Functional Consequences of Cysteine Modification in the Ligand Binding Sites of Peroxisome Proliferator Activated Receptors by GW9662

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Received November 19, 2001; Revised Manuscript Received March 19, 2002

ABSTRACT: In the course of a high throughput screen to search for ligands of peroxisome proliferator activated receptor-γ (PPARγ), we identified GW9662 using a competition binding assay against the human ligand binding domain. GW9662 had nanomolar IC_{50} versus PPARγ and was 10- and 600-fold less potent in binding experiments using PPARα and PPARδ, respectively. Pretreatment of all three PPARs with GW9662 resulted in the irreversible loss of ligand binding as assessed by scintillation proximity assay. Incubation of PPAR with GW9662 resulted in a change in the absorbance spectra of the receptors consistent with covalent modification. Mass spectrometric analysis of the PPARγ ligand binding domain treated with GW9662 established Cys^{285} as the site of covalent modification. This cysteine is conserved among all three PPARs. In cell-based reporter assays, GW9662 was a potent and selective antagonist of full-length PPARγ. The functional activity of GW9662 as an antagonist of PPARγ was confirmed in an assay of adipocyte differentiation. GW9662 showed essentially no effect on transcription when tested using both full-length PPARδ and PPARα. Time-resolved fluorescence assays of ligand-modulated receptor heterodimerization, coactivator binding, and corepressor binding were consistent with the effects observed in the reporter gene assays. Control activators increased PPAR:RXR heterodimer formation and coactivator binding to both PPARγ and PPARδ. Corepressor binding was decreased. In the case of PPARα, GW9662 treatment did not significantly increase heterodimerization and coactivator binding or decrease corepressor binding. The experimental data indicate that GW9662 modification of each of the three PPARs results in different functional consequences. The selective and irreversible nature of GW9662 treatment, and the observation that activity is maintained in cell culture experiments, suggests that this compound may be a useful tool for elucidation of the role of PPARγ in biological processes.

The peroxisome proliferator activated receptors (PPARs)[1] are members of the steroid−thyroid hormone superfamily of ligand-activated transcription factors. To date, three human PPARs termed PPARα (NR1C1), PPARγ (NR1C3), and PPARδ (NR1C2) have been described (*1*). Evidence implicating roles for these receptors in control of glucose and lipid metabolism has been recently reviewed (*2*−*6*).

PPARγ is highly expressed in adipose tissue where it is a key regulator of adipocyte gene expression and cell differentiation (*7*). Genetic ablation of PPARγ leads to embryonic lethality in mice due to a defect in placental development (*8*−*10*). However, a study of a PPARγ null mouse rescued via placental reconstitution (*9*) and of chimeric PPAR$\gamma^{+/+}$-PPAR$\gamma^{-/-}$ mice (*11*) demonstrated that PPARγ is required for adipocyte differentiation in vivo. The PPARs bind to specific DNA response elements, termed DR-1, as heterodimers with RXR. These PPAR response elements consist of a direct repeat of the hexanucleotide sequence AGGTCA separated by a single nucleotide. (*12*). The binding of activator ligands has been shown to increase PPARα:RXRα and PPARδ:RXRα heterodimer formation (*13*)

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[1] Abbreviations: APC, allophycocyanin; AR, androgen receptor; BSA, bovine serum albumin; GW9662, 2-chloro-5-nitrobenzanilide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CBP, CREB binding protein; 15d-PGJ_2, 15-deoxy $\Delta^{12,14}$-prostaglandin J_2; DTT, dithiothreitol; Erβ, estrogen receptor β; EDTA, ethylenediaminetetraacetic acid; FXR, farnesoid X receptor; GR, glucocorticoid receptor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; LBD, ligand binding domain; LCMS, liquid chromatography−mass spectrometry; L-FABP, liver fatty acid binding protein; LXR, liver X receptor; nanoES, nanoelectrospray; N-CoR, nuclear receptor co-repressor; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator activated receptor response element; PBS, phosphate-buffered saline; PXR, pregnane X receptor; PR, progestin receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SPA, scintillation proximity assay; SPAP, secreted placental alkaline phosphatase; TZD, thiazolidinedione; TR, thyroid receptor; Tris, tris(hydroxymethyl)aminomethane.

10.1021/bi0159581 CCC: $22.00
Published on Web 04/30/2002

Similarly, time-resolved fluorescence resonance energy transfer assays have demonstrated that both TZDs and farglitazar (GI262570) increase PPARγ:RXRα heterodimer formation (*14*). Comparison of the crystal structures of rosiglitazone bound to PPARγ (*15*) and to PPARγ:RXRα heterodimer (*16*) showed that the conformation of the bound ligand differed in the two complexes (*16*).

Additionally, regulation of gene transcription via ligand binding to nuclear receptors involves specific accessory proteins termed coactivators and corepressors. Agonist-induced conformation changes result in increased coactivator and decreased corepressor association with receptor (*17*−*20*). Short sequence motifs that mediate both coactivator (*21*) and corepressor (*22*) binding have been identified. Synthetic peptides based on the sequence of the natural coactivator motif retain the ability to bind to receptor in a ligand-modulated fashion, and ligand-sensing assays that monitor changes in the receptor−coactivator complex using both coactivator proteins (*23*, *24*) and peptides (*14*, *25*) have been reported.

The discovery of high-affinity synthetic ligands for the PPARs has led to rapid progress in understanding the physiological roles of these orphan receptors in lipid and glucose metabolism (*26*). Members of the TZD class of antidiabetic agents bind and activate PPARγ, and their potency in rodent models of Type 2 diabetes is correlated with the potency of PPARγ activation (*27*, *28*). Farglitazar, a non-TZD tyrosine-based (*29*−*31*) compound, which was optimized for PPARγ binding and activation, has been described as a potent anti-diabetic agent in humans (*32*). Naturally occurring PPAR ligands include dietary fatty acids (*33*, *34*) and eicosinoid metabolites, such as 15d-PGJ_2 (*35*), which exhibit binding affinities in the micromolar range. However, the physiological relevance of these low-affinity ligands remains unknown. In particular, the biological effects of 15d-PGJ_2 may be due to its inhibition of NF-κB signaling pathways as well as activation of PPARγ (*36*, *37*).

Study of the role of PPARγ in multiple cellular processes has been facilitated by the availability of high-affinity ligands. The diverse classes of ligands mentioned above are all activators of PPARγ. Identification of PPARγ antagonists would provide powerful tools to study PPARγ signaling pathways. We have described GW0072, a partial agonist of PPARγ that inhibits both rosiglitazone-induced activation of PPARγ and adipocyte differentiation (*38*). However, no potent antagonists of PPARγ have been described to date. In this report, the discovery and characterization of GW9662, a potent antagonist of PPARγ, is described. GW9662 covalently modifies a cysteine residue in the ligand binding site of PPARγ. L-764406 has previously been described as a covalent partial agonist of PPARγ (*39*). By contrast, GW9662 acts as full antagonist in both cell-based and cell-free assays. Evidence is presented that GW9662 also covalently modifies both PPARδ and PPARα at concentrations significantly higher than those required for inhibition of PPARγ.

MATERIALS AND METHODS

Synthesis of GW9662 (2-Chloro-5-nitrobenzanilide). To a stirred solution of 2-chloro-5-nitrobenzoyl chloride (5.03 g, 22.9 mmol) and triethylamine (3.51 mL, 25.1 mmol) in CH_2Cl_2 maintained under nitrogen at 0 °C was added dropwise aniline (2.19 mL, 24.0 mmol). The resulting solution was stirred for 5 min at 0 °C and then for 15 min at room temperature. This solution was then diluted with EtOAc (300 mL) and washed sequentially with 1.0 M HCl, water, 1.0 M $NaHCO_3$, and brine (100 mL each). The organic solution was then dried over $MgSO_4$ and concentrated by rotary evaporation to give a light yellow solid (5.32 g) which was recrystallized from EtOAc to provide the title compound as a white solid (3.34 g, 53%): mp 155−156 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.63 (d, 1H, J = 2.7), 8.28 (dd, 1H, J = 2.7, 8.9), 7.81 (br s, 1H) 7.68−7.63 (m, 3H), 7.42 (t, 2H, J = 7.9), 7.23 (t, 1H, J = 7.5); MS (ES^-) *m*/*e* 275.1 ($M-H)^-$; Anal. Calcd. for $C_{13}H_9Cl_1N_2O_3$: C, 56.43; H, 3.28; N, 10.13; Found: C, 56.33; H, 3.30; N, 10.03.

Protein Expression. CBP (residues 57−454) and the LBDs of human PPARα (residues 192−468), PPARγ (residues 195−497), PPARδ (residues 137−441), RXRα (225−462), and ERβ (residues 205−485) were expressed in *E. coli* strain BL21(DE3) as amino-terminal polyhistidine-tagged fusion proteins. Expression was under the control of an IPTG-inducible T7 promoter. DNA encoding for recombinant protein was subcloned into the pRSET-A expression vector (Invitrogen). The sequence of the polyhistidine tag was fused in-frame to the sequence coding for each of the proteins.

Conditions for expression of each of the proteins were as follows: PPARα was typically grown in 10 L fermentation batches in Rich Phosphate media containing 0.2% glucose and 0.1 mg/mL ampicillin at 25 °C for 16−20 h, to an OD_{600} of 5. The culture was cooled to 10 °C, followed by the addition of IPTG to 0.25 mM and glucose to 0.2%. Induction proceeded for 8−10 h at 10 °C, to a final OD_{600} of 6−7. For PPARδ, cells were grown in 2× LB media with VB salts containing 0.1% glycerol and 0.1 mg/mL ampicillin at 37 °C for 2 h, to an OD_{600} of 0.3. The culture was cooled to 20 °C (over 3−4 h), and growth proceeded (no induction, leaky expression) for 8−10 h at 20 °C, to a final OD_{600} of 24−30. Ten liter fermentation batches for PPARγ were grown in 2× YT media with 0.1 mg/mL ampicillin at 30 °C for approximately 3 h, to an OD_{600} of 0.6. Then 0.25 mM IPTG was added and induction proceeded for 3−5 h at 30 °C, to a final OD_{600} of 5−6. RXRα cells were grown in Rich Phosphate media with 0.1 mg/mL ampicillin at 25 °C for 12 h. The culture was cooled to 9 °C and growth continued for 36 h (OD_{600} = 14); 0.25 mM IPTG was added and induction proceeded for 24 h to a final OD_{600} of 16. Cells expressing ERβ were grown in 2× LB media with 0.1 mg/mL ampicillin at 25 °C, and growth proceeded (no induction, leaky expression) for 12−14 h at 20 °C, to a final OD_{600} of 5−6. For CBP, cells were grown in LB media with 0.1 mg/mL ampicillin at 22 °C to an OD_{600} of 14 (16 h). Then 0.25 mM IPTG was added and induction proceeded for 4 h to a final OD_{600} of 16.

Cells were harvested by centrifugation (20 min, 3500*g*, 4 °C), and concentrated cell slurries were either stored in PBS at −80 °C or processed immediately.

Protein Purification. Typically, an aliquot of the appropriate cell paste obtained from 3−5 L of cell culture was thawed and resuspended in 200−300 mL of buffer. Cells were lysed, and cell debris was removed by centrifugation (40 min, 22000*g*, 4 °C). The supernatant was filtered through coarse pre-filters and diluted 4-fold with buffer, and imidazole was

added to a final concentration of 50 mM. The buffers used were as follows: PBS for PPARα, PPARγ, and PPARδ; 25 mM Tris, pH 7.2, 150 mM NaCl for RXRα; and 50 mM HEPES, pH 7.5, 150 mM NaCl, 5% 1,2-propanediol for ERβ. A protease inhibitor mixture comprised of micromolar APMSF, aprotinin, bestatin, leupeptin, and pepstatin was added to the lysis buffers except in the case of RXRα. In each case, the lysate was loaded onto a column (6 × 8 or 3 × 8 cm) packed with Sepharose [Ni^{2+} charged] Chelation resin (Pharmacia) and preequilibrated with the appropriate buffer containing 50 mM imidazole. The column was washed with buffer to remove unbound protein. After the column flow-through returned to baseline absorbance, the column was developed with a linear gradient of 50−500 mM imidazole in buffer. Column fractions were pooled and concentrated using Centri-prep 10K (Amicon). The concentrated protein was subjected to size exclusion chromatography, using a column (3 × 90 cm) packed with Sepharose S-75 resin (Pharmacia), which was preequilibrated with the appropriate buffer.

Protein Biotinylation. A similar biotinylation protocol was developed for all of the proteins. Purified protein was buffer-exchanged into PBS, and the protein concentration was adjusted to 10−20 μM with PBS. A 5-fold molar excess of NHS-LC−Biotin (Pierce) was added in a minimal volume of PBS. This solution was incubated with occasional gentle mixing for 30−60 min at ambient temperature. The reaction was terminated by the addition of a 2000-fold molar excess of Tris-HCl, pH 8. The modified LBD was buffer-exchanged into PBS containing 5 mM DTT, 2 mM EDTA, and 2% (w/v) sucrose. Biotinylated LBD was subjected to mass spectrometric analysis to reveal the extent of modification by the biotinylation reagent. Optimally, approximately 95% of the protein had at least a single site of biotinylation; and the overall extent of biotinylation followed a normal distribution of multiple sites, ranging from one to nine. Minimally, the proteins were biotinylated such that 50% of the protein was modified at a single site.

Binding Assays. SPAs for all three PPARs were performed as previously described for PPARγ (*40*). In brief, the human PPARα, PPARγ, and PPARδ ligand binding domains (LBDs) were expressed in *E. coli* as polyhistidine-tagged fusion proteins. Receptors were immobilized on SPA beads (Amersham Pharmacia) by addition of the desired receptor (15 nM) to a slurry of streptavidin-modifed SPA beads (0.5 mg/mL) in assay buffer. The mixture was allowed to equilibrate for at least 1 h at room temperature, and the beads were pelleted by centrifugation at 1000*g*. The supernate was discarded, and the beads were resuspended in the original volume of fresh assay buffer with gentle mixing. The centrifugation/resuspension procedure was repeated, and the resulting slurry of receptor-coated beads was used immediately or stored at 4 °C for up to 1 week before use. [^{3}H]GW2331 (*33*), [^{3}H]rosiglitazone (*28*), and [^{3}H]GW2443 (*41*) were used as radioligands for determination of competition binding to PPARα, PPARγ, and PPARδ, respectively. Unless otherwise indicated, the buffer used for all assays was 50 mM HEPES (pH 7), 50 mM NaCl, 5 mM CHAPS, 0.1 mg/mL BSA, and 10 mM DTT. For some experiments, the HEPES (pH 7) was replaced with 50 mM Tris (pH 8).

Ultraviolet−Visible Spectroscopy. Spectra were collected using a Shimadzu UV2100U spectrophotometer. All spectra were recorded at 25 °C in quartz cuvettes. Further details are given in the legend of Figure 2.

Preparation of GW9662-Modified PPARγ for Mass Spectral Analysis. A stock solution of GW9662 in dimethyl sulfoxide was added to a 20 μM solution of PPARγ in 50 mM Tris (pH 8), 50 mM NaCl, 5 mM CHAPS, 0.1 mg/mL BSA, and 10 mM DTT. The final concentration of GW9662 was 40 μM. The solution was incubated at 4 °C followed by mass spectral analysis as described below.

Protein Mass Measurement. Mass measurement of intact protein was determined using LCMS. A 250 μm i.d. × 15 cm Poros R2/H column (Perceptive Biosystems, Framingham, MA), packed in-house, was used to desalt the protein. Mobile phases were (A) water with 0.05% trifluoroacetic acid and (B) 90% acetonitrile (aq.) with 0.035% trifluoroacetic acid. Ten microliters of the mixture was injected onto the column. The protein was eluted using a gradient of 15% B to 65% B in 20 min, followed by a hold at 65% B. The mobile phases were delivered with a HP 1090 chromatograph (Hewlett-Packard, Palo Alto, CA) at a flow rate of 0.15 mL/min. A splitter was used to give a flow rate through the column of 40 μL/min. The mobile phase was coupled to a SCIEX API I mass spectrometer (PE Sciex, Concord, ON, Canada) equipped with a pneumatically assisted electrospray (ion spray) interface. Spectra were acquired in the positive ion mode. Full scans were obtained over the mass range 900−1400 Da scanned with a 0.2 Da step and a dwell time of 1 ms. Operating voltages were 5000 V needle voltage (ISV), 70 V orifice voltage (OR), and 30 V Qo voltage (RO).

Glu-C Digestion of GW9662-Modified PPARγ. Prior to digestion, unbound ligand was separated from the modified PPARγ using the chromatographic conditions described previously. The protein fraction was collected and concentrated to approximately 4 μL on a SpeedVac. The concentrated PPARγ fraction was then diluted to 50 μL with 0.1 M ammonium bicarbonate, pH 8.0. Next, 1.5 μL of a 2 μg/μL Glu-C solution was added to the protein solution. The digest proceeded at room temperature. After 12 h, an additional aliquot (0.5 μL) of the Glu-C stock was added to the digest. The digestion was stopped by freezing the sample at −20 °C. The resulting sample was stored frozen until analysis by nanoelectrospray mass spectrometry.

Nanoelectrospray Mass Spectrometry. MS and MS/MS spectra were obtained on a SCIEX API III fitted with a nanoES ion source (*42*). Prior to mass spectrometric analysis, an aliquot of the Glu-C digest was desalted/preconcentrated using a micro-column made by packing 200−400 nL of Poros 50 R2 into a fritted gel-loader pipet tip. The procedure has been described by Blackburn (*43*) and Erdjument-Bromage et al. (*44*). Briefly, 10 μL of the digest was loaded onto the Poros R2 micro-column and desalted by washing with 150−200 μL of 1% formic acid. Next, the peptides were eluted in 1.5 μL of methanol/water/formic acid (70:25:5) directly into a spraying capillary for nanoES analysis.

Preparation of Europium Chelate-Labeled PPARs. Fluorescently labeled PPARs were prepared by incubation of the desired LBD with an equimolar concentration of LANCE europium-labeled (W8044) streptavidin (Perkin-Elmer Life Sciences) in 50 mM Tris (pH 8), 50 mM NaCl, 1 mM CHAPS, 1 mM EDTA, 0.1 mg/mL BSA, and 10 mM DTT. After a 30 min incubation at room temperature, excess biotin

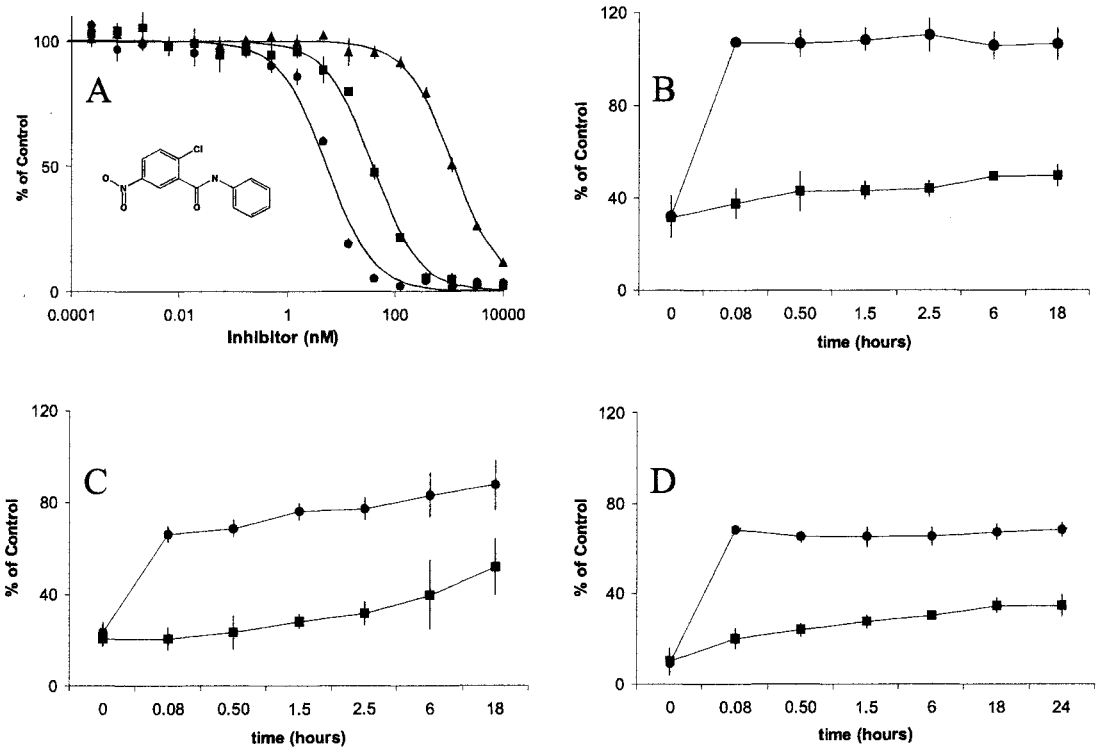

FIGURE 1: Inhibition of ligand binding to PPARs by GW9662. (A) GW9662 inhibition of radioligand binding to PPARγ (●), PPARα (■), and PPARδ (▲) was determined by scintillation proximity assay. A typical experiment is shown. Data points are the mean of triplicate determinations. IC_{50}s determined from a linear least-squares fit of the data (solid lines) assuming simple competitive inhibition were 5.4 ± 0.6 nM (pIC_{50} = 8.27) for PPARγ, 39 ± 4 nM (pIC_{50} = 7.41) for PPARα, and 1.2 ± 0.1 μM (pIC_{50} = 5.92) for PPARδ, respectively. (B) PPARγ immobilized on SPA beads was incubated with 1 μM GW9662 (■) or rosiglitazone (●) for 1 h; pretreating ligand was removed by two sequential steps of centrifugation followed by resuspension of the beads in fresh buffer. Recovery of binding was assessed by addition of [^{3}H]rosiglitazone to an aliquot of the washed bead suspension followed by scintillation proximity assay to monitor the recovery of radioligand binding as a function of time. (C) Recovery of PPARα binding after treatment with 1 μM GW9662 (■) or GW2331 (●). The method was performed as described in panel B except that the radioligand used was [^{3}H]GW2331. (D) Recovery of PPARδ binding after treatment with 1 μM GW9662 (■) or GW2433 (●). The method was performed as described in panel B except that the radioligand used was [^{3}H]GW2433.

was added to block any residual unoccupied biotin binding sites, and incubation was continued for a further 30 min.

N-CoR Peptide. A biotinylated peptide corresponding to amino acids 2251−2275 of human N-CoR was synthesized by SynPep. The peptide sequence was biotin-HN-GHSFAD-PASNLGLEDIIRKALMGSF-$CONH_2$.

Preparation of Allophycocyanin-Labeled RXRα, CBP, and N-CoR. APC-labeled RXRα was prepared by incubation of equimolar concentrations of biotinylated RXRα LBD and APC-labeled streptavidin (Molecular Probes, Eugene, OR). After a 30 min incubation at room temperature, excess biotin was added to block any residual unoccupied biotin binding sites, and incubation was continued for a further 30 min. APC-labeled CBP and N-CoR peptide were prepared in the same manner.

Ligand-Induced Receptor Heterodimerization and Receptor:Cofactor Complex Formation. The desired concentrations of europium-labeled receptor, APC-labeled RXRα, CBP, or N-CoR and ligand were mixed in individual wells of 96 well plates. The plates were incubated for at least 3 h at room temperature. Samples were protected from light during the incubation period. Time-resolved fluorescence intensities were determined in a Victor 1420 Multilabel Counter. Plots of fluorescence intensity versus ligand concentration were constructed. Further details are given in the legend of Figure 5.

Cell-Based Reporter Assays. The ability of GW9662 to activate PPAR-mediated reporter gene transcription was assessed using GAL4 chimeras of the human receptors and a $(UAS)_5$-tk-SPAP reporter plasmid as previously described for PPARγ (*28*), PPARα (*33*), and PPARδ (*41*). GW9662 antagonism of ligand-induced gene transcription was measured as previously described (*38*). Antagonism of agonist-induced reporter gene transcription was done by titrating varying concentrations of GW9662 in the presence of a constant concentration of activating ligand. The activating ligands used were 100 nM rosiglitazone for PPARγ, 8 nM GW7647 for PPARα, and 0.55 μM GW2433 for PPARδ, respectively.

The effects of GW9662 on activation of PPARγ, PPARα, and PPARδ were also assessed using full-length human receptors and a reporter construct, $(L\text{-}FABP)_4$-tk-Dual-LUC, containing four copies of the L-FABP PPRE upstream of the minimal herpesvirus thymidine kinase promoter and a luciferase reporter gene. The receptor plasmids contained the appropriate full-length PPAR cDNA plus 9 base pairs of Kozak consensus sequence cloned into the TOPO site of pcDNA3.1-TOPO (Invitrogen). Briefly, HEK293 cells were cultured in Minimum Essential Media (Life Technologies) containing 10% fetal calf serum, 1% penicillin/streptomycin, and 1% fungizone in a humidified incubator (5% CO_2 in air) at 37 °C. The cells were seeded at 2×10^4 cells per

well in 96 well culture plates the day prior to assay execution. Transfection was accomplished using PolyFect (Qiagen) according to the manufacturers' instructions. Transfection mixtures for each well contained 0.167 μg of PPAR plasmid, 0.167 μg of LFABP reporter, and 0.167 μg of a *renilla* luciferase plasmid as transfection control. Cells were incubated with the transfection mixture for 5 h before treatment with either compound or vehicle for 48 h. Culture plates were assayed using the Dual Luciferase assay system (Promega) according to the manufacturers' instructions.

Activation of other nuclear receptors by GW9662 was assessed using GAL4 chimeras of the LBDs as previously described (*45*). All determinations were performed in quadruplicate, and the statistical significance of activation both by an appropriate standard and by GW9662 with respect to control was assessed with Dunnett's multiple comparisons test using Version 4.04 of JMP Statistical Discovery Software (SAS Institute, Cary, NC).

Adipogenesis Assays. C3H10T1/2 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Subconfluent cultures were treated with GW9662 or rosiglitazone, and PPARγ-mediated ligand-induced differentiation was assessed by Oil Red O staining and analysis of aP2 expression by northern analysis as previously described (*28*, *38*, *46*, *47*).

RESULTS

GW9662 Is a Selective Ligand for PPARγ. The use of a scintillation proximity assay to identify potential ligands for PPARγ resulted in the identification of GW9662. Analysis of the *apparent* binding affinity of this compound using standard SPA showed that GW9662 inhibited radioligand binding to PPARγ, PPARα, and PPARδ with pIC_{50}s of 8.48 $\pm$ 0.27 (IC_{50} = 3.3 nM; n = 10), 7.49 $\pm$ 0.17 (IC_{50} = 32 nM; n = 9), and 5.69 $\pm$ 0.17 (IC_{50} = 2000 nM; n = 3), respectively. As will be discussed below, GW9662 irreversibly inhibits binding to all three PPARs. As a result, the observed concentrations of GW9662 that resulted in half-maximal inhibition of radioligand binding do not reflect equilibrium binding constants and are therefore referred to as pIC_{50} rather than as pK_i.

GW9662 Irreversibly Inhibits Ligand Binding to PPARs. The structure of GW9662 suggested the possibility that it had the potential to form a covalent adduct with nucleophilic amino acid side chains.

To examine the possibility of irreversible modification of the PPAR subtypes, the individual biotinylated receptors immobilized on streptavidin-coated SPA beads were exposed either to vehicle, 1 μM GW9662, or to a control inhibitor. Rosiglitazone, GW2331, and GW2433 were used as the controls for PPARγ, PPARα, and PPARδ, respectively. Following incubation for 1 h, free GW9662 was removed from the immobilized receptors, and the appropriate radioligand was added. Recovery of binding as a function of time was assessed by SPA. When PPARγ was treated with GW9662 in this manner, persistent inhibition of [^{3}H]-rosiglitazone binding to the receptor was observed (Figure 1B). Little recovery of binding activity was observed at times up to 24 h after removal of GW9662, suggesting that the inhibition was irreversible. In contrast, full receptor binding activity was recovered in parallel experiments where the receptor was incubated with rosiglitazone instead of GW9662 (Figure 1B).

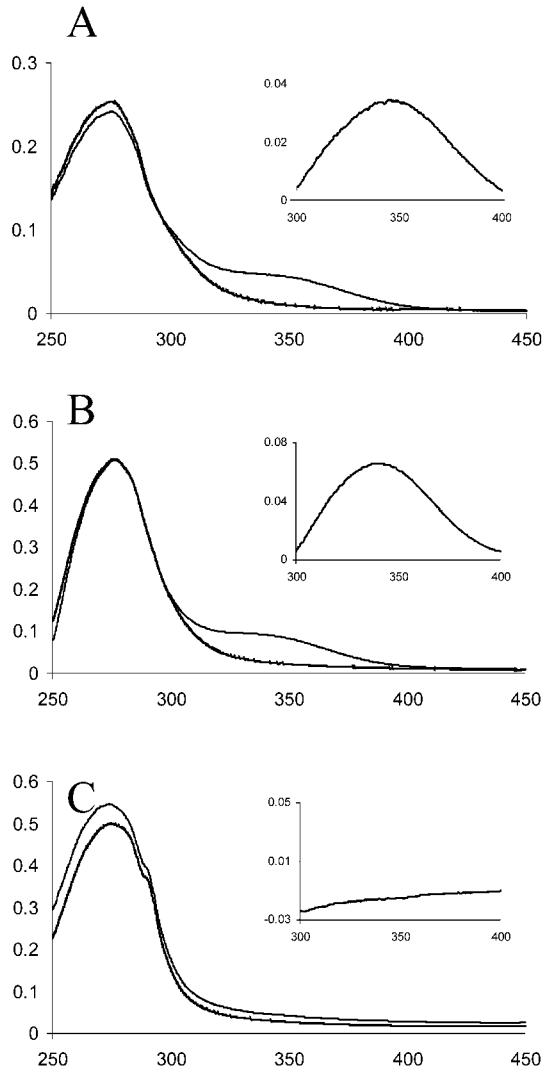

FIGURE 2: Absorbance changes associated with GW9662 treatment. For each panel, spectra of the indicated concentrations of receptor protein and GW9662 were obtained separately. Backgrounds were subtracted, and the two spectra were added together to give a composite spectrum for the unreacted sample (lower curve in each panel). GW9662 was then added to each receptor sample, and a spectrum of the treated receptor was obtained (upper curves). (A) 5 μM PPARγ and 5 μM GW9662; (B) 10 μM PPARδ and 10 μM GW9662; (C) 10 μM ERβ and 10 μM GW9662.

As was observed for PPARγ, GW9662 inhibition of radioligand binding to both PPARα and PPARδ persisted after removal of pretreating ligand. Figure 1C shows that [^{3}H]GW2331 binding to PPARα increased to 66% of the untreated control level immediately after removal of unlabeled GW2331. In contrast, rapid recovery of binding was not observed for PPARα exposed to GW9662. Only a slow upward drift was observed, and the slope of this increase paralleled that for the GW2331 control.

Although GW9662 was a much weaker inhibitor of radioligand binding to PPARδ than to either PPARγ or PPARα (Figure 1A), irreversible inhibition of [^{3}H]2433 binding was observed when GW9662 was incubated with PPARδ (Figure 1D). Thus, treatment of all three PPAR subtypes with GW9662 resulted in irreversible inhibition of ligand binding.

Covalent Modification of PPAR by GW9662. Covalent modification of proteins with nitro aryl halides such as

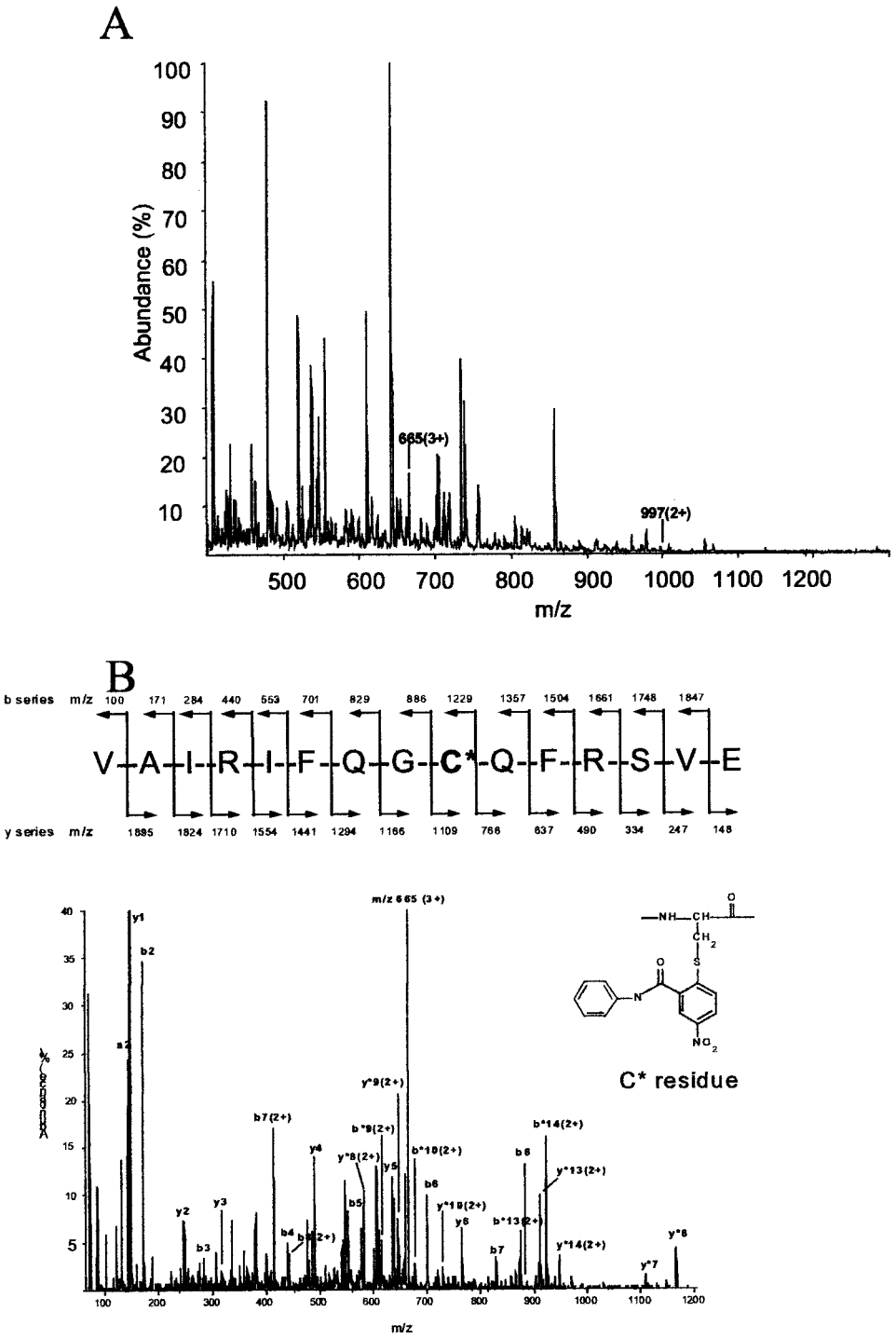

FIGURE 3: Mass spectral analysis of GW9662-modified PPARγ LBD. (A) Nanoelectrospray analysis of Glu-C digests of PPARγ treated with GW9662. The ions indicated at *m*/*z* 665 and 997 correspond to multiple charged states of the peptide modified by GW9662. (B) MS/MS fragment ion spectrum of the *m*/*z* 665 species. Peaks corresponding to the ions resulting from fragmentation of the shown sequence are indicated. The sequence of the *m*/*z* 665 species derived from this study was VARIFQ**C***QFRSVE. The inset shows the proposed structure of C*, cysteine modified by reaction with GW9662.

dinitrofluorobenzene has been shown to result in an increase in absorption at 330−360 nm (*48*, *49*). As shown in Figure 2A, treatment of 5 μM PPARγ LBD with 1 equiv of GW9662 resulted in an increase in absorbance centered at 350 nm. The absorbance change was rapid; the maximal increase occurred in the time required to mix the sample and begin recording of the spectrum. A rapid increase in absorbance at ~350 nm was also observed on treatment of PPARδ with GW9662 (Figure 2B). The magnitude of this change (0.0066/μM) was similar to that observed for modification of PPARγ (0.0067/μM), suggesting that the extent of modification of both proteins was similar. A similar result was obtained when PPARα was treated with GW9662 (data not shown). The magnitude of absorbance changes indicated that the >600-fold difference in apparent potency of GW9662 observed in the competition binding assays to the PPARs was likely not due to differences in the extent of protein modification. In contrast to the results observed with the PPARs, no change in absorbance was detected on treatment of the LBD of the ERβ with GW9662 (Figure 2C).

To conclusively demonstrate that treatment with GW9662 resulted in covalent modification of PPARγ, a sample of receptor was exposed to GW9662 and subjected to high-pressure liquid chromatography−electrospray ionization mass spectrometry. A second, untreated sample of receptor served as control. Untreated receptor gave a molecular mass of 32 542 Da, consistent with the value calculated based on the known amino acid sequence of the receptor construct used. In contrast, receptor treated with GW9662 gave a molecular mass of 32 784 Da (not shown). The observed difference of 242 Da corresponds to the molecular mass of GW9662 minus HCl.

GW9662 Modifies Cys of PPARγ. Samples of GW9662-modified PPARγ were subjected to digestion by the proteolytic enzyme Glu-C. Figure 3A shows the mass spectrum obtained from nanoES analysis of the Glu-C digest. Analysis of the spectrum confirmed the presence of all peptides predicted upon Glu-C proteolysis of PPARγ except for a peptide of sequence VARIFQCQFRSVE (not shown). The ions at *m*/*z* 665 and 997 are multiple charged states of a peptide that weighs 1993 Da, which corresponds to 240 Da greater than the predicted mass of the peptide of sequence VARIFQGCQFRSVE. This peptide contains the single cysteine residue in the PPARγ LBD sequence. Therefore, the presumed site of GW9662 modification was tentatively assigned as Cys^{285}. To confirm this assignment, MS/MS analysis of the *m*/*z* 665 species was performed. Figure 3B shows the resulting fragment ion spectrum from the collision-induced dissociation of the *m*/*z* 665 ion. The results indicate that the site of GW9662 modification was Cys^{285}.

Functional Activity of GW9662. Concentration−response analysis of GW9662 in cell-based functional assays for the three PPAR subtypes was performed using the previously described GAL4 chimera system. GW9662 failed to significantly activate PPARγ-GAL4-mediated transcription. By contrast, GW9662 antagonized the response elicited by 100 nM rosiglitazone with an apparent IC_{50} of 7.6 nM, a value in good agreement with the apparent IC_{50} obtained in the binding assay (Figure 4A). The rosiglitazone response was inhibited essentially to baseline levels, indicating that GW9662 acted as a full antagonist of PPARγ.

GW9662 acted as a partial agonist of the PPARα (LBD)-GAL4 chimeric receptor with an EC_{50} of 22 nM, and a maximal response equal to 42% of that observed for the agonist GW7647 (Figure 4B). Consistent with this partial agonist profile on the chimeric receptor, GW9662 was also able to inhibit agonist-induced reporter activity by 63% with an IC_{50} of 630 nM, a value an order of magnitude weaker than the apparent IC_{50} determined by competition binding.

No significant activation of the PPARα (LBD)-GAL4 chimeric receptor was observed at concentrations of GW9662 up to 10 μM (Figure 4C). However, GW9662 was an antagonist of the PPARδ (LBD)-GAL4 chimeric receptor with an IC_{50} of 4.1 μM, in good agreement with the IC_{50} of 2 μM measured by SPA.

To assess the potential activity of GW9662 in native cells, its functional activity was determined on the full-length PPARs using a reporter gene containing four copies of the PPRE from the L-FABP promoter. As was observed for the experiments with LBD, GW9662 was a full antagonist of the full-length PPARγ, inhibiting the activation induced by rosiglitazone with an IC_{50} of ~1 nM (Figure 4B, inset).

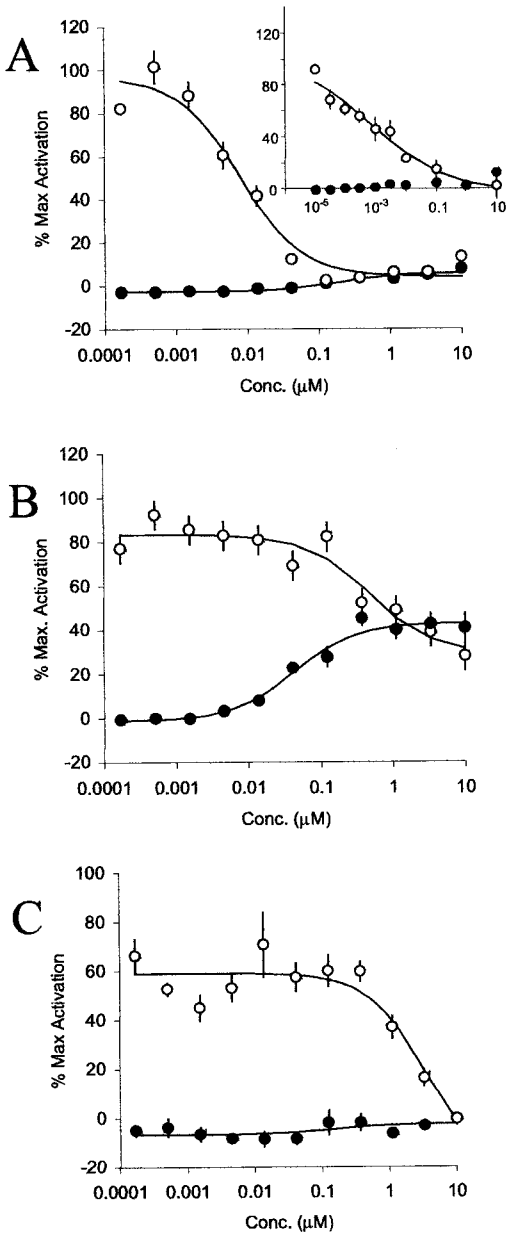

FIGURE 4: Functional analysis. (A) GW9662 antagonizes the activation of GAL-4 PPARγ induced by 100 nM rosiglitazone (○), but has little effect when added alone (●). Inset: Effect of GW9662 on full-length PPARγ in the absence (●) and presence (○) of 3 μM rosiglitazone. (B) Reporter gene activity of GAL-4 PPARα observed with varying concentrations of GW9662 alone (●) or in the presence of 8 nM GW7647 (○). (C) Effect of GW9662 on activation of PPARδ in the absence (●) and presence (○) of 0.55 nM GW2433.

However, in contrast to its partial agonist activity on the PPARα (LBD)-GAL4 chimeric receptor, GW9662 failed to activate either full-length PPARα or full-length PPARδ in parallel experiments. At a concentration of 10 μM, mean values (±SEM) for activation of full-length PPARδ and PPARα were 0.9 ± 1.0% and 2.6 ± 8.2%, respectively, of the activity observed for standard activators. Furthermore, GW9662 showed no antagonist activity against the full-length PPARδ (data not shown).

GW9662 Is a Selective PPARγ Ligand. As discussed above, although GW9662 binds irreversibly to the LBDs of all three PPAR subtypes, it is a potent PPARγ antagonist with at least 100−1000-fold functional selectivity in cells

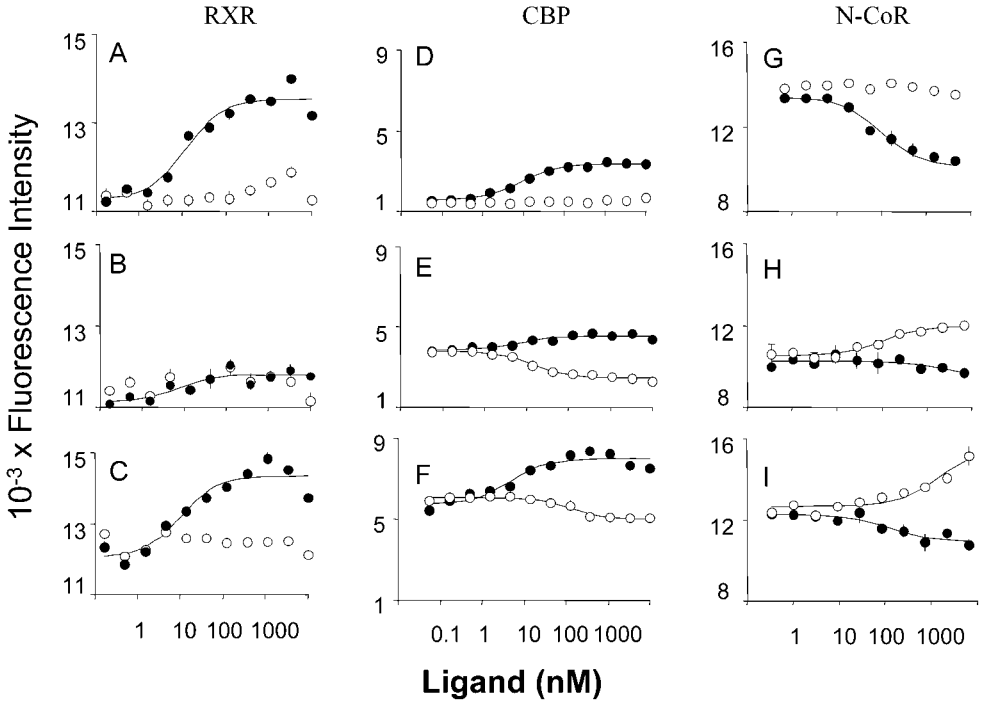

FIGURE 5: GW9662 modulation of PPAR heterodimer and cofactor complexes. Complex formation was monitored by time-resolved fluorometry using fluorescently labeled receptor LBDs and cofactors as described under Materials and Methods. (A−C) PPAR:RXR heterodimer formation. Changes in heterodimer levels of (A) 10 nM PPARγ, (B) 10 nM PPARα, and (C) 10 nM PPARδ, respectively, by GW9662 (○) or reference agonists (●) are shown. The concentration of RXR was 10 nM for all panels. The reference agonists were rosiglitazone for PPARγ, GW2331 for PPARα, and GW2433 for PPARδ, respectively. (D−F) Effect of GW9662 (○) and reference agonist (●) on levels of (D) PPARγ:CBP, (E) PPARα:CBP, and (F) PPARδ:CBP complex formation. The reference agonists were as indicated for the RXR heterodimerization experiments. Concentrations of each PPAR were 10 nM, and the concentration of CBP was 20 nM. (G−I) Effect of GW9662 (○) and reference agonist (●) on levels of (G) PPARγ:N-CoR, (H) PPARα:N-CoR, and (I) PPARδ:N-CoR complex formation. The reference agonists were as indicated for the RXR heterodimerization experiments. Concentrations of each PPAR were 10 nM, and the concentration of N-CoR peptide was 250 nM.

Table 1: Activation of GAL4-LBD Nuclear Receptor Chimeras by GW9662

	fold activation (±SEM)	
receptor	standard[a]	GW9662[b]
AR	13 ± 8	0.3 ± 0.2
FXR	46 ± 17	17 ± 7*
GR	14 ± 3	1.2 ± 0.3
LXRα	10 ± 2	1.3 ± 0.3
PR	56 ± 4	1.1 ± 0.1
PXR	3.8 ± 0.6	4.2 ± 0.6*
RAR	8.2 ± 0.9	0.1 ± 0
RXR	5.2 ± 1.5	1.9 ± 0.3
TRα	73 ± 6	1.3 ± 0.2
TRβ	15 ± 7	0.6 ± 0.3

[a] All values observed for standards were statistically different from the untreated controls at $p < 0.05$. [b] Values statistically different from control at $p < 0.05$ are indicated by an asterisk (*).

over PPARα and PPARδ. The selectivity of GW9662 was further investigated by testing its ability to activate a number of nuclear receptors using GAL4-LBD chimeras in CV-1 cells. As shown in Table 1, GW9662 (10 μM) failed to activate LXRα, retinoid (RAR, RXRα), thyroid (TRα, TRβ), and steroid (AR, GR, PR) receptors. GW9662 profiled as an agonist of human PXR and a partial agonist of FXR at a concentration of 10 μM. The reversibility of the PXR and FXR activity has not been established.

Modulation of Receptor Heterodimer Formation. All three PPAR subtypes are known to bind to DNA as heterodimers with RXR. Furthermore, it is known that the receptor LBDs can also form heterodimers with the RXR LBD in cell-free systems. The ability of ligands to induce changes in PPAR−RXR heterodimer formation was assessed by time-resolved fluorescence resonance energy transfer using fluorescently labeled purified LBDs of RXRα and the PPAR subtypes. A robust increase in the fluorescence signal indicative of an increase in PPARγ:RXR heterodimer was observed when rosiglitazone was added to a solution containing both the PPARγ and RXR LBDs (Figure 5A). The response was saturable, and the observed EC_{50} was similar to the known affinity for rosiglitazone binding to PPARγ. In contrast, GW9662 had no detectable effect on PPARγ:RXR heterodimer formation (Figure 5A).

Titration of GW2331 with PPARα and RXR resulted in only a small increase in fluorescence intensity in the heterodimerization assay. GW9662 showed no effect on PPARα:RXR heterodimer formation (Figure 5B). The small amplitude for GW2331 in the assay could reflect either a decreased formation of PPARα: RXR heterodimer in response to ligand binding, or a lower efficiency of fluorescence energy transfer in the PPARα:RXR assay, relative to that observed for PPARγ:RXR. Although we could not distinguish between these possibilities, it should be noted that GW9662 also failed to *increase* PPARα:RXR heterodimer formation.

Ligand modulation of PPARδ:RXR heterodimer formation was similar to that observed for PPARγ. The amplitude of the response was positively modulated by the PPARδ activator GW2433, but unaffected by GW9662 (Figure 5C). Thus, GW9662 had no effect on the ability of any of the PPARs to form heterodimers with RXR.

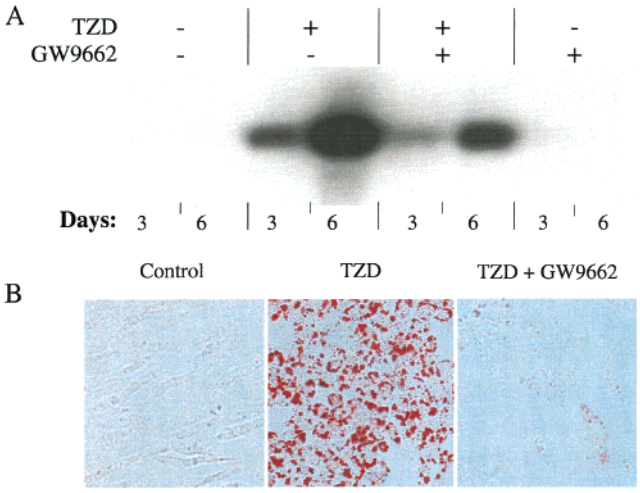

FIGURE 6: Inhibition of adipogenesis by GW9662. (A) C3H10T1/2 cells were cultured with differentiation cocktail (1 μM insulin + 250 nM 9-*cis*-retinoic acid, IR) without TZD (Control), IR with 150 nM rosiglitazone (TZD), or a combination of TZD and 1.5 μM GW9662 (TZD + GW9662). After the indicated time in culture, mRNA was prepared, and Northern analysis for the adipocyte-specific marker aP2 was performed. (B) Cells treated as described in panel A were stained with Oil Red O after 6 days in culture. Intense staining indicative of accumulation of lipid droplets was evident in the cells treated with rosiglitazone (TZD). Addition of GW9662 together with rosiglitazone diminished the staining essentially to that observed for control cultures.

Modulation of Coactivator and Corepressor Binding. The ability of GW9662 to modulate binding of the coactivator CBP and a corepressor N-CoR to each of the three PPARs was examined using time-resolved fluorescence ligand-sensing assays similar to the one employed to monitor PPAR:RXR heterodimer formation. As shown in Figure 5D−F, titration of PPARγ, PPARα, and PPARδ with rosiglitazone, GW2331, and GW2433, respectively, resulted in a concentration-dependent increase in the association of CBP bound to the receptors as measured by an increase in time-resolved fluorescence intensities. In contrast, titration with GW9662 under the same conditions showed no change in formation of the PPARγ:CBP complex and concentration-dependent decreases in PPARα:CBP and PPARδ:CBP complexes. The failure of GW9662 to increase CBP binding to any of the PPARs was consistent with the activity of GW9662 observed in the cell-based transfection assays.

To further characterize the effect of GW9662 on the three PPARs, binding of a peptide based on the sequence of residues 2251−2275 of the corepressor N-CoR to each of the three receptors was examined in the presence of GW9662. The known activators rosiglitazone, GW2331, and GW2433 caused concentration-dependent decreases in receptor:N-CoR complex for PPARγ, PPARα, and PPARδ, respectively. In contrast, GW9662 had little effect on PPARγ:N-CoR complex formation and *increased* both PPARα:N-CoR and PPARδ:N-CoR interaction (Figure 5G,H).

GW9662 Functions as an Adipogenic Antagonist. PPARγ agonists are known to promote differentiation of a fibroblast and pluripotent cells into a mature adipocyte phenotype. The effect of the PPARγ antagonist GW9662 was studied in an adipocyte differentiation assay. Treatment of murine C3H10T1/2 cells with rosiglitazone resulted in differentiation of these cells to adipocytes as assessed by an increase in mRNA for aP2 and by Oil Red O staining. In contrast, 1 μM GW9662 did not increase aP2 expression (Figure 6A) or the appearance of lipid as assessed by Oil Red O staining (not shown). When cells were cultured in the presence of GW9662, the rosiglitazone-induced increases in aP2 and Oil Red O staining were completely blocked (Figure 6). Thus, GW9662 acts as a full antagonist of PPARγ in an adipogenesis assay.

DISCUSSION

GW9662 was identified from a high throughput screen of the corporate compound collection for PPARγ ligands. Scintillation proximity assays showed that GW9662 was 10-fold and 600-fold more potent at inhibiting radioligand binding to PPARγ versus PPARα or PPARδ, respectively. The structure of GW9662 suggested the possibility that the high *apparent* affinity of this compound for binding to PPARγ might be the result of covalent modification of the receptor. The X-ray crystal structure of the PPARγ LBD showed that the ligand binding pocket contained a single Cys residue in helix 3 that was directed into the ligand binding cavity (*15*). A PPARγ partial agonist, L-764406, was recently reported to bind irreversibly to PPARγ through arylation of this residue (*39*). Several experimental observations established that GW9662 also binds irreversibly to PPARγ through covalent modification of Cys^{285}. First, inhibition of [^{3}H]rosiglitazone binding by GW9662 was not reversed after removal of free GW9662. Second, UV−visible spectroscopy of PPARγ treated with GW9662 showed an absorbance increase at ~350 nm consistent with covalent modification of the receptor. Last, the identification of Cys^{285} as the site of covalent modification of PPARγ by GW9662 was established using mass spectroscopy.

GW9662 also binds irreversibly to PPARα and PPARδ, but with significantly lower *apparent* affinity. Reaction of GW9662 with all three PPAR subtypes is consistent with known sequence and structural information for these receptors. Notably, Cys^{285}, the modified residue in PPARγ, is conserved as Cys^{384} and Cys^{249} in PPARα and PPARδ, respectively. The X-ray crystal structure of the PPARγ LBD shows that Cys^{285} in helix 3 is directed into the ligand binding cavity (*14*). In the case of PPARδ, the crystal structure reveals a "Y"-shaped binding pocket with the conserved Cys forming part of the wall of one arm of this "Y". Further, the cocrystal structure of GW2433 bound to PPARδ reveals that GW2433 makes significant interactions with residues in all three arms of the "Y" (*34*). The degree of covalent modification of all three PPAR subtypes was equivalent in the UV−visible spectroscopic assays where the concentration of GW9662 was in excess of the IC$_{50}$s observed for inhibition of radioligand binding. Therefore, the apparent selectivity in receptor binding affinity is most likely due to differences in the kinetics of the arylation reaction. Irreversible inhibition of ligand binding to the PPARs as a consequence of arylation by GW9662 is consistent with structural information available for PPARγ and PPARδ.

GW9662 was a full antagonist of PPARγ in cell-based reporter assays using both GAL4-LBD and full-length receptor. The observation that GW9662 inhibits PPARγ-induced adipocyte differentiation in C3H10T1/2 cells is of significance as this assay assesses the activity of PPARγ ligands on endogenous, full-length receptor acting through a naturally occurring PPRE. Unlike the agonist rosiglitazone, GW9662 had no effect on PPARγ:RXR heterodimer, PPARγ:CBP, and PPARγ:N-CoR complex formation. In contrast to

the results observed with GW9662, arylation of Cys^{285} of PPARγ by L-764406 resulted in a partial agonist profile (*39*). Thus, arylation of Cys^{285} leads to different receptor activation profiles depending on the modifying agent used. This is not unlike the case for reversible ligands, where occupation of the PPARγ binding pocket results in agonism, partial agonism, or antagonism depending on the structure of the particular ligand. A recent NMR spectroscopic study of PPARγ found evidence that the apo-receptor is conformationally mobile and that ligand binding causes a stabilization of receptor conformation (*51*). The authors concluded that agonists, partial agonists, and antagonists may result in differing extents of stabilization of receptor conformation. This view appears to be consistent with the observations for both reversible and irreversible ligands, such as GW9662 and L-764406.

No significant activity of GW9662 was observed with full-length PPARδ, though there was weak antagonism using the GAL4-LBD. Whereas treatment with the compound had no effect on PPARδ:RXR heterodimer formation, a concentration-dependent decrease in the CBP complex and an increase in the N-CoR complex was observed.

Covalent modification of PPARα by GW9662 had no activity against full-length receptor, but led to weak partial agonist activity when assessed with GAL4-LBD in cell-based reporter assays. The effects on heterodimer and cofactor binding to PPARα LBD were qualitatively similar to those observed on treatment of PPARδ.

GW9662 modifies all three PPAR LBDs as indicated by irreversible inhibition of binding (Figure 1). The effects of GW9662 on the PPARα and PPARδ GAL4-4LBDs are consistent with the binding results. The lack of effect of GW9662 on full-length PPARα and PPARδ may reflect a decreased reactivity of the binding site Cys relative to the LBDs. Alternatively, GW9662 may modify the full-length receptors with a less pronounced effect on receptor conformation than was evident for the LBDs. Thus, the effects of GW9662 on PPARδ and PPARα observed in assays using isolated LBDs were not detected in cell-based assays using full-length receptors. The result was that the apparent selectivity of GW9662 for PPARγ was underestimated in the experiments using isolated LBDs.

A number of experimental factors may contribute to the different effects of GW9662 observed for the three receptors in the time-resolved fluorometry assays. First, the fluorescence intensities across assays cannot be used to draw conclusions concerning relative levels of receptor complexes, as the efficiency of energy transfer for the various receptor complexes need not be constant. Furthermore, the amplitudes of the responses for these assays are dependent on the basal level of PPAR:cofactor complex in the absence of added ligand. As a result, the small increase in fluorescence amplitude observed for PPARα:RXR heterodimer formation may be a reflection of assay sensitivity rather than of limited complex formation. Despite these limitations, the results of heterodimer and cofactor binding assays provide further evidence for the consequences of GW9662 arylation of the PPAR LBDs.

GW9662 was inactive when tested against a number of other nuclear receptors in a transactivation assay. At a concentration of 10 μM, significant activity was observed only against FXR and PXR. In the case of FXR, GW9662 activity was only one-third of that seen for the positive control. Because the IC_{50} of GW9662 versus PPARγ was 7.6 nM, the compound is selective for PPARγ versus FXR; 10 μM GW9662 also activated PXR to the same extent as was observed for the positive control, rifampicin. Because PXR is well established as binding a broad variety of xenobiotic ligands (*51*, *52*), the activation of PXR by GW9662 was not surprising. PXR is only expressed at high levels in liver and intestine (*53*), tissues important in xenobiotic metabolism. As a result, the activity of GW9662 on PXR should not, in most cases, preclude the use of this compound as a tool to study the role of PPARγ in diverse biological processes. In summary, GW9662 is a potent, selective PPARγ antagonist.

Recently, bisphenyl A diglycidyl ether (BADGE) has been reported as a weak antagonist of PPARγ (*54*). Although BADGE inhibits adipocyte differentiation in cell culture, its use as an antagonist to elucidate the importance of PPARγ in biological processes may be limited by the low potency of binding. An IC_{50} of ∼100 μM was reported, and incomplete inhibition of binding, apparently due to compound solubility, was observed (*54*). The limited solubility also complicates the interpretation of the receptor selectivity. By contrast, GW9662 is at least 10 000 times more potent than BADGE as measured by inhibition of [^{3}H]rosiglitazone binding to PPARγ, and an estimate of selectivity versus the other PPARs has been established. GW9662 covalently modifies the PPARγ, and inhibition is observed even in the presence of high concentrations of reducing agent or exogenous protein (e.g., fetal bovine serum used in the transfection experiments). As the antagonistic activity of GW9662 toward PPARγ is retained in cell culture systems, this compound may be a useful tool for elucidation of the role of PPARγ in biological processes. Complex behavior has been reported for some PPARγ ligands, such as BADGE and LG100641. These compounds act as antagonists in some assays or cell lines, but agonists in others (*55*, *56*). By contrast, GW9662 was a subtype-selective PPARγ antagonist under the conditions examined. The irreversible nature of GW9662 may facilitate studies of PPARγ by enabling use of low doses and/or simplified dosing regimens.

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BI0159581