Photoaffinity Labeling of Human Retinoid X Receptor β (RXRβ) with 9-*cis*-Retinoic Acid: Identification of Phytanic Acid, Docosahexaenoic Acid, and Lithocholic Acid as Ligands for RXRβ[†]

Anna Radominska-Pandya* and Guangping Chen

Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Received December 4, 2001; Revised Manuscript Received January 30, 2002

ABSTRACT: We utilized [20-*methyl*-^{3}H]-9-*cis*-retinoic acid ([^{3}H]9-*cis*-RA) as a direct photoaffinity probe for the characterization of human recombinant retinoid X receptor β protein (RXRβ). The photoaffinity labeling was light- and concentration-dependent, saturable, and protected by unlabeled 9-*cis*-RA in a concentration-dependent manner, indicating that binding occurred in the RXR retinoid binding site. *all-trans*-Retinoic acid (atRA) did not affect labeling with the 9-cis derivative, confirming that atRA does not compete for the 9-*cis*-RA binding site. Several retinoid, fatty acid, and bile acid ligands were evaluated for their ability to recognize the 9-*cis*-RA binding site. Retinol, atRA glucuronide, 13-*cis*-RA, dolichol, 5,6-epoxy-RA, and vitamin D_3 did not compete for the 9-*cis*-RA binding site. However, the saturated diterpenoid phytanic acid (PA) and docosahexaenoic acid, which have been recently shown to activate the nuclear receptor, RXR, competed with 9-*cis*-RA labeling, showing high affinity for the 9-*cis*-RA binding site. Oleic acid, arachidonic acid, and butyric acid did not interact. However, the bile acid lithocholic acid competed efficiently with 9-*cis*-RA for the binding site. These data validated the photoaffinity assay as an excellent system for the identification and evaluation of ligands for RXR.

Retinoids are vitamin A derivatives that regulate cellular growth, differentiation, development, and apoptosis (*1, 2*). Retinoic acids exert their many biological effects by interaction with nuclear proteins such as the retinoic acid receptor (RAR)[1] and RXR. Retinoid accessibility to the nuclear receptors may be modulated by two cytoplasmic proteins, cellular retinoic acid binding protein I (CRABPI) and cellular retinoic acid binding protein II (CRABPII) (*3*). These proteins also bind atRA with high affinity and have been proposed to limit nuclear levels of atRA by sequestration and/or promotion of *all-trans*-retinoic acid metabolism (*4*). Recently, a direct interaction between atRA and protein kinase C has been demonstrated (*5*). This study raised the possibility that, in addition to CRABPs and nuclear receptors, other important cellular proteins can be affected by direct interactions with retinoids in pharmacological concentrations.

Retinoid nuclear receptors are ligand-inducible, trans-acting transcription factors which can modulate the expression of specific target genes by interacting with cis-acting DNA sequences, the retinoid response elements. RARs and RXRs have a modular structure consisting of six domains (A−F), each of which has been assigned specific functions (*6*). The E-domain plays a central role in ligand binding, receptor dimerization, and ligand-dependent transactivation. The details of this interaction are unknown due to the lack of pure functional receptor proteins and fast and reliable ligand binding assays. The X-ray crystal structures of the ligand binding domains of apo-RXR-α and holo-RAR-γ have been reported (*7, 8*).

RXR was characterized as a nuclear receptor which demonstrates a highly restricted substrate specificity. Until recently, 9-*cis*-RA was defined as the most potent RXR activator. No other endogenous retinoid was known to be accepted by RXRs. Several recent studies indicated that certain fatty acids, such as PA and DHA, activate RXR and PPAR. Weinberger and colleagues (*9*) and Lemotte and collaborators (*10*) simultaneously carried out studies which showed that PA and other phytol-derived derivatives activated RXR; however, this activation occurred at much higher concentrations as compared to 9-*cis*-RA. The most recent studies by Zomer et al. (*11*) confirmed that PA is a natural ligand for human RXRα. These studies also showed that PA is a naturally occurring ligand for the peroxisomal proliferator-activated receptor α (PPARα). Elegant studies by de Urquiza et al. (*12*) presented strong evidence that another unique fatty acid, DHA, is an effective ligand for RXRα in mouse brain. PPARs, RXRs, and the nuclear hormone

[†] This work was supported in part by NIH Grants DK51971, DK45123, and DK49715 (A.R.-P.).

* Correspondence should be addressed to this author at the University of Arkansas for Medical Sciences, 4301 W. Markham, Slot 516, Little Rock, AR 72205. Phone: (501) 686-5414. Fax: (501) 603-1146. E-mail: RadominskaAnna@exchange.uams.edu.

[1] Abbreviations: RAR, retinoic acid receptor; RXR, retinoid X receptor; CRABP, cellular retinoic acid binding protein; atRA, *all-trans*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; 4-OH-RA, 4-hydroxy-*all-trans*-retinoic acid; 4-OH-RAc, 4-hydroxy-*all-trans*-retinyl acetate; 5,6-epoxy-RA, 5,6-epoxy-*all-trans*-retinoic acid; PA, phytanic acid; DHA, *cis*-4,7,10,13,16,19-docosahexaenoic acid; LCA, lithocholic acid (5β-cholanoic acid-3α-ol); CDCA, chenodeoxycholic acid (5β-cholanoic acid-3α,7α-diol); DCA, deoxycholic acid (5β-cholanoic acid-3α,12α-diol); VD_3, 1α,25-dihydroxy-vitamin D_3; RAG, *all-trans*-retinoic acid glucuronide; OA, oleic acid (*cis*-9-octadecenoic acid); AA, arachidonic acid (5,8,11,14-eicosatetraenoic acid); BA, butyric acid.

10.1021/bi0121151 CCC: $22.00
Published on Web 03/19/2002

receptors have been shown to function as transcription factors in the control of peroxisomal enzyme expression (*13*−*15*). The fact that dietary fatty acids can activate RXRs suggests an involvement of RXR, in addition to PPARα, in the control of fatty acid metabolism.

Bile acids are recognized as endogenous compounds which may exhibit high toxicity toward hepatocytes, enterocytes, and blood cells (*16*). It is recognized that bile acids regulate the expression of a number of enzymes involved in their biosynthesis and several bile acid transporting proteins (*17, 18*). Recently, it was established that bile acids carry out these functions via two nuclear receptors, the farnesoid X receptor (FXR) and the steroid/pregnane X receptors (SXR/PXR) (*19*). Using a combination of knockout and transgenic animals, it was demonstrated that SXR and PXR are bile acid receptors and play a crucial role in the detoxification of bile acids via induction of cytochromes P450 (CYP450s) (*20*) and possibly UDP-glucuronosyltransferases (UGTs). Studies with the FXR/BAR (bile acid receptor) null mice demonstrated that FXR is critical as an endogenous bile acid receptor and a major regulator of bile acid homeostasis (*21*). It has not been shown whether RXR can also accept bile acids as ligands.

In this study, we identified [^{3}H]9-*cis*-RA as a novel photoaffinity probe for the photoaffinity labeling of human recombinant RXRβ protein and characterization of its putative ligands. We used direct photoaffinity labeling to demonstrate that purified, recombinant RXRβ binds 9-*cis*-RA acid with high affinity and that this radiolabel is directed into the 9-*cis*-RA binding site. We showed that data obtained from our photoaffinity studies correlate with RXR activation experiments (*9*−*12*). Several compounds of retinoid origin, fatty acids and bile acids, were checked as potential ligands of RXR. These data confirmed a very strict retinoid specificity for RXR toward the single retinoid, 9-*cis*-RA, and that the fatty acids, PA and DHA, are RXR ligands. In addition, we showed that bile acids are novel ligands for this nuclear receptor.

Taken together, the results represent the first evidence for direct, high-affinity photolabeling of RXRβ by 9-*cis*-RA, and this methodology will be further utilized for the characterization of new ligands, the interaction with other nuclear receptors such as PPAR, and the exact localization of amino acids in the 9-*cis*-RA binding site in RXRs.

EXPERIMENTAL PROCEDURES

Materials. Recombinant (human) retinoic X receptor β protein was obtained from Affinity Bioreagents, Inc. (Golden, CO). This recombinant protein was obtained from nuclear extracts by centrifugation and lysis of Sf9 cells infected with the rH-2RIIBP (human RXRβ) virus.

[^{3}H]9-*cis*-RA and [11,12-^{3}H]atRA ([^{3}H]RA) were purchased from Perkin-Elmer LifeScience (Boston, MA). The retinoid derivatives atRA, 9-*cis*-RA, retinol (ROH), and 13-*cis*-RA were purchased from Sigma (St. Louis, MO). 5,6-Epoxy-RA, 4-OH-RA, and the methyl derivatives of atRA, 5,6-epoxy-RA, and 4-OH-RA were synthesized in our laboratory as described by Samokyszyn (*22*). atRA glucuronide (RAG) was synthesized in our laboratory as described in (*23*). All retinoids were stored at −80 °C under argon. Stock solutions of the retinoids were prepared fresh in methanol (Fisher Scientific, Pittsburgh, PA), and all procedures involving these compounds were performed in the dark or under yellow light. PA, *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), butyric acid (BA), arachidonic acid (AA), and oleic acid (OA) were from Sigma. The structures of these compounds are shown in Figure 1. The bile acids lithocholic acid (LCA), chenodeoxycholic acid (CDCA), and deoxycholic acid (DCA) were from CalBiochem (La Jolla, CA). Vitamin D_3 (VD_3) and dolichol were purchased from Sigma. NuPAGE 4−12% Bis-Tris gels, sample buffer, and running buffer were from Novex (San Diego, CA). Solvents were all HPLC grade (Fisher Scientific). All other reagents were of the highest quality commercially available.

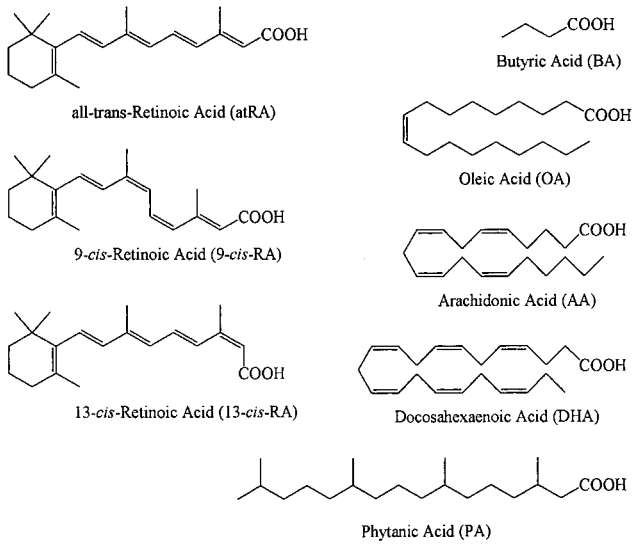

FIGURE 1: Structures of retinoids and fatty acids used in the present study.

Photoaffinity Labeling with [^{3}H]9-cis-RA and [11,12-^{3}H]-atRA. Direct photoaffinity labeling with [^{3}H]9-*cis*-RA and atRA was done using modifications of the method described by Bernstein for labeling with atRA (*24*). For photoaffinity labeling of RXRβ, 3 μg of purified recombinant RXRβ protein in 20 mM HEPES, final volume 10 μL, pH 7, was incubated for 2 min at room temperature with or without competing ligands at the concentrations indicated in the figure legends. To establish the concentration dependence of photolabeling, [^{3}H]9-*cis*-RA and [^{3}H]atRA (0, 5, 25, and 125 μM, final concentration) were added in ethanol with a final concentration of 2% ethanol in all samples. For protection experiments, 3.3 μM [^{3}H]9-*cis*-RA was used, and unlabeled potential ligands (retinoids, fatty acids, bile acids, and other compounds) were added in 0.5 μL of ethanol at varying concentrations (0, 5, and 25 μM). For controls (zero concentration), 0.5 μL of ethanol was added. Samples were incubated for 10 min on ice prior to addition of the probe and photolabeling on ice with a hand-held long-wavelength (366 nm) UV lamp (UVP-21, Ultraviolet Products, San Gabriel, CA) for 15 min. Proteins were denatured by addition of NuPAGE denaturing buffer (Novex) followed by sonication, boiling for 1 min, and, finally, centrifugation at 13 000 rpm in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY). Proteins were separated on 1.5 mm NuPAGE minigels.

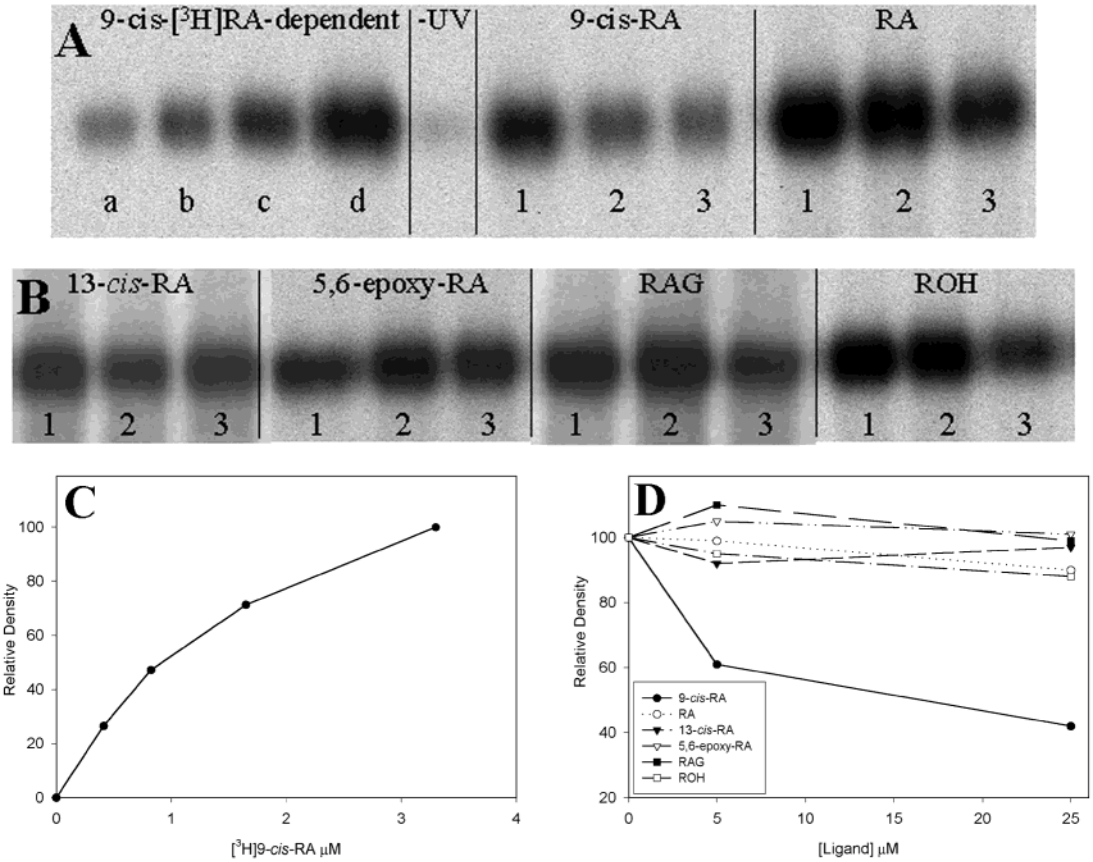

FIGURE 2: (A) Photoaffinity labeling of RXRβ by [^{3}H]9-*cis* RA and protection with 9-*cis*-RA and atRA. Direct photoaffinity labeling with [^{3}H]9-*cis*-RA was carried out as described under Experimental Procedures. Lanes a−d: RXRβ was photolabeled with increasing concentrations of [^{3}H]9-*cis*-RA (0.4, 0.8, 1.6, 3.3 μM) followed by a control lane with no UV. Protection of RXRβ from photolabeling with 3.3 μM [^{3}H]9-*cis*-RA with unlabeled 9-*cis*-RA and unlabeled atRA. For each ligand, lanes 1−3 represent increasing concentrations of retinoid (0, 5, 25 μM). (B) Photoaffinity labeling of RXRβ by [^{3}H]9-*cis*-RA and protection with various retinoids. RXRβ was photolabeled with 3.3 μM [^{3}H]9-*cis*-RA following preincubation with unlabeled 13-*cis*-RA, 5,6-epoxy-RA, RAG, and ROH. For each ligand, lanes 1−3 represent increasing concentrations of retinoid (0, 5, 25 μM). (C) The graph shows the results of quantitation of the concentration dependence of RXRβ labeling with [^{3}H]9-*cis*-RA by densitometry. (D) The graph is a plot of relative density, as determined by densitometry of autoradiograms, versus concentration of potential retinoid ligands of RXRβ.

Following electrophoresis, gels were stained with Coomassie blue, destained, washed thoroughly with water, treated with Autofluor autoradiography enhancer (National Diagnostics, Manville, NJ), dried, and subjected to autoradiography at −80 °C for 4−7 days. Results were quantitated by densitometric analysis of the autoradiograms using an AlphaInnotech IS-1000 Digital Imaging System (AlphaInnotech, San Leandro, CA).

Proteolytic Hydrolysis of [^{3}H]9-cis-RA-Labeled RXRβ with Endoproteinase Lys-C. To obtain the protease hydrolysis profile of RXRβ after photoaffinity labeling, 150 μg of RXRβ was preincubated with either PA or ethanol and photolabeled with [^{3}H]9-*cis*-RA. Protein was precipitated with TCA at a final concentration of 10%, and centrifuged at 15000*g*. The pellets were washed once with ice-cold ethanol and then dissolved in 30 μL of buffer (50 mM Tris, pH 8.0, and 0.1% SDS). Then 500 μL of charcoal/dextran solution [1% activated charcoal and 0.1% dextran (w/v) in 10 mM Tris, 0.25 M sucrose, and 1 mM EDTA, pH 7.4] was added. After shaking at 4 °C for 10 min, the charcoal was separated by centrifugation. Endoproteinase Lys-C (3.0 μg) was added, and the mixture was incubated at room temperature for 48 h before HPLC separation was carried out.

HPLC Separation of Peptides from Endoproteinase Lys-C-Hydrolyzed Photolabeled RXRβ. For HPLC separation, a reversed-phase C18 column was used. Gradient solvent A contained 2% acetonitrile and 0.06% trifluoroacetic acid in water; solvent B contained 70% acetonitrile and 0.06% trifluoroacetic acid in water. A linear gradient from 98% A to 98% B was used over 100 min at a flow rate of 1 mL/min. The column eluate was monitored for UV absorption at 215 nm. Fractions of 1 mL were collected, and an aliquot of 0.1 mL from each fraction was used for determination of radioactivity by scintillation counting.

RESULTS

Characterization of Photoaffinity Labeling of RXRβ with [^{3}H]9-cis-RA. Human RXRβ was used as a model protein for studying the use of photoaffinity labeling to determine the interaction of potential new ligands with this protein. As shown in Figure 2A, photoaffinity labeling of recombinant RXRβ with [^{3}H]9-*cis*-RA was light- and concentration-dependent (Figure 2A, lanes a−d). Competition experiments with unlabeled 9-*cis*-RA showed that the photoincorporation of [^{3}H]9-*cis*-RA into RXRβ was protected in a concentration-dependent manner as shown by the decrease in photolabeling

as the concentration of unlabeled 9 *cis*-RA increased from 0 to 5 and 25 μM (Figure 2A, 9-*cis*-RA, lanes 1−3). Figure 2A also demonstrates that atRA and ROH competed with [^{3}H]9-*cis*-RA for RXRβ binding at high concentrations, but the protection (~5%) was negligible (Figure 2A, atRA, lanes 1−3). This might be explained by these two retinoids having some affinity for the 9-*cis*-RA binding site at nonphysiological concentrations. The RXR photoaffinity labeling system was evaluated with several other retinoids to establish the validity of the competitive binding assay (Figure 2B, lanes 1−3). An oxidized derivative of atRA, 5,6-epoxy-RA, and other retinoids, such as atRA glucuronide and 13-*cis*-RA, were not effective as RXRβ ligands. These studies also confirmed that the carboxyl function of 9-*cis*-RA is essential for binding to RXRβ. Methyl-9-*cis*-RA, which contains a methyl group on the terminal carboxyl function of 9-*cis*-RA, did not compete with [^{3}H]9-*cis*-RA for the 9-*cis*-RA binding site of RXRβ (data not shown). Dolichol, which lacks the typical retinoid ring but has the familiar isoprenyl chain with a substituted -OH group, and VD_3 were not ligands for RXRβ. The lack of inhibition by several atRA derivatives has additional significance. It addresses a common concern in photoaffinity studies: the possibility that competitive ligands will quench the input light energy, thus preventing cross-linking and providing false results. This type of quenching appears to be ruled out by the studies described in Figure 2 since atRA, 5,6-epoxy-RA, 13-*cis*-RA, and ROH, even at high concentrations, did not inhibit photoincorporation. Especially important is the lack of competition between [^{3}H]9-*cis*-RA labeling and 13-*cis*-RA. The conformation of the isoprenyl chain and, therefore, the position of the terminal carboxyl group in 9-*cis*-RA are different than in 13-*cis*-RA and atRA. It is likely that this is one of the factors that determines RXR and RAR ligand binding specificity. Therefore, the single most important determinant of ligand specificity appears to be the conformation of the terminal carboxyl group.

Evaluation of Fatty Acids and Bile Acids as Potential Ligands for RXRβ. Photoaffinity labeling competition studies with several fatty acids were done to determine if fatty acids compete for the binding site of RXRβ with 9-*cis*-RA. Current evidence suggests that RXRα is activated by two fatty acids, PA and DHA (*9*−*12*), and these fatty acids were used in our studies (Figure 3). RXRβ was preincubated with increasing concentrations (0, 1, 5, and 25 μM) of PA, DHA, OA, and AA versus a fixed amount of the photoaffinity label. All the samples were photolyzed and analyzed. At 1 μM PA and DHA, the labeling with 9-*cis*-RA was inhibited 70% and 50%, respectively. Photoaffinity labeling, in general, does not allow the calculation of K_d and K_I values; however, it can provide comparative numbers for IC_{50}. Table 1 shows the apparent IC_{50} values, and this value for 9-*cis*-RA is 6 μM, for PA it is 0.2 μM, and for DHA it is 1 μM. These results suggest that PA and DHA might be much more effective competitors of RXRβ labeling with [^{3}H]9-*cis*-RA than 9-*cis*-RA itself.

The final class of compounds used for evaluation as RXRβ ligands were bile acids: the primary bile acid, CDCA, and two secondary bile acids, DCA and LCA. CDCA and DCA did not interfere with [^{3}H]9-*cis*-RA binding to the RXRβ. Only LA appears to be able to inhibit binding of [^{3}H]9 *cis*-RA with complete inhibition at 50 μM. However, the exact mechanism of this competition with 9-*cis*-RA for binding to RXRβ is not known.

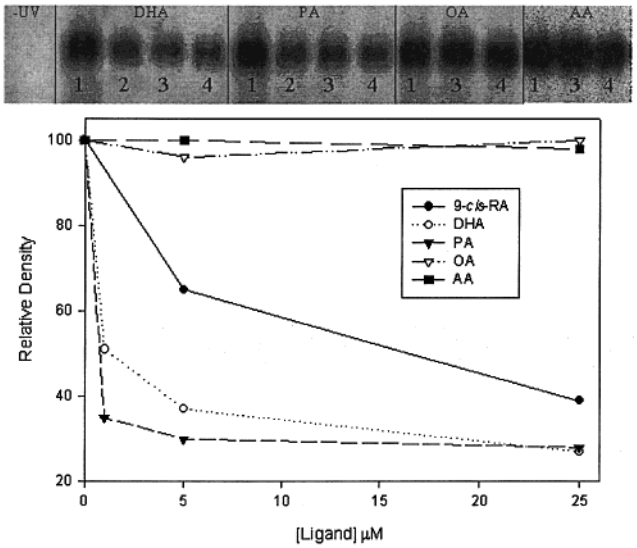

FIGURE 3: Photoaffinity labeling of RXRβ by [^{3}H]9-*cis*-RA and protection by fatty acids. RXRβ was photolabeled with [^{3}H]9-*cis*-RA following preincubation with DHA, PA, OA, and AA. Four lanes are shown for each ligand, representing increasing concentrations of fatty acid (0, 1, 5, 25 μM).

Table 1: Estimated Concentration of Ligand at 50% Protection (IC_{50}) of Photoincorporation of [^{3}H]9-*cis*-RA into RXRβ[a]

ligand	PA	DHA	9-*cis*-RA	LA
IC_{50} (μM)	0.2	1.0	6	65

[a] Experiments were carried out as described in the text using 3 μg of RXRβ and 3.3 μM [^{3}H]9-*cis*-RA. Unlabeled potential ligands were added in 0.5 μL of ethanol at varying concentrations (0.2, 1.0, 5.0, 25, and 125 μM for PA; 5, 25, 50, and 125 μM for all other ligands).

Photoaffinity Labeling of RXRβ with [^{3}H]atRA. The use of [^{3}H]atRA as a photoaffinity probe was described in our laboratory for the characterization of CRABPI and -II (*25*). In those studies, conditions for photoaffinity labeling were developed, and it was demonstrated that atRA upon irradiation binds to the atRA binding site on CRABPI and -II with high affinity. In the present work, recombinant RXRβ was used as a model protein for photoaffinity labeling with [^{3}H]-atRA. Carried out under the experimental conditions described for [^{3}H]9-*cis*-RA labeling, the [^{3}H]atRA labeling of RXRβ was negligible, occurred only at very high concentrations of the radiolabeled probe, and was not fully protected by unlabeled atRA, indicating partial unspecific labeling (data not shown).

Proteolytic Digestion and HPLC Separation of the Radioactive Peptides Derived from Endoproteinase Lys-C-Catalyzed Hydrolysis of [^{3}H]9-cis-RA-Labeled RXRβ. In this experiment, presented in Figure 6, two samples of 150 μg of RXRβ proteins were photolabeled with [^{3}H]9-*cis*-RA directly (control) or after preincubation with 100 μM unlabeled PA. After precipitation and removal of unbound [^{3}H]9-*cis*-RA, both protein pellets were dissolved in buffer and subjected to hydrolysis with endoproteinase Lys-C. The digestion was monitored on an SDS gel, and after 48 h, total hydrolysis was achieved. Following hydrolysis, radiolabeled polypeptides were separated by reverse-phase HPLC, and fractions were collected and radioactivity was measured in

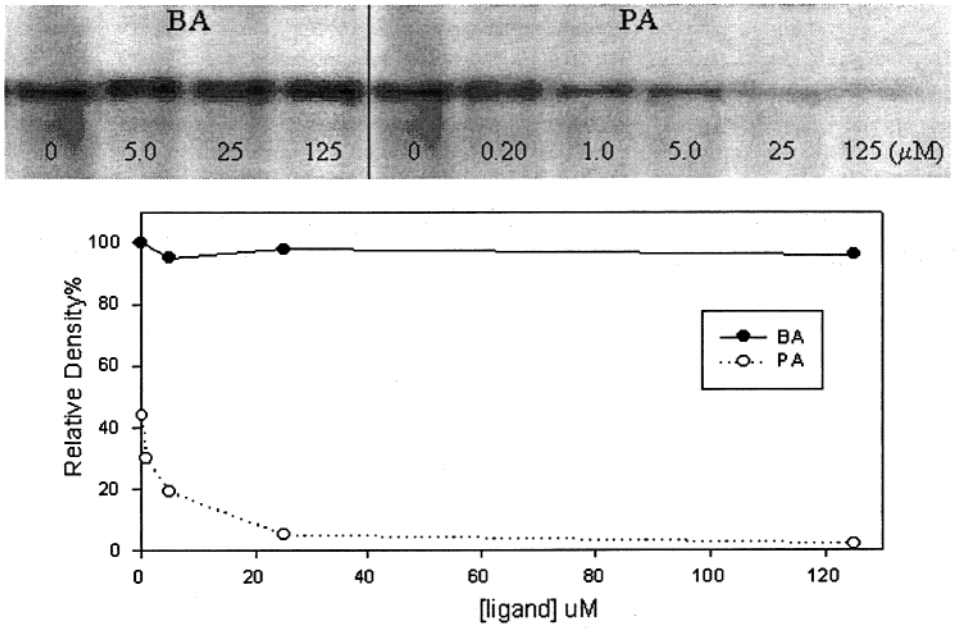

FIGURE 4: Photoaffinity labeling of RXRβ by [^{3}H]9-*cis*-RA and protection with butyric and phytanic acids. RXRβ was photolabeled with [^{3}H]9-*cis*-RA following preincubation with BA and PA. Four lanes are shown for BA, and six lanes are shown for PA, representing increasing concentrations of fatty acid (0, 5, 25, and 125 μM for BA and 0, 0.2, 1.0, 5.0, 25, and 125 μM for PA). The graph shows the results of quantification of photoaffinity labeling by densitometry.

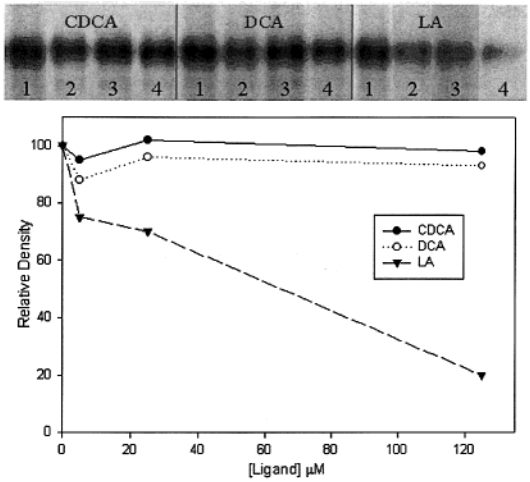

FIGURE 5: Photoaffinity labeling of RXRβ by [^{3}H]9-*cis*-RA and protection with CDCA, DCA, and LA. RXRβ was photolabeled with [^{3}H]9-*cis*-RA following preincubation with CDCA, DCA, and LA. Four lanes are shown for each ligand, representing increasing concentrations of bile acid (0, 5, 25, 130 μM). The graph shows the results of quantification of photoaffinity labeling by densitometry.

the fractions. Radioactive profiles for the HPLC separation of both samples are shown in Figure 6A. Two radioactive peaks, the major one localized between fractions 61 and 66 and a minor one localized between fractions 30 and 32, were obtained. The enlarged scale profile of two radioactive peaks observed for the control and PA preincubated samples is shown in Figure 6B,C. The profiles of photolabeled polypeptides of digested photolabeled RXRβ and the fraction protected by PA have the same retention time but different magnitudes. This indicates that both 9-*cis*-RA and PA may bind to the same amino acids of RXRβ. Fractions 60−68 of the radioactive profile were selected for peptide sequencing. The N-terminal protein sequencing was carried out at the Protein Microanalysis Facility at the University of Texas at Austin. The sequencing was unsuccessful due to the small amount of radiolabeled polypeptide available. Experiments with large-scale preparations are currently being carried out.

DISCUSSION

RXRs were initially identified as orphan nuclear receptors that bind and respond to 9-*cis*-RA (*9*). Subsequent studies that focused on the identification of compounds that may serve to modulate the activity of RXR in vitro and in vivo resulted in reports that several naturally occurring fatty acids can bind to RXRs and activate them in the context of transactivation assays (*11*, *19*). However, a persistent problem in accepting fatty acids as RXR ligands has been that the reported concentrations of PA required for binding, their binding affinities, and the activation potencies between RXR and putative fatty acid ligands were clearly orders of magnitude different than the corresponding values for 9-*cis*-RA. PA induced RXR-dependent transcription at concentrations between 4 and 64 μM and displaced 9-*cis*-RA from RXR with K_i values of 4.0 μM (*9*). The efficiency of the displacement was interpreted as transcriptional effects that are mediated by direct receptor interactions. The most recent studies by Zomer et al. (*11*) added to and supported the data that PA is a natural ligand for human RXRα.

In the present work, we used direct photoaffinity labeling with [^{3}H]9-*cis*-RA to evaluate several putative RXRβ ligands. Direct photoaffinity labeling is a process characterized by using an unmodified ligand for covalent binding to the active sites. This technique has been used previously with [^{3}H]atRA in the photolabeling of atRA binding proteins found in the cytosol and membranes from bovine tissues (*24*). Recently, we applied this technique to the characterization and identification of amino acids involved in the RA binding site of CRABPI (*25*). The main advantage of photoaffinity labeling is that it is the most direct method for the characterization of enzymes and transporters, including the

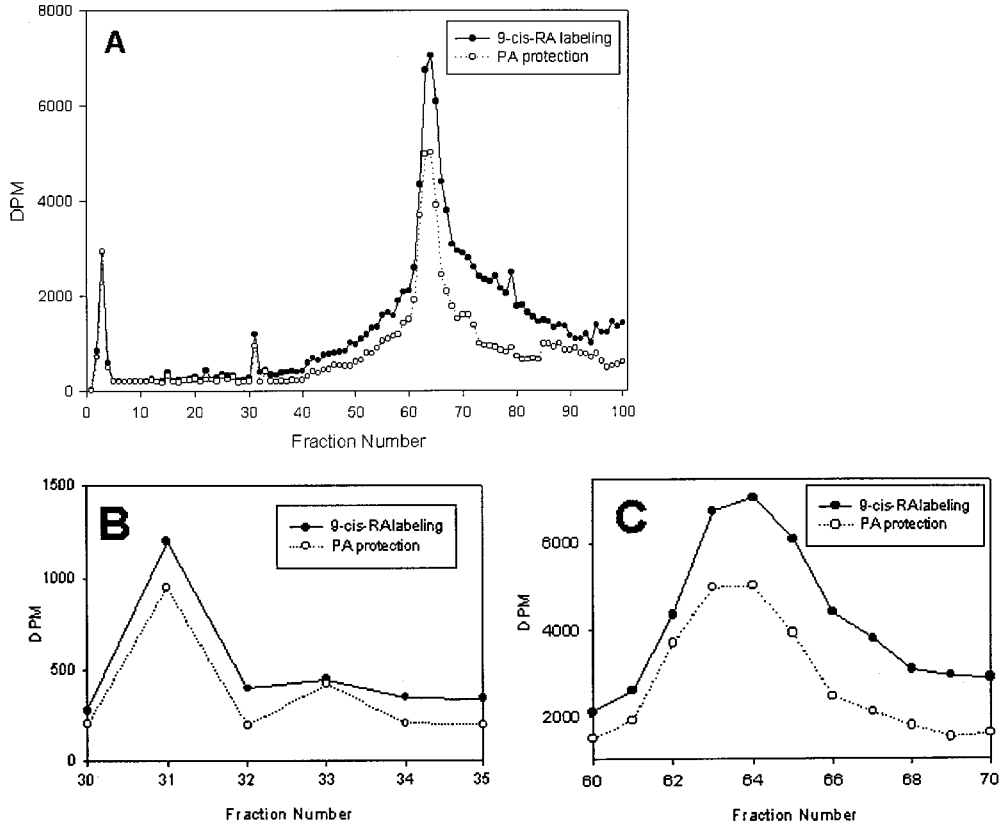

FIGURE 6: HPLC profile for the separation of radioactive peptides. [^{3}H]9-*cis*-RA-labeled RXRβ (150 μg of protein) was preincubated with ethanol (control) or 100 μM PA. Radiolabeled proteins were hydrolyzed with sequencing grade endoproteinase Lys-C. The hydrolyzed peptides were separated on a reversed-phase C18 column. (A) The column eluate was monitored by UV absorption at 215 nm. Fractions of 1 mL (flow rate, 1 mL/min) were collected, and an aliquot of 0.1 mL from each fraction was used for scintillation counting. The fractions at the maximum of the radioactivity peaks in the HPLC profile were 30−32 and 61−66. (B and C) Enlarged profiles of the major and minor peaks.

identification of crucial amino acids in the active sites of these proteins. The application of this technique to characterization of nuclear receptors RXR and RAR is very limited. Thus far, two fluorescent retinoids with the properties of photoaffinity probes have been synthesized and characterized with RARs (*26*). In that study, a tritium-labeled photoreactive retinoid (ADAM-3) was synthesized and used for photoaffinity labeling of recombinant protein MBP-RARα/E, revealing two photoaffinity-labeled sites in the ligand binding domain of RARα. However, no photoaffinity probes have been designed for RXR characterization. In this work, we first validated the method of direct photoaffinity labeling with [^{3}H]9-*cis*-RA and then investigated several retinoids as putative ligands for human recombinant RXRβ. These data demonstrated that the labeling was dependent on UV irradiation, concentration-dependent, and effectively protected by unlabeled 9-*cis*-RA, confirming the presence of a 9-*cis*-RA binding site in the RXRβ molecule. This showed covalent modification of RXRβ protein within the 9-*cis*-RA binding site and provided direct evidence for 9-*cis*-RA binding to RXRβ. atRA, 5,6-epoxy-RA, RAG, and ROH did not compete with 9-*cis*-RA for binding, demonstrating that these retinoids derived from atRA are not accepted as ligands for RXRβ. Moreover, the protection experiments with various retinoids demonstrated that RXRβ exhibits absolute specificity toward only one retinoid, 9-*cis*-RA, and that retinoid derivatives with configurations different from 9-*cis*-RA are not accepted by this nuclear receptor. Experiments with a methylated derivative of 9-*cis*-RA (data not shown) indicated that the presence of a carboxyl function on the ligand was an obligatory requirement for binding to RXRβ. Thus, our data strongly suggest that 9-*cis*-RA binds to RXRβ through a 9-*cis*-RA binding site and this process is highly specific. Our work clearly demonstrates that direct photoaffinity labeling is highly suitable for the identification of new ligands for RXR.

Next, we applied this technique to characterize the ability of RXRβ to interact with nonretinoid ligands, such as fatty acids and bile acids, and the ability of these compounds to compete with 9-*cis*-RA for binding to RXRβ. The rationale for the selection of the competing compounds was based on several recent studies discussed above, which demonstrated the involvement of two unique fatty acids in activation of RXR and PPAR (*9*−*12*). PA and DHA are physiologically important dietary components and were identified as natural ligands for those two receptors. The selection of OA for our studies was based on the fact that this compound was present in the ligand binding pocket of RXRα during its crystallization (*7*). AA is a polyunsaturated fatty acid (24:4), an important natural ligand for PPARs, and a second messenger of several signaling pathways (*27*−*31*). Finally, butyric acid (BA), the simplest fatty acid, is a naturally occurring compound and appears to be an important differentiating agent (*32*). Figure 3 shows that there was a strong competition between 9-*cis*-RA and PA and DHA for binding to RXRβ and no competition with OA and AA. Figure 4 demonstrates that BA, even in 125 μM, did not bind to RXRβ as opposed to the observed concentration-dependent com-

petition of PA which, in 0.2 μM concentration, totally protected against 9-*cis*-RA binding. These results confirmed that PA binds specifically to RXRβ and competes with 9-*cis*-RA for binding and that the postulated binding sites are identical or localized in close proximity. There is no evidence in the literature that RXR is regulated by bile acids. However, with the recent discovery of the interaction of bile acids with SXR and FXR (*20, 21*), the regulation of bile acid synthesis by PPARα (*33*), and the fact that the single most important determinant of RXR specificity appears to be the conformation of the carboxyl group, we have included bile acids as potential ligands for RXRβ. The evaluation of bile acids as potential ligands for RXRβ showed that CDCA and DCA did not bind to RXRβ; however, relatively strong interactions were seen for LCA, and, therefore, the role of LCA as a regulator of RXR must be investigated further.

A particularly intriguing result to emerge from our present studies and those of others (*9*−*12*) is the fact that there are no structural similarities between 9-*cis*-RA and the fatty acid ligands. 9-*cis*-RA and DHA contain several double bonds in their hydrocarbon chains while PA is a fully saturated, branched diterpenoid (see Figure 1). Nevertheless, all of these compounds contain a carboxyl function which is an essential element for binding to amino acids in the ligand binding pocket of RXR. The recent X-ray crystal structure of the ligand binding domain of RXRα (*7*) and site-directed mutagenesis studies with RAR (*34*) revealed that positively charged amino acids are involved in 9-*cis*-RA binding. The demonstration of fatty acids as RXRβ ligands clearly indicates that the active site of this protein has significant potential for adaptation of its active site to accommodate fatty acid ligands.

To further characterize interactions between 9-*cis*-RA and PA within the binding site of RXRβ, we carried out additional experiments in which the protein was preincubated with and without PA, photolabeled with [^{3}H]9-*cis*-RA, and subjected to proteolytic digestion (Figure 6). The radioactive polypeptides from both experiments were separated by HPLC and analyzed. Data presented in Figure 6 clearly demonstrate that only one major radioactive polypeptide was produced by proteolytic digestion of RXRβ radiolabeled by covalently attached [^{3}H]9-*cis*-RA. This labeled polypeptide was obtained in the absence and presence of competing PA. This can be interpreted as both 9-*cis*-RA and PA binding to the same or very closely related amino acids in the RXRβ ligand binding domain. It is most probable that the binding occurs via electrostatic interactions of the carboxyl groups with positively charged amino acids. Similar results were obtained by us with direct photoaffinity labeling and proteolytic digestion of the CRABP which was photolabeled with [^{3}H]-at-RA (*25*). Our first attempts to identify the specific amino acids which bind 9-*cis*-RA and PA were unsuccessful because the RXR protein is not available in sufficient amount and homogeneous form to successfully carry out those experiments. We are currently involved in the purification of RXR and RAR protein for these purposes.

An important question that remains open relates to the possible physiological outcome of the fatty acid ligand binding selectivity of RXR. The two fatty acids that were found to be the highest affinity ligands for this receptor are obtained from the diet. PA, a saturated diterpenoid, is a branched-chain fatty acid which occurs in dairy products, meat, and fish. DHA, a polyunsaturated fatty acid, is a major constituent of the human diet and constitutes up to 50% of total fatty acids in mammalian brain. Since both PA and DHA are provided exclusively by the diet, they may represent essential nutrients that coordinate cellular metabolism through an RXR signaling pathway.

It was recently shown that PA is a naturally occurring ligand for PPARα (*11*). These data suggest the involvement of RXRβ and PPARα in the control of fatty acid metabolism. PPARs, RXRs, and the nuclear hormone receptors have been shown to function as transcripition factors in the control of peroxisomal enzyme expression (*11*). Known activators of these transcription factors include polyunsaturated fatty acids and, for RXRs, 9-*cis*-RA. This could imply that both receptors play a significant role in the disease effects resulting from defective PA catabolism. It has been established that these receptors play a critical role in modulating lipid metabolism by regulating the expression of genes that are involved in lipid storage, lipid catabolism, and adipose differentiation (*35, 36*). PPARα upregulates the expression of genes that encode for enzymes involved in β-oxidation of fatty acids and in proliferation of peroxisomes. PPARγ plays an important role in lipid storage and adipose differentiation. Identification of dietary FAs as ligands for RXRβ also indicates that it has a role in lipid metabolism.

In summary, the present work clearly demonstrates the suitability of photoaffinity labeling for the identification of new ligands. This photoaffinity-based competitive binding assay can also be applied to the characterization of other retinoid binding proteins, such as RAR. Finally, this is the most reliable method for the identification of amino acids in active sites of proteins.

REFERENCES

1. De Luca, L. M. (1991) *FASEB J. 5*, 2924−2933.
2. Gudas, L. J. (1994) *J. Biol. Chem. 269*, 15399−15402.
3. Li, E., and Norris, A. W. (1996) *Annu. Rev. Nutr. 16*, 205−234.
4. Fiorella, P. D., and Napoli, J. L. (1994) *J. Biol. Chem. 269*, 10538−10544.
5. Radominska-Pandya, A., Chen, G., Czernik, P. J., Little, J. M., Samokyszyn, V. M., Carter, C. A., and Nowak, G. (2000) *J. Biol. Chem. 275*, 22324−22330.
6. Chambon, P. (1996) *FASEB J. 10*, 940−954.
7. Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature 375*, 377−382.
8. Renaud, J. P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature 378*, 681−689.
9. Kitareewan, S., Burka, L. T., Tomer, K. B., Parker, C. E., Deterding, L. J., Stevens, R. D., Forman, B. M., Mais, D. E., Heyman, R. A., McMorris, T., and Weinberger, C. (1996) *Mol. Biol. Cell 7*, 1153−1166.
10. Lemotte, P. K., Keidel, S., and Apfel, C. M. (1996) *Eur. J. Biochem. 236*, 328−333.
11. Zomer, A. W., van der Burg, B., Jansen, G. A., Wanders, R. J. A., Poll-The, B. T., and van der Saag, P. T. (2000) *J. Lipid Res. 41*, 1801−1807.
12. de Urquiza, A. M., Liu, S., Sjoberg, M., Zetterstrom, R. H., Griffiths, W., Sjovall, J., and Perlman, T. (2000) *Science 290*, 2140−2144.
13. Sorensen, H. N., Treuter, E., and Gustafsson, J. A. (1998) *Vitam. Horm. 54*, 121−166.
14. Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K., and Rosenfeld, M. G. (1991) *Cell 67*, 1251−1266.
15. Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) *Nature 358*, 771−774.

16. Radominska, A., Treat, S., and Little, J. (1993) *Semin. Liver Dis. 13*, 219−234.
17. Bahar, R. J., and Stolz, A. (1999) *Gastroenterol. Clin. N. Am. 28*, 27−58.
18. Hylemon, P. B., Stravitz, R. T., and Vlahcevic, Z. R. (1994) *Prog. Liver Dis. 12*, 99−120.
19. Makishima, M., Okamoto, A. Y., Repa, J. J., Tu, H., Learned, R. M., Luk, A., Hull, M. V., Lustig, K. D., Mangelsdorf, D. J., and Shan, B. (1999) *Science 284*, 1362−1365.
20. Xie, W., Radominska-Pandya, A., Shi, Y., Simon, C. M., Nelson, M. C., Ong, E. S., Waxman, D. J., and Evans, R. M. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 3375−3380.
21. Sinal, C. J., Tohkin, M., Miyata, M., Ward, J. M., Lambert, G., and Gonzalez, F. J. (2000) *Cell 102*, 731−744.
22. Samokyszyn, V. M., Gall, W. E., Zawada, G., Freyaldenhoven, M. A., Chen, G., Mackenzie, P. I., Tephly, T. R., and Radominska-Pandya, A. (2000) *J. Biol. Chem. 275*, 6908−6914.
23. Becker, B., Barua, A. B., and Olson, J. A. (1996) *Biochem. J. 314*, 249−252.
24. Bernstein, P. S., Choi, S. Y., Ho, Y. C., and Rando, R. R. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 654−658.
25. Chen, G., and Radominska-Pandya, A. (2000) *Biochemistry 39*, 12568−12574.
26. Sasaki, T., Shimazawa, R., Sawada, T., Iljima, T., Fukasawa, H., Shudo, K., Hashimoto, Y., and Iwasaki, S. (1996) *Biol. Pharm. Bull. 19*, 659−664.
27. Ford, S. R., Chenault, K. D., Hall, M. S., Pangburn, S. J., and Leach, F. R. (1994) *Arch. Biochem. Biophys. 314*, 261−267.
28. Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J., and Wahli, W. (1996) *Nature 384*, 39−43.
29. Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Wilson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997) *Proc. Natl. Acad Sci. U.S.A. 94*, 4318−4323.
30. Lin, Q., Ruuska, S. E., Shaw, N. S., Dong, D., and Noy, N. (1999) *Biochemistry 38*, 185−190.
31. Krey, G., Braissant, A., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M. G., and Wahli, W. (1997) *Mol. Endocrinol. 11*, 779−791.
32. Chen, Z. X., and Treitman, T. R. (1994) *Cancer Res. 54*, 3494−3499.
33. Hunt, M. C., Yang, Y., Eggertsen, G., Carneheim, C. M., Gafvels, M., Einarsson, C., and Alexson, S. E. H. (2000) *J. Biol. Chem. 275*, 28947−28953.
34. Zhang, Z. P., Gambone, C. J., Gabriel, J. L., Wolfgang, C. L., Soprano, K. J., and Soprano, D. R. (1998) *J. Biol. Chem. 273*, 34016−34021.
35. Lemberger, T., Desvergne, B., and Wahli, W. (1996) *Annu. Rev. Cell Dev. Biol. 12*, 335−363.
36. Schoonjans, K., Staels, B., and Auwex, J. (1996) *J. Lipid Res. 37*, 907−925.

BI0121151