Retinoic Acid Is a High Affinity Selective Ligand for the Peroxisome Proliferator-activated Receptor β/δ*

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Retinoic acid (RA) modulates transcription of numerous target genes, thereby regulating a myriad of biological processes. It is well established that RA functions by activating retinoic acid receptors (RARs), which, in turn, control cell differentiation, proliferation, and apoptosis. However, perplexing reports of diverse and sometime opposing actions of RA have been published. Hence, while RA induces apoptosis and inhibits cell growth in some settings, it potentiates proliferation and acts as an anti-apoptotic agent in others. These observations raise the possibility that signaling pathways other than RA may be involved in mediating RA activities. Here we show that RA is a high affinity ligand for another nuclear receptor, namely the orphan receptor peroxisome proliferator-activated receptor (PPAR) β/δ. We demonstrate that while RA does not activate PPARα and PPARγ, it binds to PPARβ/δ with nanomolar affinity, modulates the conformation of the receptor, promotes interaction with the coactivator SRC-1, and efficiently activates PPARβ/δ-mediated transcription. Transcriptional signaling by RA is thus exerted by a dual pathway, providing a rationale for understanding divergent cellular responses to this hormone.

The vitamin A metabolite all-trans-retinoic acid (RA) is a potent modulator of cell growth, differentiation, and apoptosis in various tissues both in the embryo and in the adult (1, 2). Consequently, RA plays key roles in embryonal development (3, 4) and is also used as a therapeutic agent in a number of pathologies including several cancers (5, 6). It is well established that the pleotropic activities of RA are mediated by members of the superfamily of nuclear hormone receptors known as retinoic acid receptors (RARs), which upon activation modulate the rates of transcription of numerous target genes.

Like other class 2 nuclear receptors, RARs function as heterodimers with retinoid X receptors (RXRs). These heterodimers bind to specific response elements in the promoter regions of target genes and function as ligand-inducible transcription factors (1, 7). Perplexingly, RA displays diverse and sometime opposing activities. For example, while this hormone induces apoptosis and inhibits cell growth in some settings (8–10), it potentiates proliferation and acts as an anti-apoptotic agent in others (11–14). These observations raise the possibility that signaling pathways other than RAR may be involved in mediating RA activities. In search for a mechanism that may underlie these inconsistencies, we considered the orphan nuclear receptor PPARβ/δ, for which no physiological ligand has been reported. The biological functions of PPARβ/δ are not well understood although those of its PPARα and PPARγ isoforms. It was, however, recently demonstrated that PPARβ/δ mediates differentiation in keratinocytes and oligodendrocytes (15, 16). It was also shown that by up-regulating the expression of antiapoptotic genes and down-regulating the expression of proapoptotic genes, PPARβ/δ significantly increases cell resistance to apoptosis (15, 17). We thus set out to examine whether this receptor may be activated by RA. We show that RA is a high affinity ligand for PPARβ/δ. We demonstrate that while RA does not activate PPARα and PPARγ, it binds to PPARβ/δ with nanomolar affinity, modulates the conformation of the receptor, promotes interaction with the coactivator SRC-1, and efficiently activates PPARβ/δ-mediated transcription. These observations suggest that transcriptional signaling by RA is exerted by a dual pathway, which, in turn, may lead to divergent cellular responses.

**EXPERIMENTAL PROCEDURES**

Ligands, Vectors, and Proteins—WY-14643 and troglitazone were purchased from Cayman Chemical Co. and Biomol, respectively. L165041 and LG268 were gifts from Walter Wahli and Ligand Pharmaceuticals, respectively. Bacterial expression vectors were constructed in pET 28a for mPPARα-LBD (residues 203–467) and RXRαΔH12 (residues 1–448). mPPARα-LBD and mPPARγ-LBD in pRSF were gifts from Eric Xu. Proteins were expressed and purified as described previously (18, 19). Mammalian expression vectors for mPPARα in pCMX, mPPARα in pCDNA, mPPARγ in pMOS5, and the (PPRE)-luciferase reporter were gifts from Ronald Evans. SRC-1 (in pSG5) was a gift from Christopher Glass.

Fluorescence Titrations—Bacterially expressed mPPARα-LBD, mPPARβ/δ-LBD, and mPPARγ-LBD (0.2–1 μM) were titrated directly in a cuvette with RA dissolved in ethanol. Ethanol concentration was usually below 1% and never exceeded 2%. To ensure equilibration between protein and ligand, the fluorescence was monitored until a constant value was reached. The progress of titrations was monitored by following the decrease in the intrinsic fluorescence of the protein (excitation, 280 nm; emission, 340 nm), which accompanies RA binding. Inner filtering by the ligand, reflected by linear slopes observed following saturation of the protein, was corrected for as described (20). Corrected data were analyzed to obtain equilibrium dissociation constants (Kd).

Analyses were carried out by fitting the data to equation (1) derived from simple binding theory (23),

\[
F = F_0 - \frac{1 + (P_T + R_T)K_a - (P_T - R_T)K_a^{-1} + 2P_T + R_T)K_a - 1)^{1/2} - (P_T - F_0)}{2P_T},
\]

(Eq. 1)

where F is the observed fluorescence, F, and F, are the fluorescence in the absence of ligand and at saturation, respectively, P, and R, are the total concentrations of protein and RA, respectively, and K is the association constant (K = 1/Kd).

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**In Vitro Transcription/Translation**—

$^{35}$S-Labeled PPARβ/δ and SRC-1 were synthesized using the TNT-coupled transcription/translation system (Promega).

**Proteolysis Assay**—

$^{35}$S-Labeled PPARβ/δ (2 μl) was incubated for 20 min at 25 °C in a total volume of 8 μl of 22 mM Tris, pH 8.0, 75 mM KCl, 5% (v/v) glycerol, and 2 mM dithioerythritol in the presence of vehicle or ligand (10 μM). Protein was digested with chymotrypsin (25 °C, 20 min), resolved by SDS-PAGE, and visualized by autoradiography. Addition of either L165041 (middle panel) or RA (bottom panels) gave rise to protected fragments (denoted by lines on right), but the protection patterns were distinct.

**Fluorescence titrations of PPARs with RA**. Bacterially expressed PPARα-LBD, PPARβ/δ-LBD, and PPARγ-LBD were titrated with RA and the titration followed by monitoring the decrease in the intrinsic fluorescence of the proteins ($\lambda_{\text{exc.}}$ 280 nm; $\lambda_{\text{em.}}$ 340 nm). Titration curves (empty circles) were corrected for inner filtering (linear slopes observed following saturation). Corrected data (solid circles) were fitted to a binding equation (line through data points) to obtain $K_d$ values. Representative titrations are shown. In these, the concentrations of PPARα-LBD, PPARβ/δ-LBD, and PPARγ-LBD were 1, 0.77, and 0.2 μM, respectively.

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**Pull-down** Assays—GST-PPARβ/δ-LBD was immobilized on glutathione-Sepharose beads in 20 mM Tris, pH 8.0, 100 mM NaCl, 4 mM MgCl₂, 1 mM dithioerythritol, 0.1% (v/v) Igepal CA-630, 10% (v/v) glycerol, and 5% (v/v) fatty acid-free milk powder. −5 μg of protein was mixed with 8 μl of $^{35}$S-labeled in vitro transcribed/translated SRC-1. Mixtures were incubated for 2 h at 4 °C. Beads were washed three times in 1 ml of buffer without milk powder, and bound proteins were resolved by SDS-PAGE and visualized by autoradiography. Band intensities were quantitated using the Cyclone phosphorimager system (Packard Bioscience).

**Transactivation Assays**—COS-7 cells were maintained with Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-treated newborn calf serum. Cells were transfected using FuGENE (Roche Diagnostics) with a (PPRE)₃-luciferase reporter (0.3 μg/well), expression vectors for receptors (0.3 μg/well), and pCH110 (0.2 μg/well). Medium was replaced with serum-free Dulbecco’s modified Eagle’s medium for 24 h. Cells were treated with denoted ligands for 24 h and lysed. Lysates were analyzed for reporter activity and normalized for β-galactosidase.

**RESULTS AND DISCUSSION**

Binding of RA to PPARα, PPARβ/δ, and PPARγ was studied by fluorescence titrations. This method relies on the considerable overlap of the absorption spectrum of RA with the fluorescence emission spectra of tryptophanes and tyrosines, a consequence of which is that RA-binding results in quenching of the intrinsic fluorescence of a binding protein (20–22). PPARs were titrated with RA and the ligand-induced decrease in protein fluorescence was monitored (Fig. 1). Titration curves were corrected for inner filtering and fitted to a simple binding equation (23) to obtain equilibrium dissociation constants ($K_d$, Table I). RA bound to PPARα and PPARγ with a low affinity demonstrated by $K_d$ values of 100–200 nM. In contrast, RA associated with PPARβ/δ with a $K_d$ of 17 nM, revealing both high affinity and isotype selectivity. The measured affinity of PPARβ/δ for RA is thus comparable with that of various PPAR ligands for
these receptors (24) and with that of the synthetic PPARβ/δ-selective ligand L165041 (25).

Ligand binding by nuclear receptors induces a conformational transition that results in a more compact protein fold (26) and, consequently, decreases their sensitivity to proteolysis (27). Protease sensitivity assays were carried out to compare the effects of RA to those of the synthetic PPARβ/δ-selective agonist L165041 (Fig. 2). As shown previously (28), addition of L165041 decreased the sensitivity of the receptor to chymotrypsin digestion and resulted in preservation of protease-resistant fragments (denoted by lines on the right in Fig. 2). RA also increased the resistance of the receptor to proteolysis but gave rise to a different pattern of protected fragments.

It was reported previously that different PPAR agonists may give rise to differential protection profiles (29). Nevertheless, the observations in Fig. 2, showing differences between the effects of RA and L165041, raise the question whether RA is a bona fide PPARβ/δ agonist. The hallmark of active nuclear receptor ligands is that they enable the recruitment of transcriptional coactivators (30). The ability of RA to promote association of PPARβ/δ with the coactivator SRC-1 was thus investigated by GST-pull-down assays (Fig. 3). Similarly to L165041, the interactions of SRC-1 with PPARβ/δ were significantly stabilized in the presence of RA but not upon addition of the RAR-selective ligand TTNPB (Fig. 3a). Moreover, RA-induced enhancement of SRC-1 binding by the receptor occurred in dose-dependent fashion (Fig. 3, b and c).

The ability of RA to enhance the transcriptional activity of PPARα, PPARβ/δ, and PPARγ was then directly examined by transactivation assays. In these, a luciferase reporter driven by a consensus PPAR response element (PPRE) was utilized. Cells were cotransfected with the reporter construct together with expression vectors for each of the PPAR isotypes, and the effect of RA treatment of reporter expression was studied. RA did not activate PPARγ and poorly activated PPARα. In contrast, RA efficiently enhanced the transcriptional activity of PPARβ/δ (Fig. 4), displaying an EC50 for this subtype that was an order of magnitude lower than that observed for PPARα (Fig. 4a). Control experiments showed that all three PPAR isotypes were similarly activated in these cells by synthetic isotype-selective
ligands, verifying that the inability of RA to activate PPARα and PPARγ did not stem from poor expression of these isotypes (Fig. 4b). The data thus demonstrated that RA is a potent and selective activator of PPARβ/δ.

The transcriptional activities of PPARs are mediated by their heterodimers with RXR (31). Both PPAR-RXR heterodimers and RXR homodimers bind to similar response elements comprised of two repeats separated by a single base pair (DR-1). In either complex, RXR can be activated by 9-cis-RA. It can thus be argued that RA may have been converted to an RXR ligand within the cells and that its transcriptional activity reflected activation of RXR rather than PPARβ/δ. While the observations that RA selectively activated PPARβ/δ renders this possibility unlikely, we examined it directly. An RXR construct lacking its AF-2 domain (RXRΔH12) was used. RXRΔH12 can heterodimerize with PPAR (32) and properly associate with cognate DNA (33), but it cannot activate transcription on its own. Consequently, PPAR-RXRΔH12 dimers do not respond to RXR ligands but can be activated by PPAR agonists (albeit at a somewhat lower efficiency than in the presence of WT-RXR). Cells were cotransfected with expression vectors for RXRΔH12 and PPARβ/δ, and the ability of RA to activate the PPRE-reporter was studied (Fig. 4c). As expected, the RXR-selective agonist LG268 was ineffective. In contrast, both the PPARβ/δ ligand L165041 and RA activated the reporter, demonstrating that RA acts in these assays through PPARβ/δ and not via RXR. Note as well that the observed reporter activity was not mediated by RAR, which does not function through the PPRE used in these assays (Fig. 4d).

These observations lead to the surprising conclusion that in addition to activating RAR, RA is a potent and selective ligand for the orphan receptor PPARβ/δ. The biological functions of PPARβ/δ have only begun to be elucidated as it was recently reported that this receptor mediates differentiation in keratinocytes and oligodendrocytes (15, 16). It was also demonstrated that expression and activation of PPARβ/δ increases cell resistance to apoptosis (15). This activity was traced to PPARβ/δ-mediated transcriptional up-regulation of the expression of ILK and PDK1, leading to activation of the anti-apoptotic Akt1 signaling pathway (17).

The current use of RA as a chemotherapeutic agent in several cancers is based on the pronounced growth inhibitory activities of this hormone, which are believed to be mediated by RARs. It was reported that in various carcinomas, RA induces apoptosis (34, 35), differentiation (8), cell cycle arrest (36), and that in some cells, and it inhibits proliferation by a combination of effects (37, 38). However, it has also been reported that in some settings, RA treatment may result in enhanced proliferation (11–14). For example, it was shown that RA potentiates epidermal growth factor-induced proliferation in cultured epidermal cells of mouse mammary gland (14), inhibits lobuloalveolar differentiation of cultured mouse mammary glands (13), and promotes at late stages of malignant progression in some cell variants in breast cancer tumors (39). The basis for the opposing activities of RA on cell growth is unknown at present. However, an intriguing possibility suggested by our observations is that while RA exerts growth inhibition through activating RAR, it may evoke anti-apoptotic activities, and thus promote growth via activation of PPARβ/δ. An important ques-

Articles on this topic have been published in a variety of journals, including Nature and Science. The majority of these articles focus on the mechanisms by which PPARs regulate gene expression, with a particular emphasis on the role of RXR in these processes. It is clear that understanding the regulation of PPARs is critical for the development of new therapeutic strategies, particularly in the context of cancer and inflammation. Further research will be necessary to fully elucidate the mechanisms by which PPARs function in vivo.

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