Alteration of a Single Amino Acid in Peroxisome Proliferator-Activated Receptor-α (PPARα) Generates a PPARδ Phenotype

Ichiro Takada, Ruth T. Yu, H. Eric Xu, Millard H. Lambert, Valerie G. Montana, Steven A. Kliewer, Ronald M. Evans, and Kazuhiko Umesono*

Graduate School for Biostudies
Kyoto University (I.T., R.T.Y., K.U.)
Kyoto 606-8507, Japan

Glaxo Wellcome Inc. Research and Development (H.E.X., M.H.L., V.G.M., S.A.K.)
Research Triangle Park, North Carolina 27709

Howard Hughes Medical Institute (R.M.E.)
The Salk Institute for Biological Studies
La Jolla, California 92037

Three pharmacologically important nuclear receptors, the peroxisome proliferator-activated receptors (PPARs α, γ, and δ), mediate key transcriptional responses involved in lipid homeostasis. The PPARα and γ subtypes are well conserved from *Xenopus* to man, but the β/δ subtypes display substantial species variations in both structure and ligand activation profiles. Characterization of the avian cognates revealed a close relationship between chick (c) α and γ subtypes to their mammalian counterparts, whereas the third chicken subtype was intermediate to *Xenopus* (x) β and mammalian δ, establishing that β and δ are orthologs. Like xPPARβ, cPPARβ responded efficiently to hypolipidemic compounds that fail to activate the human counterpart. This provided the opportunity to address the pharmacological problem as to how drug selectivity is achieved and the more global evolutionary question as to the minimal changes needed to generate a new class of receptor. X-ray crystallography and chimeric analyses combined with site-directed mutagenesis of avian and mammalian cognates revealed that a Met to Val change at residue 417 was sufficient to switch the human and chick phenotype. These results establish that the genetic drive to evolve a novel and functionally selectable receptor can be modulated by a single amino acid change and suggest how nuclear receptors can accommodate natural variation in species physiology. (Molecular Endocrinology 14: 733–740, 2000)

0888-8809/00/$3.00/0
Molecular Endocrinology 14(5): 733–740

INTRODUCTION

Lipid and glucose homeostasis involves the coordination of signaling pathways mediated by transcription factors, among which the peroxisome proliferator-activated receptors (PPARs) have been shown to play a major role. The PPARs are members of the nuclear receptor superfamily of ligand-activated transcription factors. Several PPAR subtypes have been described and named PPARα, PPARβ, PPARγ, and PPARδ. The different forms are expressed in tissue-specific patterns: PPARα is abundantly found in liver, kidney, heart, and muscle; PPARγ is localized in fat, large intestine, and macrophages; and PPARs β and δ are widely expressed. The PPARs form a subclass of fatty acid and eicosanoid sensors that are characterized by their distinct pharmacological profiles, a property that has allowed the identification of subtype-selective ligands including the widely used fibrate and thiazolidinedione classes of drugs (for review, see Refs. 1–4 and references therein).

The PPARβ and -δ forms posed a dilemma as to whether they constituted a single group or represented distinct subtypes. Since *Xenopus* PPARβ (xPPARβ) shares only approximately 75% amino acid identity in the ligand-binding domain with mouse and human (h) PPARδ, it was not clear whether these receptors are orthologs or paralogs. This lack of clarity was further exacerbated by the finding that human and mouse PPARδs are functionally distinct from xPPARβ in their response to ligands (5, 6). To better understand the evolutionary relationship between the PPARs, we have isolated the chick counterparts as a means for providing insight into the ancestral form of these

genes after divergence from amphibians. Our results demonstrate that chick and *Xenopus* PPARβ and mammalian PPARδ are orthologs. Moreover, we have exploited cross-species differences in the PPARβ/δ subtype to understand the molecular basis for important pharmacological differences in the ligand binding properties of the PPARs.

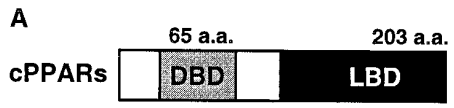
RESULTS AND DISCUSSION

Chick PPAR (cPPAR)-related gene products were obtained from cDNA libraries prepared from 2.5-day-old embryos and adult adipose tissue (see *Materials and Methods*). Isolation and characterization of multiple overlapping clones allowed the compilation of full-length cDNA sequences for all three cPPARs[1] (Fig. 1A, GenBank accession nos. AF163809, AF163810, and AF163811), and an evolutionary tree comparing the ligand-binding domains (LBDs) was constructed using the ClustalW program (Ref. 7 and Fig. 1B). The phylogenetic relationships reveal that PPARα and -γ are highly conserved from *Xenopus* to human but greater divergence exists among the β/δ subtypes, with the chick counterpart forming an intermediary link between *Xenopus* PPARβ and mouse/human PPARδ. This alignment indicates clearly that the β and δ forms constitute a single subtype as the conservation within individual subtypes is much higher than the similarity between that of a given species.

To characterize the ligand response profiles of the cPPARs, we used GAL4 fusions of the LBDs to avoid background activation from endogenous PPARs. Serum-free conditions were chosen as some nuclear receptors are modulated by the addition of FBS. The chick subtypes were found to exhibit distinct ligand response profiles (Fig. 2A; structures of compounds are shown in Table 1). Wy-14,643 and eicosatetroenoic acid (ETYA) are selective PPARα activators; in chick, ETYA activates PPARα to a greater extent than Wy-14,643, as is the case in humans and *Xenopus*, but not in mouse (8, 9). Carbaprostacyclin is active on both PPARα and -β (but $\alpha \gg \beta$) and thiazolidinediones (BRL 49653, Glaxo Wellcome, Inc., Research Triangle Park, NC) are selective for PPARγ as previously reported (5, 10). Among the fibrate derivatives, bezafibrate and GW2331 (11) were capable of activating PPARβ; other fibrates (fenofibrate and gemfibrozil) were active only on PPARα.

This ability of some of the fibrates to activate cPPARβ prompted us to compare the effect of bezafibrate, GW2331, and carbaprostacyclin in the mammalian and chick PPARβ/δs. As shown in Fig. 2B, the responses elicited by bezafibrate and GW2331 were distinct between chick (c) and human (h). The potency of bezafibrate on cPPARβ was similar to that seen with xPPARβ (6). Carbaprostacyclin activated hPPARδ and cPPARβ with equal efficiency.

To determine whether these compounds could directly bind to PPARδ/β, we used protease digestion assays. Addition of increasing concentrations of trypsin in the presence of 100 μM bezafibrate or 1 μM GW2331 to ^{35}S-labeled cPPARβ resulted in the appearance of protease-resistant fragments of approxi-



DBD	α	β/δ	γ	
Human	95	98	100	(%)
Mouse	95	95	100	
Xenopus	86	86	97	

LBD	α	β/δ	γ	
Human	96	90	96	(%)
Mouse	91	86	95	
Xenopus	92	78	90	

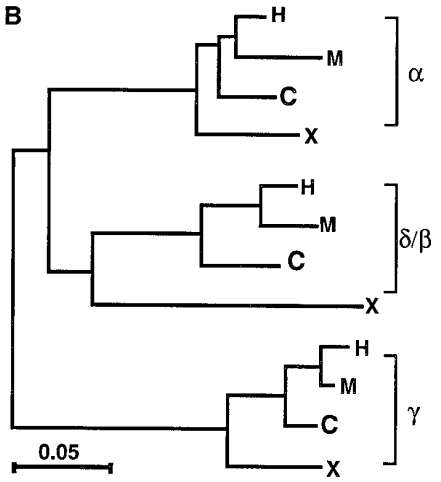


Fig. 1. Comparison of Chick PPARs with Human, Mouse, and *Xenopus* Counterparts

A, Schematic of the DBDs and LBDs of cPPARs compared with the corresponding regions of the human, mouse, and *Xenopus* receptors. *Numbers* indicate percent identity to the corresponding cPPAR. B, Tree plot comparison of human (H), mouse (M), chick (C), and *Xenopus* (X) PPAR LBDs. Human RXRα was used as the root for this tree. 0.05 indicates the frequency of amino acid change (maximum is 1). The Clustal W program (7) was used for alignment.

[1] The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF163809 (cPPARα), AF163810 (cPPARβ/δ), and AF163811 (cPPARγ)].

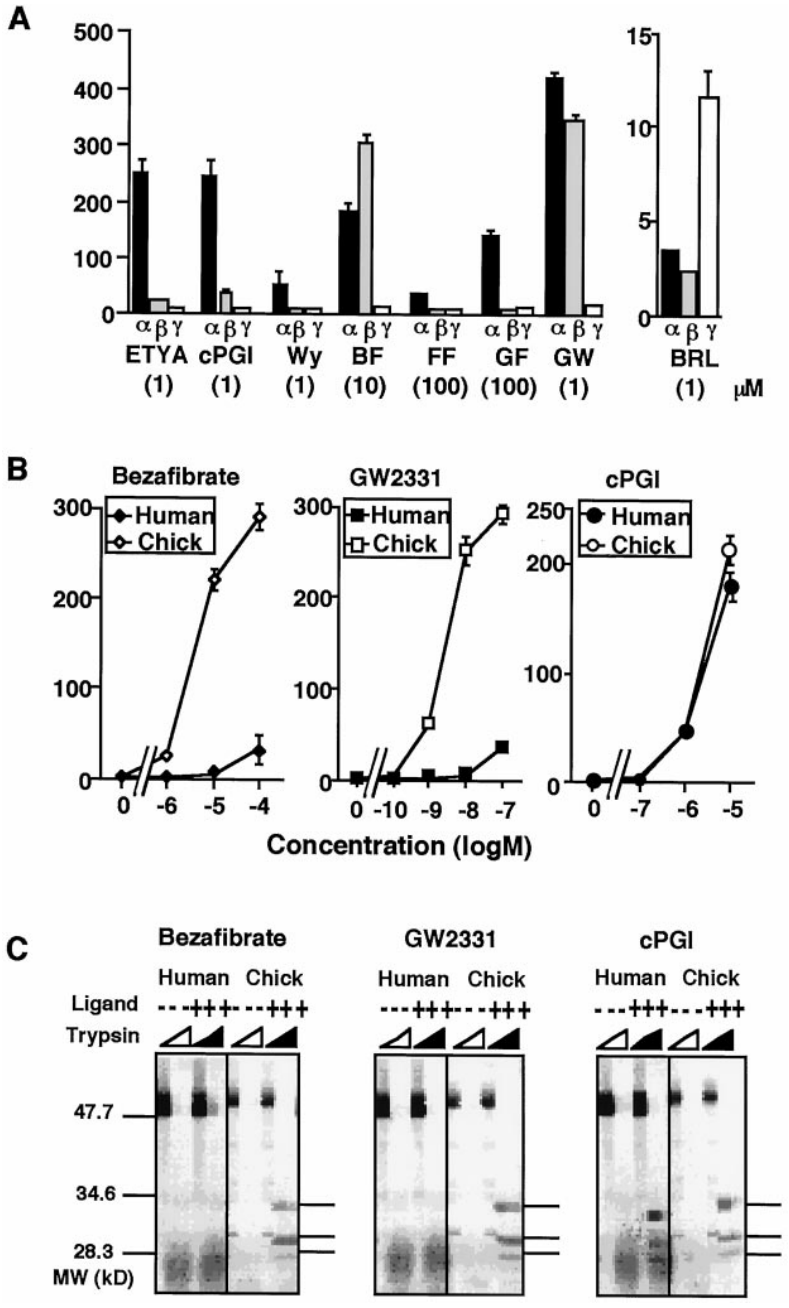
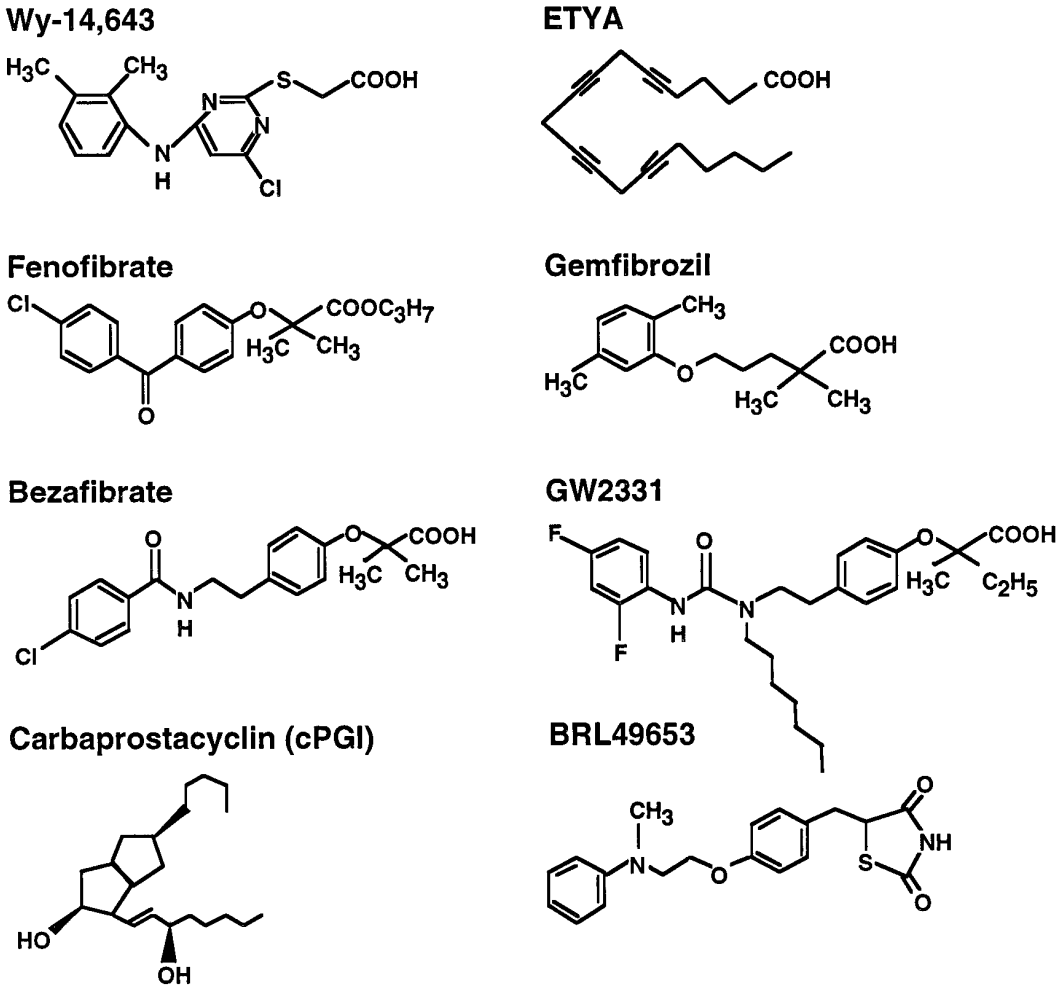


Fig. 2. Transcriptional Activity of GAL-cPPAR Fusion Receptors

CV-1 cells were cotransfected with a reporter gene containing four copies of a GAL4 binding site (MH-100x4-tk-Luc) in the presence or absence of a chimeric receptor (GAL4-cPPARα,β,γ). Activation of the luciferase reporter gene was measured in relative light units with β-galactosidase activity as a control for transfection efficiency and presented as fold activation. Ligand response data are derived from triplicate points from two independent experiments and represented as the mean ± SE; n = 6. A, Comparison of the fold activities of cPPARα, -β, and -γ by the indicated compounds. *Numbers within brackets* represent the ligand concentration in micromoles. cPGI, carbaprostacyclin, Wy; Wy-14,643, BF; bezafibrate, FF; fenofibrate, GF; gemfibrozil, GW; GW2331, BRL; BRL 49653. B, Chick and human GAL4-PPARβ/δs were analyzed in cotransfection assays with bezafibrate, GW2331, and carbaprostacyclin. Bezafibrate and GW2331 appear specific for cPPARβ. C, ^{35}S-human and chick PPARδ/β were preincubated for 30 min at 37 C with either 100 μM bezafibrate, 1 μM GW2331, or 100 μM carbaprostacyclin before addition of trypsin (final concentrations of 20 and 40 μg/ml, respectively). Proteolytic digestions were carried out at 37 C for 10 min, and then samples were denatured and electrophoresed on a 12.5% SDS-polyacrylamide gel. In the presence of bezafibrate and GW2331, only cPPARβ shows protected fragments (*arrows*). Addition of carbaprostacyclin results in protected fragments for hPPARδ and cPPARβ (*arrows*).

Table 1. Chemical Structures of the Compounds Used in This Study



mately 32 kDa, 29 kDa, and 27 kDa (Fig. 2C, *arrows*), but no protected bands were observed with hPPARβ. With carbaprostacyclin, protease-resistant fragments of similar sizes were observed with both human and chick PPARδ/β. It is thus apparent that PPARβ/δ ligands can be classified into those with species-selective activity (bezafibrate, GW2331) and those without (carbaprostacyclin).

To determine the region essential for ligand-selective recognition by PPARβ/δ, we examined the structures of the chick, *Xenopus*, mouse, and human homologs (12–14). Although cPPARβ LBD and xPPARβ LBD share only 71% amino acid identity (216/303) *vs.* 90% (272/303) between chick and human, the ligand activation properties of the cPPARβ LBD more closely resemble those of *Xenopus* (6, 11). Detailed comparison of the LBD sequences of cPPARβ with those of human, mouse, and *Xenopus* revealed that 200 amino acids (a.a.) are conserved with the remaining 103 a.a. varying between species.

Taking into consideration the similarity in ligand response between chick and *Xenopus*, we focused on 9 a.a. that are conserved between chick and *Xenopus,* but not between chick and human/mouse. A series of chimeric human and chick PPARδ/β expression constructs were made in an attempt to further localize the key residues involved in the ligand specification (Fig. 3A). Examination of the response of these receptors to bezafibrate, GW2331 and carbaprostacyclin indicated that the domain spanning from the hinge region to helix 9 is not critical for recognition of either bezafibrate or GW2331 by the cPPARβ, but that helix 10, containing a net change of 3 a.a., was essential for recognition of both compounds (Fig. 3B).

During the course of this work, we solved the crystal structure of the fibrate GW2331 bound to hPPARδ (Fig. 4). As in the case of other PPAR ligands, the carboxylic acid of GW2331 (see Table 1) was found to form an intricate series of hydrogen bonds with histi-

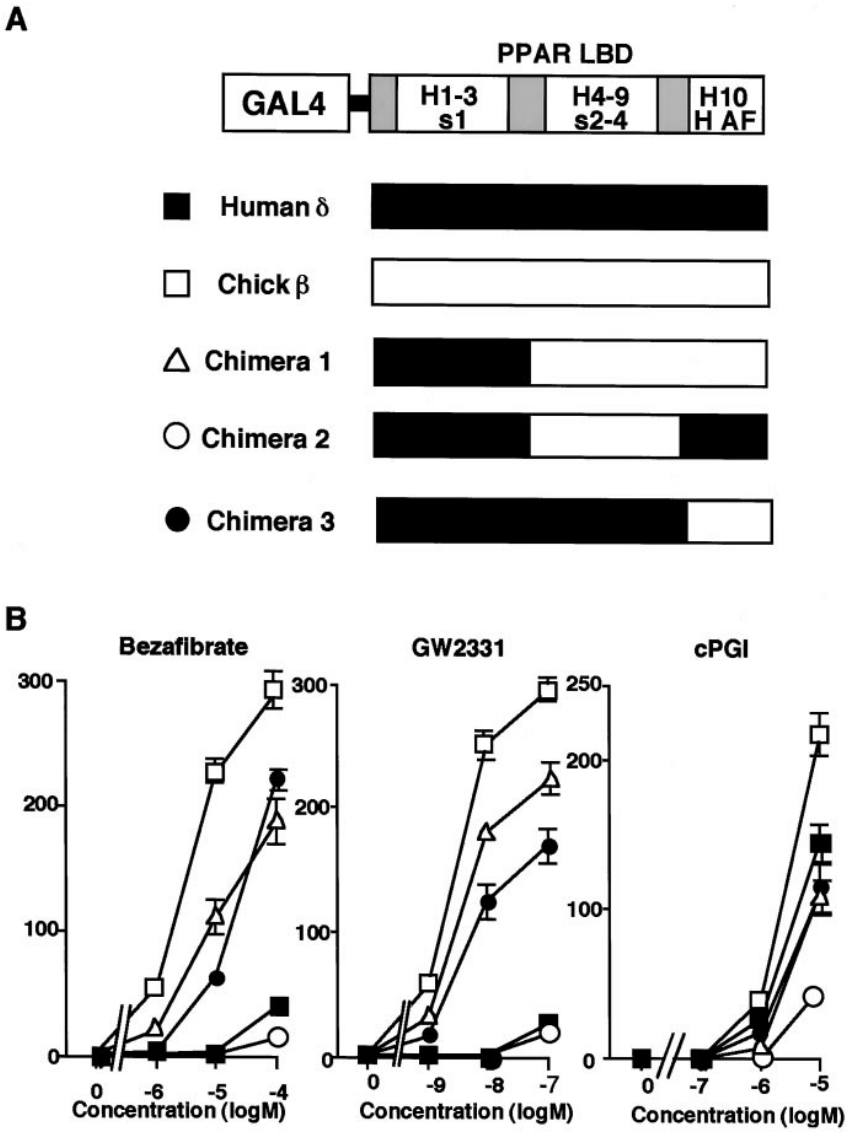


Fig. 3. The C-Terminal Region of cPPAR Is Required for Fibrate-Dependent Activity

A, Schematic representations of chimeric GAL4-hPPARδ/cPPARβ fusion proteins (*numbers* indicate the a.a. position from the first methionine). Chimera 1 (*open triangle*) is a fusion of hPPARδ (137–261) and cPPARβ (264–443), chimera 2 (*open circle*) encodes a fusion of hPPARδ (137–261), cPPARβ (264–383), and hPPARδ (382–441), and chimera 3 (*solid circle*) is a fusion of hPPARδ (137–381) and cPPARβ (384–443). B, Cotransfection experiments were performed as described in Fig. 2 with the addition of bezafibrate, GW2331, and carbaprostacyclin as indicated. Chimeras 1 and 3 showed response to bezafibrate and GW2331, and all constructs were responsive to carbaprostacyclin. *Vertical axis* represents fold activation.

dine residues in helices 5 and 10 and a tyrosine in the AF-2 helix (15, 16). We postulate that this network of interactions effectively locks the receptor into a conformation permissive for coactivator interactions. Notably, M417 in helix 10 is bent into an unfavorable conformation for accommodation of the gem-dialkyl constituent of the GW2331 fibrate headgroup. The steric interference between M417 and the fibrate may explain the relatively low-affinity binding of GW2331 to hPPARδ.

Sequence comparison revealed that both the chick and *Xenopus* PPARβ subtypes have a valine residue at the position analogous to M417. The shorter side chain of valine would be expected to better accommodate the gem-dialkyl substituent of the fibrate headgroup. These data suggested that this single residue might be a key determinant in the binding of fibrates to PPARs. To test this hypothesis, we constructed the following mutants (depicted in Fig. 5A); one in which M417 of hPPARδ was

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.