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Ligand-Mediated Regulation of Peroxisome Proliferator-Activated Receptor (PPAR) β/δ: A Comparative Analysis of PPAR-Selective Agonists and All-*trans* Retinoic Acid

Markus Rieck, Wolfgang Meissner, Simone Ries, Sabine Müller-Brüsselbach, and Rolf Müller

Institute of Molecular Biology and Tumor Research (IMT), Philipps University, Marburg, Germany

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily that modulate target gene expression in response to natural fatty acid ligands and synthetic agonists. It is noteworthy that all *trans*-retinoic acid (atRA) has recently been reported to act as a ligand for PPARβ/δ, to activate its transcriptional activity, and, in contrast to the "classic" function of atRA, to stimulate cell proliferation (Schug et al., 2007). Here, we report that in contrast to synthetic PPARβ/δ agonists, atRA failed to induce the transcriptional activity of PPARβ/δ using different types of reporter gene assays. Likewise, atRA did not affect the expression of the bona fide PPARβ/δ target genes *ADRP* and *ANGPTL4* but strongly increased expression of the retinoic acid target gene *CYP26A* under the identical experimental conditions. Consistent with these observations, atRA did not compete with established PPARβ/δ agonists in a ligand binding assay, and atRA did not enable the interaction of PPARβ/δ with a coactivator peptide in a time-resolved fluorescence resonance energy transfer assay in vitro. These results are in sharp contrast to the effect of established PPARβ/δ agonists in both in vitro assays. Taken as a whole, these data strongly suggest that atRA does not function as a ligand of PPARβ/δ in any of the experimental systems tested and that the previously reported atRA effects are more likely to reflect an uncharacterized and less direct mechanism.

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that modulate target gene expression in response to endogenous and exogenous ligand activation (Desvergne et al., 2006). Because PPARβ/δ is activated by certain fatty acids and fatty acid derivatives (Forman et al., 1997; Desvergne et al., 2006), it is thought to act as an intracellular lipid sensor. Although the DNA-binding domains of the three subtypes, PPARα, PPARβ/δ, and PPARγ, are highly similar, the ligand-binding domains of the three PPARs exhibit a higher degree of divergence (~65% identity), which probably accounts for the differential activation of PPARs by fatty acid derivatives and synthetic compounds (Forman et al., 1997; Desvergne et al., 2006; Michalik et al., 2006; Peraza et al., 2006). Differences in tissue expression patterns of PPARs provide another level of regulation.

After ligand binding, PPARβ/δ forms obligatory heterodimers with the nuclear receptor RXR and subsequently binds to peroxisome proliferator response elements (PPREs) in target genes, resulting in transcriptional activation as a result of the recruitment of specific cofactors (PGC1α, steroid receptor coactivators) and the displacement of corepressors (nuclear receptor corepressor, silencing mediator of retinoic acid and thyroid hormone receptors) (Yu and Reddy, 2007; Zoete et al., 2007). PPARβ/δ probably can also repress genes by directly interacting with specific transcription factors (Lee et al., 2003) or sequestration of RXR from other RXR-dependent nuclear receptors (Matsusue et al., 2006).

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ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; GW501516, 2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl)phenoxy-acetic acid; RA, retinoic acid; RAR, retinoic acid receptor; atRA, all-*trans* retinoic acid; LBD, ligand binding domain; PBS, phosphate-buffered saline; 9-*cis* RA, 9-*cis* retinoic acid; GW1929, (2*S*)-((2-benzoylphenyl)amino-3-(4-[2-(methylpyridin-2-ylamino)ethoxy]phenyl)propionic acid; AA, arachidonic acid; Ro 13-7410, 4-[*E*-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB); HEK, human embryonic kidney; PCR, polymerase chain reaction; TR-FRET, time-resolved fluorescence resonance energy transfer; ADRP, adipocyte differentiation-related protein; Angptl4, angiopoietin-like protein 4.

PPARβ/δ has an important role in the regulation of intermediary metabolism, in particular energy homeostasis, lipid catabolism, and glucose homeostasis (Desvergne et al., 2006). Mice lacking PPARβ/δ show an aberrant development and malfunction of the placenta (Peters et al., 2000; Barak et al., 2002; Nadra et al., 2006), presumably as a result of a lack of differentiation and metabolic malfunctioning of trophoblast giant cells (Nadra et al., 2006). *Pparb*-null mice also exhibit a defect in wound healing (Michalik et al., 2001), and, consistent with this observation, PPARβ/δ is critical for the survival, differentiation, and cell cycle control of keratinocytes (Peters et al., 2000; Di-Poï et al., 2002; Burdick et al., 2006). PPARβ/δ is also essential for mediating differentiation in the digestive tract, where it promotes the differentiation of Paneth cells in the intestinal crypts (Varnat et al., 2006). Consistent with its physiological function in differentiation and proliferation, PPARβ/δ also modulates intestinal tumorigenesis in different mouse models (Peters et al., 2000; Di-Poï et al., 2002; Burdick et al., 2006). PPARβ/δ inhibits chemically induced skin carcinogenesis (Peters et al., 2000) and exerts an essential function in the tumor stroma (Müller-Brüsselbach et al., 2007). Ligand activation of PPARβ/δ has also been shown to induce terminal differentiation in keratinocytes and colonic epithelium and to inhibit cell proliferation in a number of different cell types (Peters et al., 2000; Di-Poï et al., 2002; Burdick et al., 2006). PPARβ/δ has potent anti-inflammatory activities, including modulation of the expression of cytokines, adhesion molecules, and extracellular matrix proteins in immune cells and regulating their proliferation, differentiation, and survival (Kilgore and Billin, 2008). Therefore, PPARβ/δ represents a highly relevant drug target for the treatment of major human diseases such as obesity, metabolic syndrome, chronic inflammation, and arteriosclerosis.

The central role of PPARβ/δ in multiple disease-related processes has helped lead to the development of several synthetic drug candidates with subtype selectivity and high-affinity binding, such as GW501516 and L165,041 (Peraza et al., 2006). It is noteworthy that it was recently suggested that all *trans*-retinoic acid (atRA) is a high-affinity ligand for PPARβ/δ (Shaw et al., 2003). This finding is intriguing, because the same laboratory recently reported that the effect of atRA on cell proliferation is dependent on the type of receptor activated: when delivered to the retinoic acid receptor (RAR) by the intracellular fatty acid transporter CRABP-II atRA inhibited proliferation, whereas its delivery to PPARβ/δ via the fatty acid binding protein-5 generated a proliferative stimulus (Schug et al., 2007). As part of a study pertaining at the characterization of PPAR ligands, we also analyzed the effect of atRA on PPARβ/δ. In contrast to the former findings, we were unable to identify any effect of atRA on the transcriptional activity of PPARβ/δ in vivo in different experimental systems. Consistent with this finding, and in sharp contrast to established PPARβ/δ agonists, atRA did not enable the interaction of PPARβ/δ with a coactivator peptide in vitro, nor did it interact with PPARβ/δ in a ligand binding assay. These findings demonstrate that atRA does not function as a ligand for PPARβ/δ and does not modulate its transcriptional activity in any of the experimental systems tested.

Materials and Methods

Chemicals. GW501516 and 9-*cis*-RA were purchased from Axxora (Lörrach, Germany); atRA (purity, 98%) was obtained from Sigma (Taufkirchen, Germany); GW1929 from Biozol (Eching, Germany); and arachidonic acid (AA), linoleic acid, eicosapentaenoic acid, and TTNPB (Ro 13-7410) from Sigma-Aldrich (Steinheim, Germany). L165,041 was from Calbiochem (Merck, Darmstadt, Germany), and carbaprostacyclin was from (Cayman Chemical, Ann Arbor, MI). For details on PPARβ/δ and RXR/RAR ligands, see Peraza et al. (2006) and Altucci and Gronemeyer (2001), respectively.

Cell Culture. *PPARb*-null fibroblasts (Müller-Brüsselbach et al., 2007), NIH3T3 (obtained form D. Lowy, National Institutes of Health, Bethesda, MD), HaCAT cells (kindly provided by Drs. N. Fusenig and P. Boukamp, DKFZ Heidelberg, Germany), and HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C and 5% CO_2.

Plasmids. pCMX-mPPARβ (Forman et al., 1997) and Gal4-mPPARβ (Shi et al., 2002) were kindly provided by Dr. R. Evans (The Salk Institute, La Jolla, CA). 3×Flag-PPARβ was generated by cloning the coding sequence of mPPARβ N-terminally fused to a triple FLAG tag (Müller-Brüsselbach et al., 2007) into pcDNA3.1(+) zeo (Invitrogen, Karlsruhe, Germany). pCMX-empty has been described by Umesono et al. (1991). LexA-mPPARβ, 7L-TATAi, and 10×Gal4SVGL3 have been described previously (Jérôme and Müller, 1998; Fauti et al., 2006). pSG5-hRXRa containing the full-length RXRa cDNA was kindly provided by Dr. A. Baniahmad (Jena, Germany), respectively. The 3×PPRE-TATAi plasmid was constructed by inserting the 3×PPRE cassette (KpnI/BamHI) from PPRE×3-tk-pGl3 (Fauti et al., 2006) into TATAi-pGl3 (KpnI/BglII) (Jérôme and Müller, 1998). The pUC18 plasmid was obtained from New England Biolabs (Frankfurt am Main, Germany) and pcDNA3.1 from Invitrogen (Karlsruhe, Germany).

Luciferase Reporter Assays. Transfections were performed with polyethylenimine (average MW 25,000; Sigma-Aldrich). Cells were transfected on six-well plates at 70 to 80% confluence in Dulbecco's minimal essential medium plus 2% fetal calf serum with 5 μg of plasmid DNA and 10 μl of polyethylenimine (1:1000 dilution, adjusted to pH 7.0 and preincubated for 15 min in 200 μl of phosphate-buffered saline for complex formation). Four hours after transfection, the medium was changed, and cells were incubated in normal growth medium for 24 h. Luciferase assays were performed as described previously (Gehrke et al., 2003). Values from three independent experiments were combined to calculate averages and standard deviations.

Retrovirally Transduced Cells Expressing FLAG-PPARβ. 3×FLAG-PPARβ was cloned into the retroviral vector pLPCX (Clontech, Mountain View, CA). Phoenix cells expressing ecotropic *env* were transfected with 3×FLAG-mPPARb-pLPCX as described (http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html). Culture supernatant was used to infect *Pparb*-null fetal mouse lung fibroblasts that had previously been established from *Pparb* knockout mice by standard procedures. Cells were selected with puromycin (2 μg/ml; Sigma), and a clone expressing 3×FLAG-mPPARβ (3Fb3 cells) at moderate levels, comparable with endogenous PPARβ/δ in mouse fibroblasts, was used in the present study.

Quantitative PCR. cDNA was synthesized from 1 μg of RNA using oligo(dT) primers and the Omniscript kit (QIAGEN, Hilden, Germany). qPCR was performed in a Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) for 45 cycles at an annealing temperature of 60°C. PCR reactions were carried out using the Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany) and a primer concentration of 0.2 μM according to the manufacturer's instructions. *L27* was used as normalizer. Comparative expression analyses were statistically analyzed by Student's *t* test (two-tailed, equal variance). Primers are listed in Table 1.

Time-Resolved Fluorescence Resonance Energy Transfer Assays in Vitro. TR-TRET (Stafslien et al., 2007) was performed with the LanthaScreen TR-FRET PPARβ competitive binding assay, the Lanthascreen TR-FRET PPARβ coactivator assay, and the LanthaScreen TR-FRET RARγ coactivator assay according to the instructions of the manufacturer (Invitrogen, Karlsruhe, Germany). All assays were validated for their robustness by determining the respective Z′-factors (Zhang et al., 1999). Measurements were performed on a VICTOR3_V Multilabel Counter (WALLAC 1420; PerkinElmer Life and Analytical Sciences, Rodgau, Germany) with instrument settings as described in the manufacturer's instructions for LanthaScreen assays.

Results

Effect of Selective PPAR Ligands and atRA on the Transcriptional Activity of PPARβ/δ. To analyze the effect of specific compounds on the transcriptional activity of PPARβ/δ in mouse fibroblasts (NIH3T3), we used luciferase reporter constructs consisting of either seven LexA or 10 Gal4 binding sites upstream of a TATA-initiator module without any additional promoter elements. In the absence of ligand, these reporter plasmids show negligible luciferase activity and therefore allow for a highly sensitive detection of the transcriptional activity of a cotransfected transcriptional activator harboring a LexA DNA binding domain. In this system, the synthetic PPARβ/δ agonist GW501516 (1 μM) caused a strong transcriptional induction (average, 16.6-fold; Table 2). This transcriptional activation did not vary over concentration range of 0.1 to 1 μM ligand (Fig. 1A). Similar results were obtained with a fusion protein consisting of the PPARβ/δ ligand binding domain and the Gal4 DNA binding domain (Fig. 1A). In contrast, treatment with atRA over a wide concentration range of 0.01 to 1 μM had no effect on the transcriptional activity of LexA-PPARβ/δ and Gal4-PPARβ/δ (Fig. 1, A and B; Table I). As expected, a strong induction was also observed by two other synthetic PPARβ/δ agonists, L165,041 and carbaprostacyclin (average, 11.2- and 6.7-fold, repectively), whereas no significant effect was seen with the PPARγ ligand GW1929 (Table 2). In agreement with previous reports (Forman et al., 1997; Xu et al., 1999), the natural unsaturated fatty acid ligands AA, linoleic acid, and eicosapentaenoic acid also induced the transcriptional activity of LexA-PPARβ/δ to a weak but significant extent (average, 1.6- to 2.6-fold at 20 μM), whereas the RXR agonist 9-*cis*-RA had no effect (Table 2). These results obtained with established PPAR ligands are fully consistent with published data and therefore validate the reporter assays used in this study. To exclude cell type-specific effects, the same experiment as in Fig. 1A was also performed with HEK293 cells, which gave essentially the same results (Fig. 1C).

Failure of atRA to Induce PPRE-Driven Transcription. We next analyzed the effect of GW501516 and atRA on a luciferase reporter construct containing a PPRE-driven minimal promoter in *Pparb*-null fibroblasts, both in the presence and in the absence of a cotransfected PPARβ/δ expression vector (Fig. 2). No increase in transcriptional activity was observed after treatment with atRA irrespective of the presence of PPARβ/δ. In contrast, a significant increase in PPARβ/δ-dependent transcriptional activation by GW501516 was found that was strongly enhanced in the presence of the cotransfected PPAR dimerization partner RXRα and its agonist 9-*cis*-RA (Fig. 2). RXR proteins have also been reported to be able to bind as homodimers to PPREs and to activate PPRE-dependent transcription (IJpenberg et al., 2004), which is consistent with the data in Fig. 2. The fact that atRA treatment resulted in an increased transcriptional activity in the presence of cotransfected RXRα is presumably due to the presence of low levels of a RXR agonist in the preparation (Altucci and Gronemeyer, 2001) or metabolically produced atRA derivative(s). Taken together, these findings are highly consistent with published data except for failure of atRA to activate PPARβ/δ. These observations are surprising given recent publications suggesting that atRA is a PPARβ/δ ligand (Schug et al., 2007).

Regulation of Gene Expression by a PPARβ/δ Agonist and atRA. We next investigated the effect of GW501516, atRA, and 9-*cis*-RA on verified target genes of PPARβ/δ (*ADRP* and *ANGPTL4*) and of RAR (*CYP26A*) (Mandard et al., 2004; Schmuth et al., 2004; Loudig et al., 2005). This analysis was performed with HaCAT cells. where

TABLE 1
Primers used for qPCR analyses.

Human	
ANGPTL4_for	5′-GAT GGC TCA GTG GAC TTC AAC C
ANGPTL4_rev	5′-CCC GTG ATG CTA TGC ACC TTC
ADRP_for	5′-TGT GAG ATG GCA GAG AAC GGT
ADRP_rev	5′-CTG CTC ACG AGC TGC ATC ATC
CRABP II_for	5′-TCG GAA AAC TTC GAG GAA TTG C
CRABP II_rev	5′-CCT GTT TGA TCT CCA CTG CTG
CYP26A1_for	5′-ACA AGC AGC GCA AGA AGG
CYP26A1_rev	5′-AGC TCA GCC ACT GCT CCA
FABP5_for	5′-ATG AAG GAG CTA GGA GTG GGA
FABP5_rev	5′-TGC ACC ATC TGT AAA GTT GCA G
PPARb_for	5′-TCA TTG CGG CCA TCA TTC TGT GTG
PPARb_rev	5′-TTC GGT CTT CTT GAT CCG CTG CAT
Murine	
Angptl4_for	5′-CTC TGG GGT CTC CAC CAT TT
Angptl4_rev	5′-TTG GGG ATC TCC GAA GCC AT
Adrp_for	5′-CAC AAA TTG CGG TTG CCA AT
Adrp_rev	5′-ACT GGC AAC AAT CTC GGA CGT
CrabpII_for	5′-CCT GGA GCC GAG AAC TGA CCA A
CrabpII_rev	5′-TGG GAG GGA GGT TTG TGT CCT GTA
Fabp5_for	5′-GGA AGG AGA GCA CGA TAA CAA GA
Fabp5_rev	5′-GGT GGC ATT GTT CAT GAC ACA
murine/ L27_for	5′-AAA GCC GTC ATC GTG AAG AAC
murine/ L27_rev	5′-GCT GTC ACT TTC CGG GGA TAG

TABLE 2
Effect of different ligands on the transcriptional activity of LexA-PPARβ/δ fusion proteins in luciferase reporter assays
Values represent averages of three o eight independent experiments (± S.D.). Asterisks indicate statistically significant induction (*$P < 0.05$) or lack of significance (**$P > 0.05$) by t test. Data from different experiments were standardized to GW501516.

Compound	Properties	Concentration	-Fold Induction
		μM	
GW501516	Synthetic PPARβ/δ agonist	1.0	16.6 ± 6.0*
L165,041	Synthetic PPARβ/δ agonist	2.0	11.2 ± 4.9*
cPGI	Synthetic PPARβ/δ agonist	1.0	6.7 ± 1.7*
GW1929	Synthetic PPARγ agonist	0.3	1.3 ± 0.6**
Arachidonic acid	Pan-PPAR ligand	20	2.6 ± 1.1*
Linoleic acid	Pan-PPAR ligand	20	1.6 ± 0.3*
Eicosapentaenoic acid	Pan-PPAR ligand	20	1.9 ± 0.4*
atRA	RAR agonist	1.0	1.1 ± 0.1**
9-*cis*-RA	RAR and RxR agonist	1.0	1.0 ± 0**

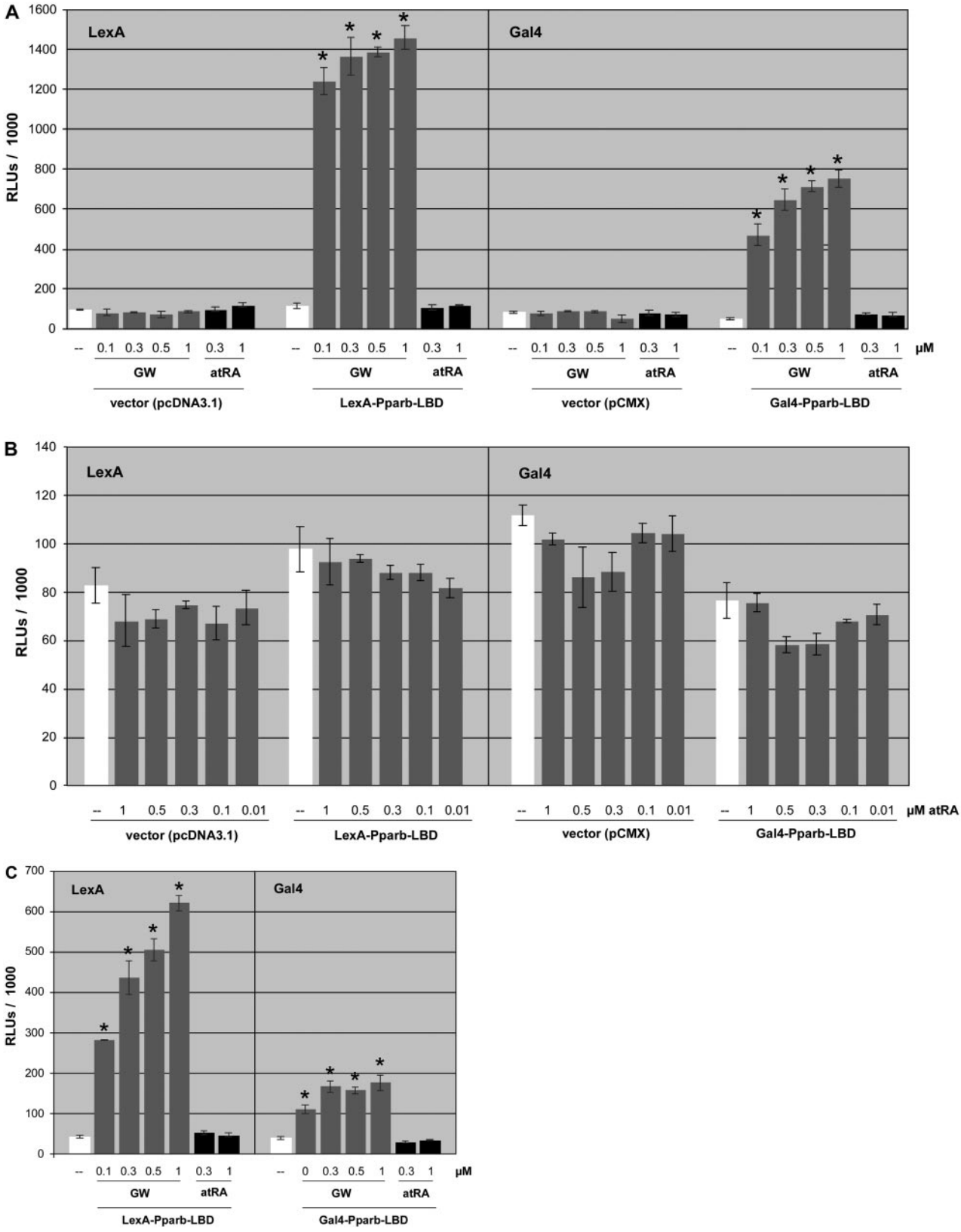

Fig. 1. Effects of GW501516 and atRA on the transcriptional activity of the PPARβ/δ ligand binding domain fused to either the LexA (left) or the Gal4 (right) DNA-binding domain. NIH3T3 (A and B) or HEK293 cells (C) were transiently transfected with an expression vector encoding the LexA-PPARβ/δ fusion protein (LexA-Pparb-LBD), the Gal4-PPARβ/δ fusion protein (Gal4-Pparb-LBD), or the empty vector (pcDNA3.1) together with a LexA or Gal4 binding luciferase reporter plasmid. Cells were treated with solvent (−), GW501516 (GW) or atRA at the indicated concentrations for 24 h before harvesting. B, the effects of a wider range of atRA concentrations was analyzed. Values represent the average of triplicates; error bars show the standard deviation. Significant differences between untreated and GW501516 treated cells are indicated by an asterisk (paired *t* test; $P < 0.001$).

increased expression of PPARβ/δ target genes has been described by others (Schug et al., 2007). The HaCAT cells used for this study strongly expressed the *FABP5* gene (Fig. 3), which has been reported to be a prerequisite for atRA-mediated PPARβ/δ activation. HaCAT cells also expressed the classic RA-binding protein and intracellular fatty acid transporter *CRABP-II* and the three target genes to be analyzed at readily detectable levels (Fig. 3). GW501516 and 9-*cis*-RA clearly induced expression of ADRP and ANGPTL4 mRNA, whereas no significant increase in expression was observed after treatment with atRA (Fig. 3). In contrast, the established RAR target gene *CYP26A* was strongly up-regulated by both atRA with the identical experimental conditions (Fig. 3). *CYP26A* induction was also found with 9-*cis*-RA (Fig. 3), which is a known agonist for both RXR and RAR (Altucci and Gronemeyer, 2001).

We also analyzed the effect of these same ligands on the *Adrp* and *Angptl4* genes in mouse fibroblasts lacking PPARβ/δ and in cells with restored PPARβ/δ expression. For this experiment, fetal lung fibroblasts from *Pparb*-null mice were infected with a retrovirus expressing FLAG-tagged PPARβ/δ (3×Flag-Pparb) or a control retrovirus (pLPCX). The data in Fig. 4 show essentially the same result as that obtained with HaCAT cells (i.e., a clear PPARβ/δ-dependent induction of both Adrp and Angplt4 by GW501516, but no detectable regulation of these PPARβ/δ target genes by atRA).

Failure of atRA to Induce Coactivator Peptide Binding to PPARβ/δ. The observations described above strongly suggest that atRA does not activate the transcriptional activity of PPARβ/δ in any of the experimental models examined. To exclude that this failure may be due to the specific cell culture systems used, we next sought to investigate the effect of different ligands on PPARβ/δ coactivator interaction in vitro. Toward this goal, we applied TR-FRET to analyze the interaction of the PPARβ/δ LBD indirectly labeled by terbium with the coactivator peptide C33 labeled with fluorescein (Stafslien et al., 2007). C33 was previously identified, by phage display, as a peptide strongly interacting with the RXR and PPAR coactivator binding sites in a ligand-dependent fashion, similar to peptides derived from the PGC1α or steroid receptor coactivators (Chang et al., 1999). This assay determines the intensity of terbium-induced fluorescence emission of the fluorescein moiety of the C33 peptide, expressed as the ratio of fluorescein-derived and terbium-derived fluorescence. As shown in Fig. 5A, both synthetic PPARβ/δ ligands, GW501516 and L165,041, induced FRET in a concentration-dependent manner with half-maximal intensity at 25 nM and 1.8 μM, respectively (EC_{50} values). A lower FRET signal was also observed with AA, which is consistent with the fact that this fatty acid is a weak PPAR agonist (Forman et al., 1997; Xu et al., 1999). In contrast, neither atRA nor 9-*cis*-RA showed any response in the same assay up to concentrations of 100 μM. As a positive control, we also performed the coactivator binding assay with the LBD of RARγ (Fig. 5B). atRA, 9-*cis*-RA, and the specific RAR agonist TTNPB all strongly induced FRET with EC_{50} values of 11, 9, and 5 nM, respectively. As a whole, these data strongly suggest that atRA is unable to induce coactivator binding to the PPARβ/δ LBD.

Failure of atRA to Compete with PPARβ/δ Agonists for LBD Binding. Finally, we analyzed the interaction of atRA and different agonists with LBD of PPARβ/δ in TR-FRET-based competitive ligand binding assay. In this assay, the terbium-labeled PPARβ/δ LBD interacts with Fluormone Pan-PPAR Green as PPAR ligand, which produces FRET. The fluorescent Fluormone Pan-PPAR Green can be displaced by unlabeled ligands resulting in a quantifiable attenuation of FRET. The results obtained with this assay (Fig. 6) are very similar to those of the coactivator binding assay above. Although GW501516, L165,041, and AA showed readily detectable competition with IC_{50} values of 3 nM, 10 nM, and 2 μM, respectively, displacement of the fluorescent Fluormone Pan-PPAR Green ligand by atRA was undetect-

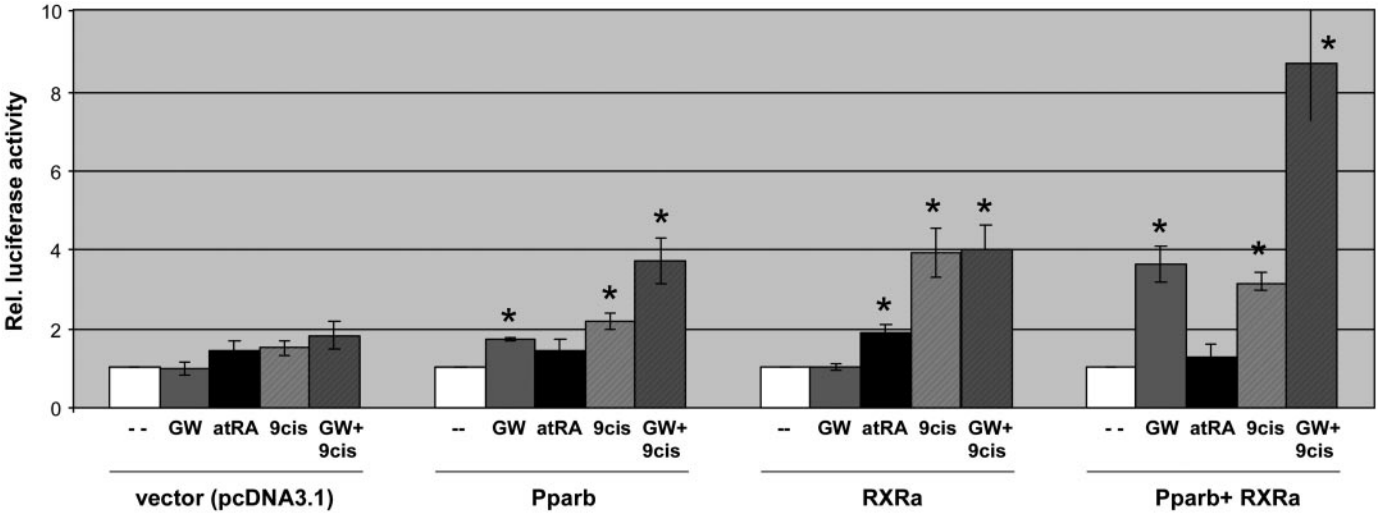

Fig. 2. Effects of GW501516, atRA, and 9-*cis*-RA on the transcriptional activity of PPARβ/δ. *Pparb*-null fibroblasts were transiently transfected with expression vectors encoding PPARβ/δ, RXRα, both vectors, or the empty vector (pcDNA3.1) together with a PPRE-driven luciferase reporter plasmid. Cells were treated with solvent (−), GW501516 (GW; 0.3 μM), atRA (0.3 μM), 9-*cis*-RA (9cis; 0.3 μM), or GW501516 plus 9-*cis*-RA for 24 h before harvesting. Values represent the average of triplicates; error bars show the S.D. Significant differences to untreated cells are indicated by an asterisk (paired *t* test; $P < 0.005$).

able. This finding strongly supports the conclusion that atRA is unable to compete for binding of both high-affinity synthetic agonists and low-affinity natural ligands (AA) with PPARβ/δ.

Discussion

Results from the present study provide strong evidence that atRA does not function as a PPARβ/δ ligand. This con-

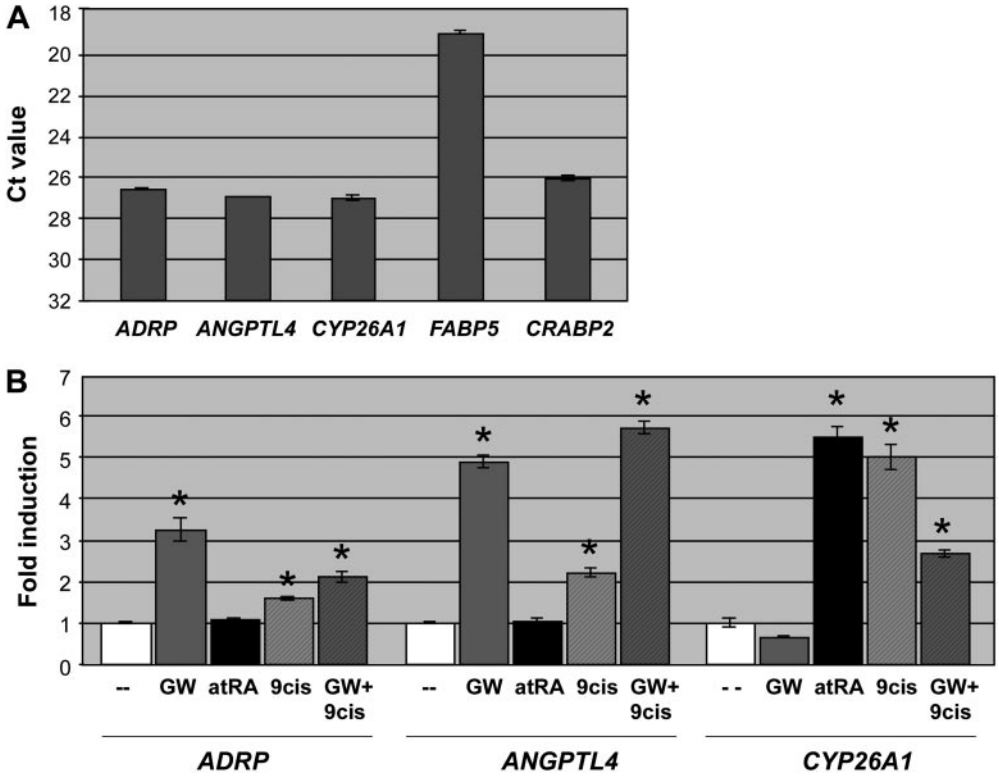

Fig. 3. A, expression of the PPARβ/δ target genes *ADRP* and *ANGPTL4*, the RA target gene *CYP26A1*, and the fatty acid binding proteins *FABP5* and *CRABP2* in HaCAT cells. Expression levels were determined by qPCR. Values represent C_t values (averages of triplicates ± S.D.) normalized to *L27* (C_t = 18) B, regulation of *ADRP*, *ANGPTL4*, and *CYP26A1* by GW501516 (GW; 0.3 μM), atRA (0.3 μM), 9-*cis*-RA (9cis; 0.3 μM), GW501516 plus 9-*cis*-RA or solvent (−). Cells were treated for 24 h, and RNA was isolated and analyzed by qPCR. Induction values were calculated relative to solvent-treated cells and represent averages of triplicates (± S.D) normalized. ∗, values significantly different ($P < 0.001$) between solvent- and ligand-treated cells.

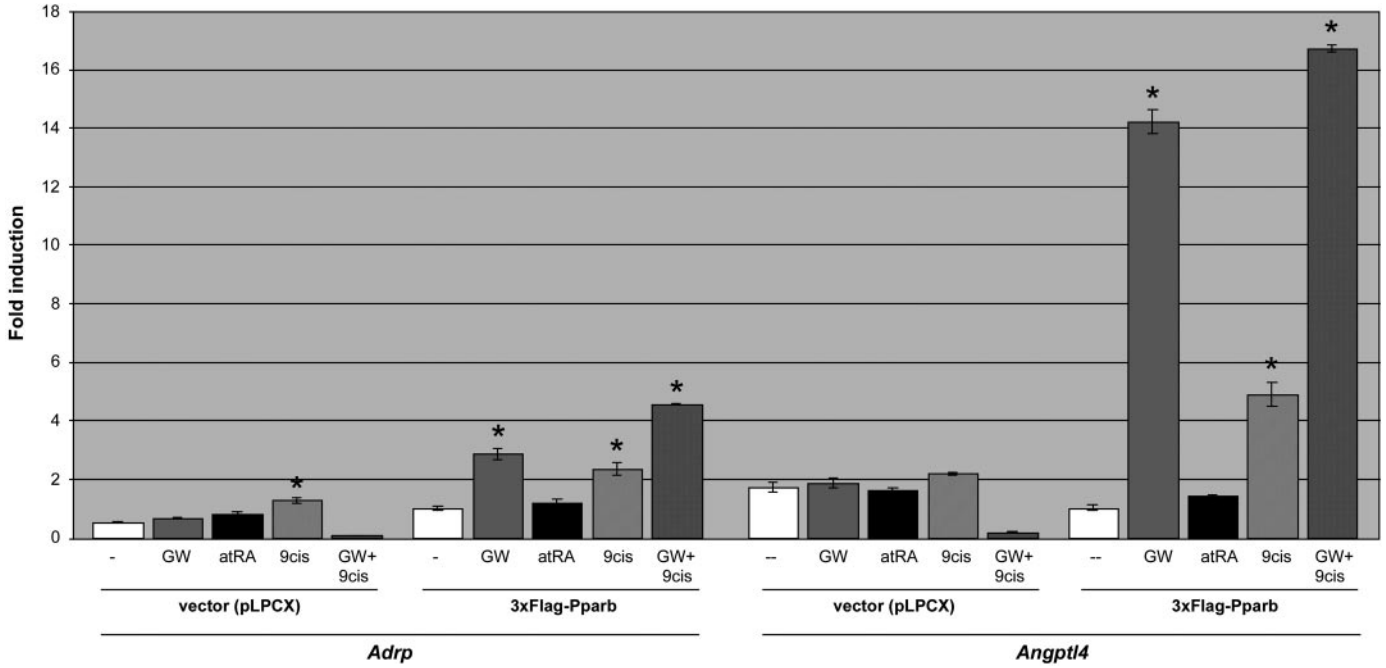

Fig. 4. A, expression of the PPARβ/δ target genes *Adrp* and *Angptl4* in mouse fibroblasts lacking PPARβ/δ and in cells with restored PPARβ/δ expression. Mouse fibroblasts from *Pparb*-null mice were infected with a retrovirus expressing FLAG-tagged PPARβ/δ (3×Flag-Pparb) or a control retrovirus (pLPCX). These cells were treated with GW501516 (GW; 0.3 μM), atRA (0.3 μM), 9-*cis*-RA (9cis; 0.3 μM), GW501516 plus 9-*cis*-RA, or solvent (−) for 24 h and analyzed for *Adrp* and *Angptl4* expression by qPCR. Induction values were calculated relative to solvent-treated cells and represent averages of triplicates (± S.D) normalized. ∗, values significantly different ($P < 0.001$) between solvent and ligand treated cells.

clusion is based on multiple experimental strategies comparing the properties of established PPARβ/δ agonists with those of atRA in various experimental models. First, unlike synthetic and natural compounds, such as GW501516, L165,041, or AA, atRA failed to induce the transcriptional activity of LexA-PPARβ/δ and Gal4-PPARβ/δ fusion proteins in luciferase reporter assays in either the mouse fibroblast cell line NIH3T3 (Fig. 1, A and B) or the human embryonic

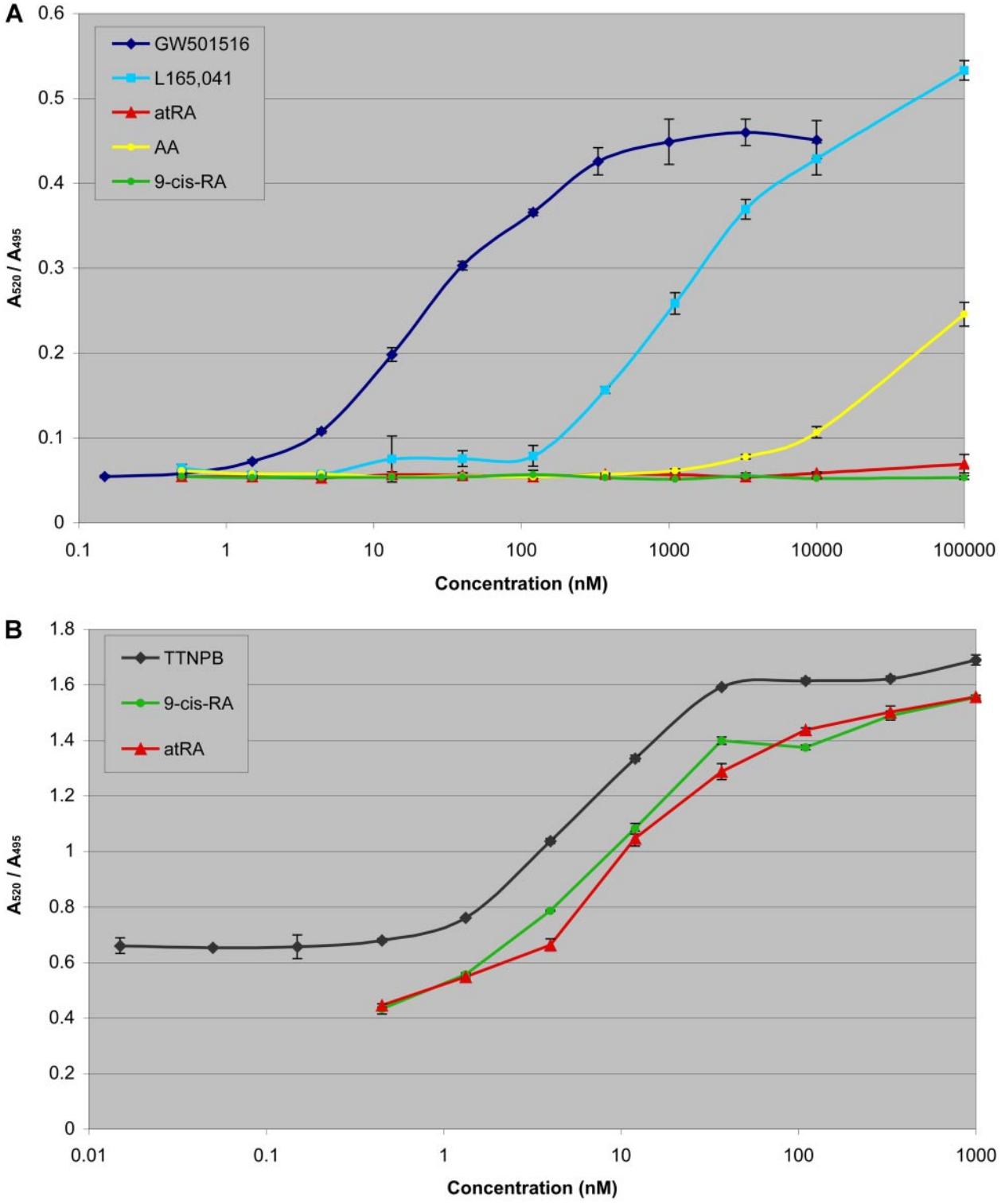

Fig. 5. Ligand-induced binding of a coactivator derived peptide to PPARβ/δ (A) or RARγ (B) in vitro. Interaction of fluorescein-labeled coactivator peptide C33 and recombinant GST-PPARβ/δ or GST-RARγ bound by a terbium-labeled anti-GST antibody was determined by TR-FRET. GW501516, L165,041, atRA, arachidonic acid (AA), 9-*cis*-RA, and TTNPB were used at the concentrations indicated. Results are expressed as the ratio of fluorescence intensity at 520 nm (fluorescein emission excited by terbium emission) and 495 nm (terbium emission). All data points represent averages of triplicates (± S.D).

kidney cell line 293 (Fig. 1C). Second, a similar discrepancy was observed when the same ligands were analyzed for their ability to induce PPARβ/δ-dependent PPRE-driven transcription in reconstituted *Pparb*-null cells (Fig. 2). Third, endogenous PPARβ/δ target genes were efficiently induced by GW501516 in two different cell lines (HaCAT keratinocytes and reconstituted *Pparb*-null fibroblasts), but not by atRA, even though atRA activated the transcription of a bona fide RAR target under the same experimental conditions (Figs. 3 and 4). These results are consistent with findings by others showing that ligand activation of PPARβ/δ by GW0742 and GW501516 increases expression of PPARβ/δ target genes, but atRA and 9cRA do not (Borland et al., 2008). The same authors also showed that both the synthetic PPARβ/δ ligand GW0742 and atRA inhibit HaCAT cell proliferation, but only inhibition of cell growth by GW0742 is dependent on the presence of PPARβ/δ. Together, observations made in HaCaT cells by two independent laboratories are particularly striking and are highly inconsistent with work by others using the same cell line (Schug et al., 2007).

Because it was possible that the observed discrepancies were related to differences in cell culture conditions, we also analyzed the effect of atRA and other ligands on recombinant PPARβ/δ in vitro. However, two independent FRET-based assays failed to show any interaction of atRA with PPARβ/δ. Whereas the established PPARβ/δ ligands GW501516, L165,041, and AA triggered the interaction of the PPARβ/δ LBD with a coactivator mimicking peptide, atRA failed to show any effect, even though it induced the recruitment of a coactivator peptide to RARγ. Furthermore, the same PPARβ/δ ligands were able to displace a fluorescent PPAR ligand from the PPARβ/δ LBD in a competitive ligand binding assay, whereas atRA failed to do so. These results are also highly inconsistent with work showing a direct interaction of atRA with PPARβ/δ and an induction of coactivator recruitment in vitro (Schug et al., 2007). Although these authors used different types of in vitro assays compared with those used in the present study, differences in the assays are unlikely to explain the observed discrepancies, because such deviations were seen in all in vivo and in vitro assays performed without exception. Whereas the results from our study are congruent with those in the accompanying article (Borland et al., 2008), they are incongruent with reports by others (Shaw et al., 2003; Schug et al., 2007).

In summary, our study and the work by Borland et al. (Borland et al., 2008) clearly demonstrate that atRA does not function as a PPARβ/δ agonist. The latter study also shows that the inhibition of keratinocyte cell proliferation by atRA does not require PPARβ/δ. Thus, the recent suggestion that atRA is a PPARβ/δ ligand, and that activation of this pathway potentiates cell growth (Shaw et al., 2003; Schug et al., 2007), should be viewed with caution. The atRA effects reported by these authors are more likely to reflect an uncharacterized and less direct mechanism.

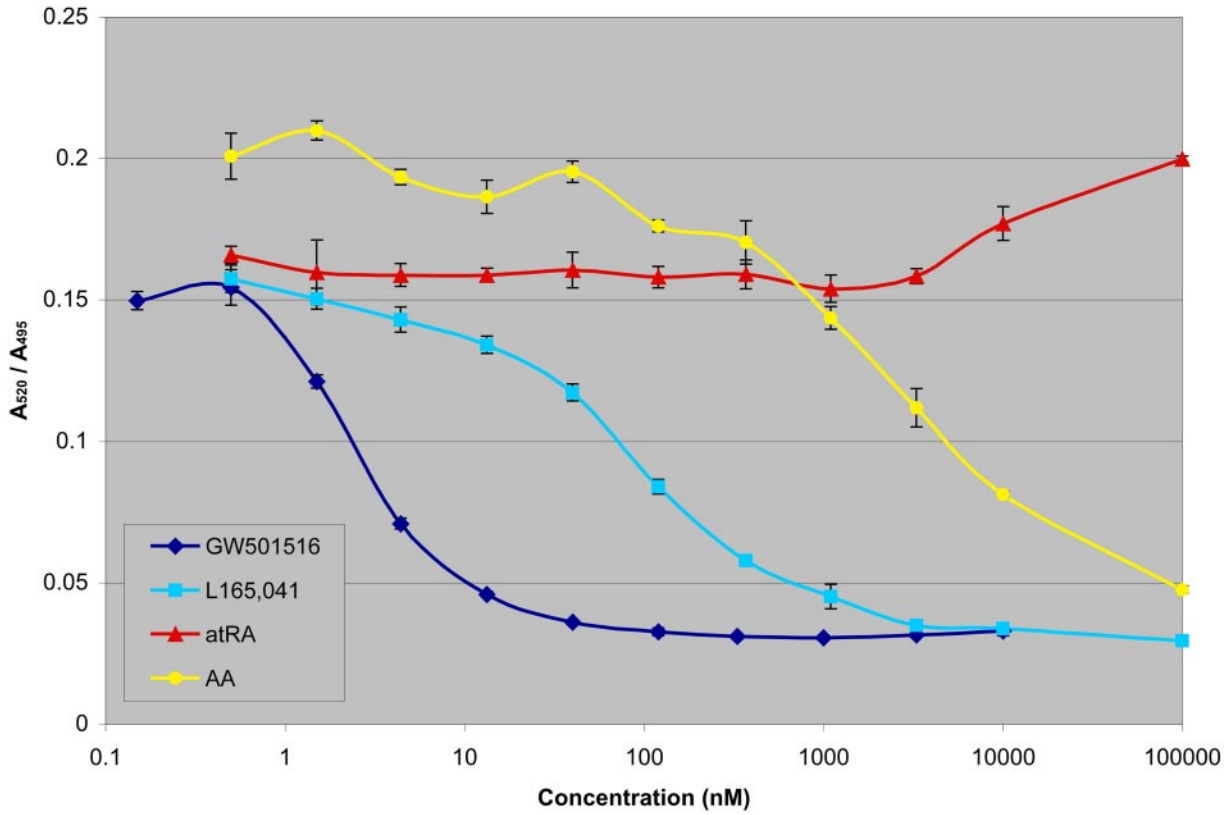

Fig. 6. Competitive in vitro ligand binding assay for PPARβ/δ. Interaction of Fluormone Pan-PPAR Green (PPAR ligand) and recombinant GST-PPARβ/δ. GW501516, L165,041, atRA, and AA were used at the concentrations indicated. Results are expressed as the ratio of fluorescence intensity at 520 nm (fluorescein emission excited by terbium emission) and 495 nm (terbium emission). All data points represent averages of triplicates (± S.D).

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Address correspondence to: Dr. Rolf Müller, Institut für Molekularbiologie und Tumorforschung (IMT), Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, 35032 Marburg, Germany. E-mail: rmueller@imt.uni-marburg.de.