 μM) and cPGI2 (5 μM) and forskolin either alone or in combination. (C) Comparison of the repressive effects of NCoR and SMRT on PPARδ-dependent transactivation. The assay was performed as in (B) except that forskolin was added either alone or in combination with the PPAR ligand and the effects of NCoR and SMRT were titrated at very low amounts of plasmid DNA (25–100 ng of NCoR or SMRT expression vectors).

not repress the transcriptional activity of the reporter constructs below the basal levels of activity.

Our recent studies [4] supported by other work [43] have shown that cAMP-elevating agents enhance PPARδ–RXR-mediated transactivation and cAMP-elevating agents and ligands synergistically induce PPARδ-mediated transactivation. Upon coexpression of NCoR, we observed a significant repression of PPARδ-mediated transactivation induced by the PPARδ-specific ligand, L165041, as well as transactivation induced by the cAMP-elevating agent forskolin, alone or in synergy with L165041. Noticeably, NCoR is capable of total repression of the activity of the liganded PPARδ (Figure 5C, left-hand panel). Most nuclear receptors appear to have a preference for interaction with either NCoR or the related corepressor, SMRT [21,28,30], and repression of PPARγ-mediated transactivation through activation of the mitogen-activated protein kinase cascade was shown to be strongly dependent on SMRT, but not on NCoR [21]. Our results clearly demonstrate that NCoR potently represses PPARδ-mediated transactivation, and hence we decided to investigate whether PPARδ differed from PPARγ or whether PPARδ-mediated transactivation could be repressed by SMRT. Figure 5C (right-hand panel) demonstrates that coexpression of SMRT led to a dose-dependent repression of PPARδ-mediated transactivation similar to that obtained with NCoR coexpression (Figure 5C). Thus, PPARδ is a target for both NCoR and SMRT-mediated transcriptional repression.

DISCUSSION

Understanding the mechanisms determining ligand-dependent exchange of corepressors for coactivators is central to the understanding of nuclear receptor-dependent control of gene expression. In the work presented here, we demonstrate physical and functional interaction between NCoR and the three known PPAR subtypes. We show that the strength of NCoR interaction with the three PPAR subtypes followed the ranking order PPARδ > PPARγ > PPARα. The PPARs bind to the same type
of DNA elements although with different affinities [44,45] and, hence, competition for binding in conjunction with ligand availability, ligand-binding affinities and differences in the affinity for binding of corepressors and coactivators will ultimately determine the activity of genes harbouring PPRE elements in their promoters. We show that the corepressors NCoR and SMRT are capable of fully repressing PPARδ-mediated transactivation induced either by ligands or by cAMP-regulated signalling pathways. This suggests corepressors as general antagonists of the various stimuli inducing PPARδ-mediated transactivation.

Isolation of NCoR as a PPARδ-binding protein and the discovery that it represses ligand-induced, PPARδ-mediated transactivation raise the question as to how this repression takes place at the molecular level, taking into account that binding of ligand switches the structure of related nuclear receptors from an inactive/repressive form to an active form (reviewed in [14]). With the elucidation of the crystal structures of the liganded and unliganded forms of the LBDs of PPARγ [13] and later of PPARα [12] and PPARβ [12,46,47], it became clear that the PPARs deviate structurally from related nuclear receptors in several ways. The ligand-binding hydrophobic cavity of the PPAR LBDs is much larger and more spacious than those of other nuclear receptors, which explains the ability of the PPARs to accommodate a wide variety of structurally different classes of ligands. It also appeared that the PPAR ligands are oriented so that the polar headgroup of the ligand faces the AF2 helix and makes an intricate series of hydrogen bonds with amino-acid residues of the receptors including a conserved tyrosine residue in the C-terminal AF2 helix. This is in contrast with the structure determined for other liganded receptors, where the polar group(s) is facing away from the AF2 helix. Several studies have shown that different PPAR ligands mediate a differential pattern of interaction with cofactors [48–50], suggesting that each ligand may stabilize the otherwise rather unstable LBD [51] in a particular conformation defined by the structure of the ligand. Direct evidence of a high degree of structural flexibility has been obtained by NMR spectroscopy of the PPARγ LBD [51]. This flexibility is consistent with a highly dynamic structure of the unliganded receptors as it was predicted based on the fact that the LBDs of the PPARs contain several additional helices and substructures compared with other crystallized receptors [12,13,47]. The notion of a very flexible PPAR structure was further supported by the discovery of a separate class of synthetic PPAR ligands which prevent both coactivator and corepressor interaction by freezing the PPARγ LBD in a conformation similar to that observed in the structure of the crystallized unliganded PPARγ LBD [24]. Thus corepressor as well as coactivator binding may require/prefer a conformation of the PPARs distinct from the ‘canonical’ structure of the unliganded receptors. Further evidence in favour of the notion that corepressor binding induces a particular conformation of nuclear receptors was obtained in studies showing that corepressor binding imposed a structural alteration in the TR LBD. This alteration included a stabilized interaction between helix 1 and the remainder of the TR LBD, similar to that observed in ligand binding to the TR LBD [52].

Assembly of the AF2 helix into the active AF2 structure appears necessary for stable coactivator recruitment, whereas it precludes corepressor interaction with the steroid receptors and strongly antagonizes coactivator interaction with the RARs and TRs [27]. Thus, it would appear that the flexibility of the AF2 helix is a determinant of the relative affinity of a receptor for corepressors and coactivators. We found that NCoR interacted strongly with PPARδ. This interaction was unaffected by the presence of ligands in vitro, showing that ligand binding by itself did not preclude or significantly antagonize NCoR interaction. On the other hand, when tested in cell-based assays we observed a ligand-induced reduction of the PPARα–NCoR interaction. Hence, in the cell-based assays the effect of ligands must be mediated by factors absent or present at insufficient levels in the in vitro assays. Addition of ligand increases the affinity of PPARδ for coactivators, and it is therefore plausible that coactivator recruitment may be required for completion of the release of NCoR initiated by ligand binding. This idea is further supported by our observation that ligand-induced PPARδ-mediated transactivation in NIH 3T3 cells was repressed by corepressor coexpression in a dose-dependent manner, suggesting that corepressors when present at sufficiently high levels may exert a significant competition against ligand-induced coactivator binding to the PPARδ. Previously, it has been anticipated that the conformation of ligand-activatable nuclear receptors was mainly determined by the presence of ligands, which then dictated interactions with cofactors. We propose a model for PPARδ function where ligands may pose the PPARδ for receptor-mediated transactivation, whereas the actual magnitude of the transactivation depends on the equilibrium between binding of the actual coactivators and corepressors. This mechanism allows fine-tuning of PPARδ-mediated transactivation induced by various signalling pathways.

We thank Dr M. A. Lazar for NCoR plasmids, and Novo Nordisk, Bagsværd, Denmark and Merck Research Laboratories, Rahway, NJ, U.S.A. for providing ligands. This work was conducted at the Center for Experimental Biinformatics and supported by the Danish Biotechnology Program, the Danish Natural Science Research Council and the Novo Nordisk Foundation.

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