Substitution of a conserved amino acid residue alters the ligand binding properties of peroxisome proliferator activated receptors

Mirsada Causevic, C. Roland Wolf, Colin N.A. Palmer*

Biomedical Research Centre and ICRF Molecular Pharmacology Unit, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, UK

Received 5 November 1999

Edited by Philip Randle

Abstract Mutation of glutamic acid 282 of PPARα to glycine has been shown to result in an increased EC_{50} for a wide variety of PPAR activating compounds. This has suggested that mutant receptor has a reduced ability to bind ligand. In this study we show that this mutation reduces the affinity of mPPARα and hPPARγ for the fluorescent fatty acid, *cis*-parinaric acid and that the mutant hPPARγ protein has a reduced affinity for the radiolabelled compound, SB236636. These data confirm the role of this glutamic acid in ligand binding and support recent crystal structure observations regarding a proposed novel mode of ligand entry into the PPAR ligand binding cavities.

Key words: Nuclear receptor; Peroxisome proliferator; Fatty acid; Fluorescence; Thiazolidinedione; Diabetes

1. Introduction

The peroxisome proliferator activated receptors (PPAR) belong to the nuclear receptor family of ligand activated transcription factors. These receptors regulate lipid metabolism in response to fatty acids and eicosanoids and are targets for drugs used in the treatment of hyperlipidemia and diabetes. PPARα is expressed at high levels in the rodent liver and is activated by the fibrate family of hypolipidemic drugs [1,2]. PPARα also mediates the pathological effect known as peroxisome proliferation caused by these drugs in the rodent liver. The absolute requirement for PPARα in these events has been confirmed in mouse containing a targeted disruption of the PPARα gene [3–5]. The PPAR subfamily contains two other members known as PPARδ and PPARγ. The biological activity of PPARδ is relatively obscure, although a role in embryonic implantation has been proposed [6]. PPARγ, on the other hand, has been intensively investigated with proposed roles in diabetes [7], atherosclerosis [8], inflammation [9], and cancer [10–12]. PPARγ is expressed in adipose tissue and has been shown to be an important determinant in the differentiation of adipocytes from fibroblasts and myoblasts [13–15]. PPARγ is activated by the isoprostanoid 15-deoxy $\Delta^{12,14}$ PGJ2 [16,17] and the thiazolidinedione family of insulin sensitising drugs [18]. It has recently been proposed that the action of PPARγ in insulin sensitisation and controlling inflammation is due to the inhibition of the expression of a wide range of cytokines including TNFα [9,19]. PPARs are molecular switches that bind activating compounds and upon doing so changes occur in the protein conformation mediating an increase in the transcription of target genes. In studies with mouse PPARα, it was noted that PPARα displayed extensive activation of transcription without the addition of activating compound and, upon the addition of fatty acids and fibrate drugs, only very small responses were observed [20,21]. This was not the case when a PCR-derived mutant form of PPARα was used in the same experiments. This mutant, which contained a substitution of a glycine for glutamic acid 282, displayed very little transcriptional activation in the absence of added drugs and responded very well to the addition of fatty acids and fibrate drugs. Careful examination of the concentration dependency of the activation of both forms of the receptor revealed that the mutant receptor required higher concentrations of all ligands studied for maximal activation when compared to the wild type protein [20]. This gave rise to the hypothesis that the GLU282GLY substitution resulted in a diminished affinity for ligand binding and that this lowered affinity was no longer sufficient to permit activation by the endogenous ligand that provided the constitutive activation of the wild type receptor. This observation was supported by the finding that increased levels of ligands were required to stimulate mutant PPARα binding to DNA in a heterodimer with retinoid X receptor (RXR) [22].

In this study we show that the GLU282GLY substitution in mouse PPARα leads to a lower efficiency of binding to a fluorescent fatty acid, *cis*-parinaric acid (CPA), and that the corresponding mutation in human PPARγ leads to reduced binding of both CPA and the high affinity ligand, SB236636.

2. Materials and methods

2.1. Expression of PPAR ligand binding domains (LBD) in Escherichia coli

The PPAR coding sequences were isolated by the polymerase chain reaction using primers that generated a *Nde*I site/ATG initiation codon at the 5′ end and a *Bam*HI site immediately after the terminator codon. For mPPARα, nucleotides 674–1573 of GenBank accession number X57638 were amplified using the upper primer: GGAATTCCATATGGCAATTCGCTTTGGAAGAATGCC and the lower primer: CGGGATCCTCAGTACATGTCTCTGTAGATCTC. The resulting sequence encodes amino acid 170–468 of mouse PPARα. For hPPARδ, nucleotides 761–1663 of GenBank accession number L07592 were amplified using the upper primer: GGAATTCCATATGGCTATCCGTTTTGGTCGGATG and the lower primer:

*Corresponding author. Fax: (44)-1382-669993.
E-mail: palmerc@icrf.icnet.uk

Abbreviations: CPA, *cis*-parinaric acid; EPA, eicosapentanoic acid; PPAR, peroxisome proliferator activated receptor; ER, oestrogen receptor; TR, thyroid hormone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; LBD, ligand binding domain

PII: S0014-5793(99)01618-X

CGGGATCCTTAGTACATGTCCTTGTAGATCTCCTG. The resulting sequence encodes amino acid 142–442 of human PPARδ. For mPPARγ, nucleotides 577–1428 of GenBank accession number U10374 were amplified using the upper primer: GGAATTCCATATGGCGGAGATCTCCAGTG and the lower primer: CGGATCCTAATACAAGTCCTTGTAGATCTCCTG. The resulting sequence encodes amino acids 192–475 of mouse PPARγ. The amplified products were cloned into *Nde*I/*Bam*HI digested pET15b and transformed into *E. coli* strain BL21(DE3) pLYSs. Expression of the His-tagged LBD was induced with 0.5 mM IPTG at 25°C for 2 h. Lysates from these cultures were prepared and the expressed protein purified by nickel/agarose affinity chromatography.

2.2. Mutation of a human PPARγ ligand binding domain

The portion of the human PPARγ cDNA encoding the LBD was isolated by PCR from a human kidney cDNA library (Clonetech) as previously described [23]. The amplification product was cloned into pET15b as described for the other PPAR cDNAs. Oligonucleotide directed mutagenesis was used to substitute a glycine for a glutamic acid at codon 291.

2.3. CPA fluorescence assays

Purified PPAR LBDs were mixed with CPA in 25 mM Tris–HCl (pH 7.5) at room temperature and the resulting fluorescence was measured immediately in a Perkin Elmer fluorimeter using a excitation wavelength of 318 nm and an emission wavelength of 410 nm. The fluorescence resulting from protein alone and CPA alone were totaled and deducted from the experimental value. Graphs were plotted using Graphpad Prism 2.0 for Macintosh and the data subjected to a non-linear regression analysis based on a sigmoidal dose response. This provided the K_d and standard error. All K_ds stated in the text were obtained using concentrations of ligand between 4 and 10 fold below the EC_{50} in order to avoid stoichiometric binding.

2.4. Rapid filtration binding assays

Purified mutant and wild type PPAR LBDs (5 nM) were incubated with increasing concentrations of I^{125} labelled SB236636 as previously described [24]. The mixtures were incubated on ice for 2 h and then applied to 25 mm Millipore HAWP filters on a vacuum manifold. The filters were washed twice with 5 ml of ice cold binding buffer, dried and the bound radioactivity was determined by liquid scintillation spectrophotometry. All assays represent means of triplicate samples minus the value determined for non-specific binding to the filter. The K_d is calculated as the slope of the Scatchard plot.

3. Results

Expression of the soluble PPAR LBDs in *E. coli* was achieved using the pET15b expression vector as previously described [23]. PPARα and PPARδ consistently provided

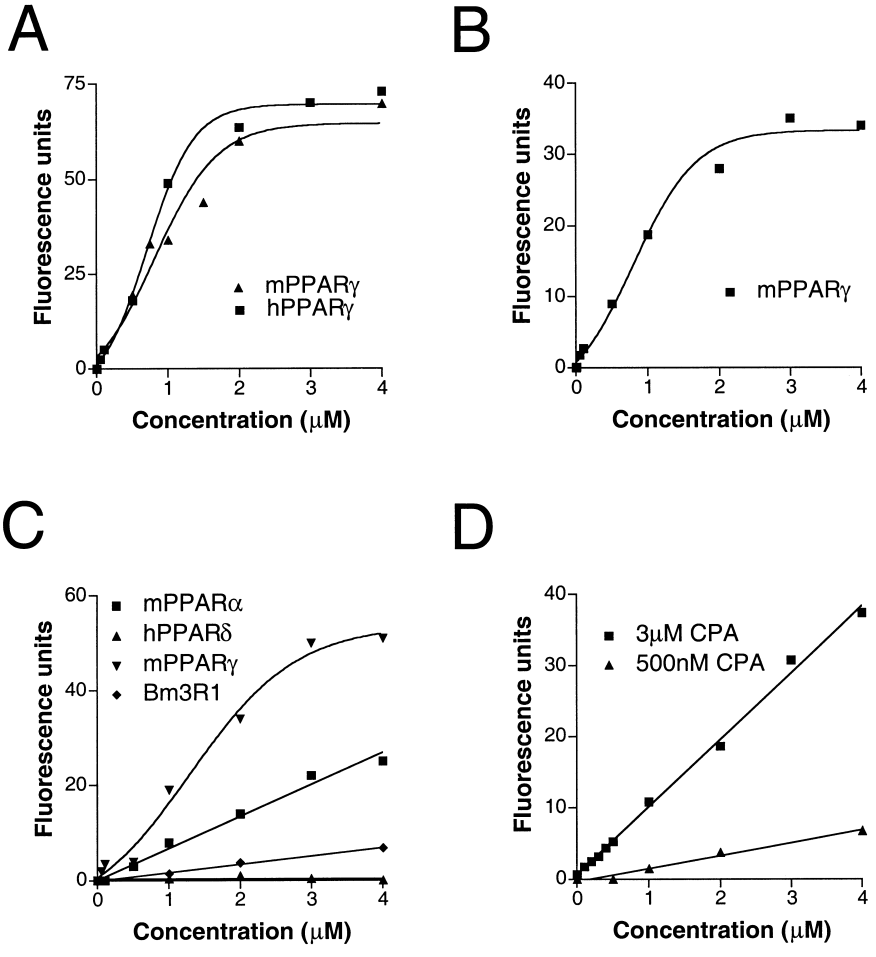

Fig. 1. The fluorescent lipid probe, CPA, binds to PPARs. A: Increasing concentrations of human and mouse PPARγ LBDs were mixed with CPA (500 nM). The resulting fluorescence is shown. B: Increasing concentrations of the mouse PPARγ LBD were mixed with CPA (200 nM). The resulting fluorescence is shown. C: Purified PPAR LBDs were mixed with CPA (400 nM) in 25 mM Tris–HCl (pH 7.5). The fluorescence values obtained using mouse PPARα and γ, and human PPARδ are shown. Trypsinogen (5 μM) served as a negative control and Bm3R1, a bacterial fatty acid sensor serves as a positive control. D: Increasing concentrations of purified Bm3R1 were mixed with either 500 nM or 3 μM CPA. Saturation binding was not observed under either conditions suggesting the K_d of Bm3R1 binding to CPA is very high. Indeed, the K_d of Bm3R1 binding to CPA is approximately 25 μM as judged by the ability of CPA to disrupt a Bm3R1/DNA complex in vitro (data not shown).

less soluble protein when compared to either mouse or human PPARγ, and culture conditions for the induction of expression of these proteins had to be performed at below 25°C (data not shown). The proteins were all highly purified by nickel-agarose affinity chromatography and assayed for their ability to bind the fluorescent fatty acid CPA. When assayed for fluorescence in the presence of 500 nM CPA, both mouse and human PPARγ LBDs produced saturable increases in fluorescent signal (Fig. 1A). The binding efficiency is similar for the mouse and human proteins. This was confirmed in further assays using 200 nM CPA where a K_d of 800 ± 100 nM was obtained for mPPARγ (Fig. 1B) and this may be compared with a K_d of 600 nM for the human isoform (Fig. 1A and reference [23]).

The mouse PPARα LBD also produced fluorescence in the presence of CPA, however the hPPARδ did not produce significant fluorescence under these conditions (Fig. 1C). Weak binding to hPPARδ was, however, observed at high concentrations of protein and CPA (data not shown). Trypsinogen used as a control produced no significant fluorescence even at greater concentrations of CPA and protein than those used for the PPAR proteins (data not shown). Bm3R1, a fatty acid sensor from *Bacillus megaterium*, displayed weakly increasing fluorescence over the protein concentrations studied (Fig. 1C). Increasing the concentration of CPA to 3 μM gave much stronger fluorescence with Bm3R1 but was not saturated at the concentrations of protein used, indicating a K_d well above the concentrations studied (Fig. 1D). Fatty acids disrupt the binding of Bm3R1 to its operator DNA sequence and in vitro DNA binding assays can be used to measure the concentrations at which Bm3R1 may interact with the fatty acids. Such in vitro assays indicate that Bm3R1 has a K_d for CPA of about 25 μM (C.Palmer, unpublished data). Bm3R1, however, binds other polyunsaturated fatty acids with a K_d of around 1 μM [25,26]. In summary, the rank order of affinities for CPA are hPPARγ ≥ mPPARγ > mPPARα > Bm3R1 > hPPARδ ⋙ trypsinogen.

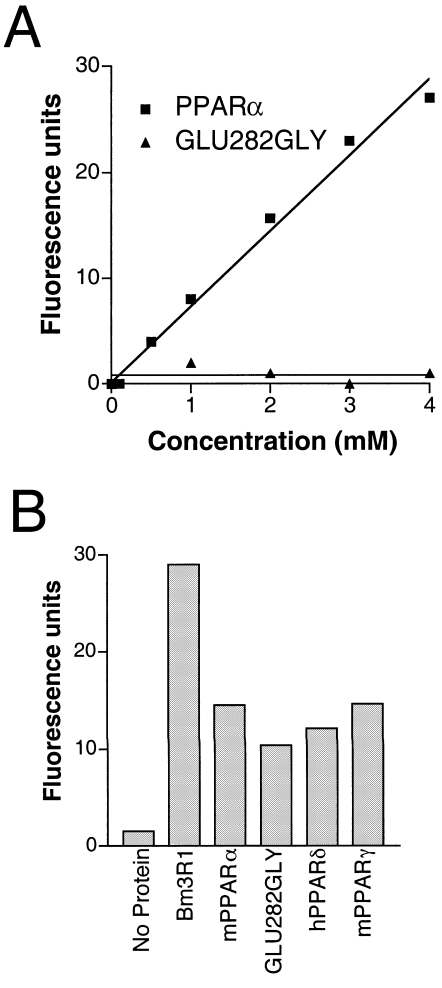

Fig. 2. mPPARα-Glu282Gly displays reduced binding to CPA. A: Increasing concentrations of purified LBDs were mixed with CPA (400 nM). The fluorescence values obtained using mouse PPARα and the mPPARα-Glu282Gly are shown. A reduced binding of the GLU282GLY mutant form of mPPARα relative to the wild type form is evident. B: Purified PPAR LBDs (2 μM) were mixed with 12-AO (1 μM) in 25 mM Tris–HCl (pH 7.5). The resulting fluorescence is shown.

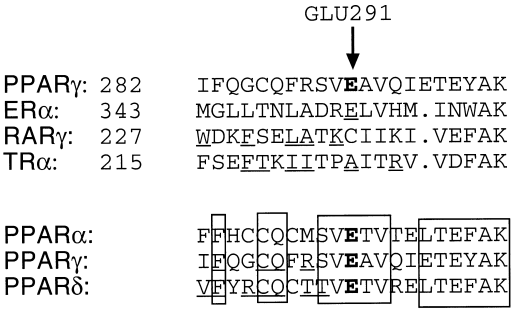

Fig. 3. Helix 3 is a conserved ligand binding site for all nuclear receptors. Shown is an alignment of the helix 3 sequences from several nuclear receptors. Known ligand interacting residues are underlined and conserved regions of PPAR helix 3 are boxed. The conserved glutamic acid is shown in bold.

The fluorescent property of CPA was exploited in order to determine whether or not the GLU282GLY mutant of mouse PPARα is impaired in its ability to bind ligands. When the wild type and mutant mPPARα proteins were compared it was evident that the GLU282GLY mutant bound very poorly to CPA (Fig. 2A), thus confirming the hypothesis that the transcriptional effects seen with this mutant are due to a disruption of the ligand binding properties of the encoded protein. However, due to the weak interaction of both forms of PPARα it was impossible to quantitate the difference in affinity. Also, it was possible that the mutation resulted in a protein defective in some non-specific manner. In order to address this issue and determine the specificity of the binding of CPA, we used the fluorescent fatty acid 12-anthracene oleic acid (12-AO). This fatty acid binds to the fatty acid-binding pocket of the adipocyte fatty acid-binding protein in a manner not affected by mutations in the residues responsible for the binding of the fatty acid carboxylic acid group [27]. This is due to additional hydrophobic interactions between the hydrophobic anthracene moiety and the binding pocket. Bm3R1 has a K_d of 625 nM for 12-AO [23] and was therefore used as a positive control in this assay. Bm3R1 appeared to bind more efficiently when compared to the PPAR proteins. All the PPAR proteins bind to 12-AO with a similar efficiency (Fig. 2B), suggesting that the selectivity observed with CPA was not due to the functionality of the expressed proteins.

In order to further investigate and quantify the role of the glutamic acid in ligand binding, the cDNA encoding the hu-

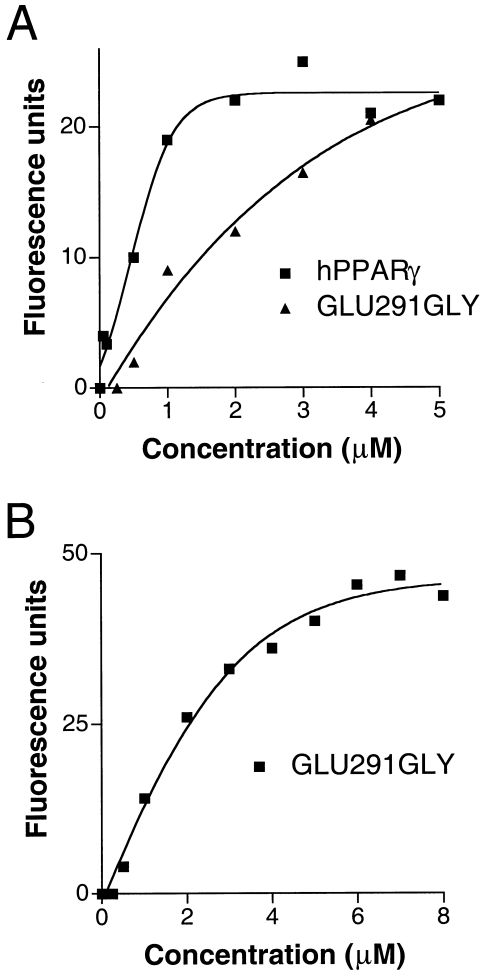

Fig. 4. PPARγ-Glu291Gly displays a reduced efficiency of binding for CPA. Increasing concentrations of mutant and wild type proteins were added to CPA (150 nM) as before. Higher concentrations of the mutant protein were required to obtain a fluorescent signal when compared to the wild type protein. Both proteins were of similar purity and yield as judged by SDS-PAGE (data not shown). PPARγ-Glu291Gly assayed in the presence of 300 nM CPA.

man PPARγ LBD was modified using site directed mutagenesis to encode a glycine substitution at position 291. GLU291GLY corresponds to the GLU282GLY mutation already studied in mouse PPARα (Fig. 3). This residue is conserved within all members of the PPAR subfamily and therefore it is possible that it may affect the binding of many PPAR ligands (Fig. 3).

The wild type and mutant forms of hPPARγ were then prepared and assayed for fluorescence in the presence of CPA (Fig. 4A). It is apparent that the wild type protein yields fluorescent signal at much lower protein concentrations than observed for the mutant protein; thus, demonstrating that the GLU291GLY substitution attenuates the physical binding of fatty acid ligands to PPARγ as well as to PPARα. The values obtained using 150 nM CPA were 560 ± 70 nM for the wild type and 1.82 ± 0.3 μM for the mutant protein. The assays were also performed for the mutant at 300 nM due to the higher apparent K_d of this protein (Fig. 4B). This analysis gave a K_d of 1.9 ± 0.19 μM in good agreement with the assays performed with 150 nM CPA. Therefore, there is a 3.5 fold difference in apparent K_d for CPA between the wild type and mutant proteins.

PPARγ is a high affinity receptor for the thiazolidinedine group of insulin sensitising drugs such as troglitazone and BRL49653. In order to determine whether the GLU291GLY substitution affected the binding of synthetic drugs, we performed rapid filter binding studies using I^{125}-labelled

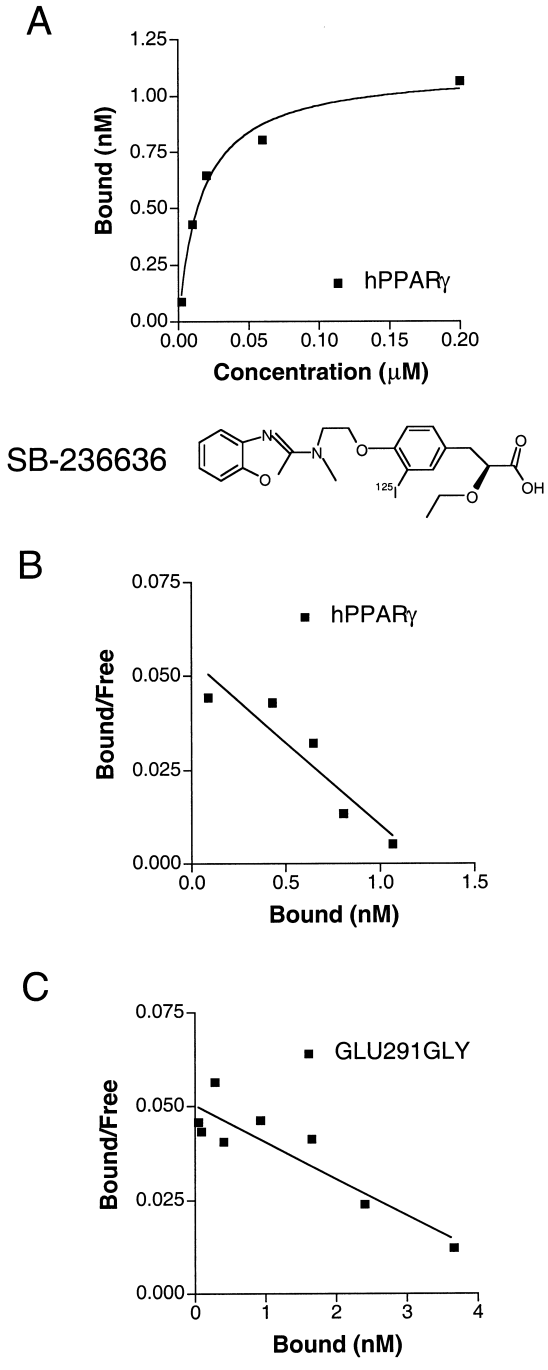

Fig. 5. PPARγ-Glu291Gly has a reduced binding affinity for the the high affinity drug, SB236636. Purified mutant and wild type PPAR LBDs were incubated with increasing concentrations of I^{125} labelled SB236636 and assayed by rapid filtration as described in Section 2. Saturating binding was observed (A). Scatchard analysis of the wild type (B) and mutant protein (C) binding to SB236636 reveals that the mutant receptor displays about a four fold reduction in binding affinity for SB236636.

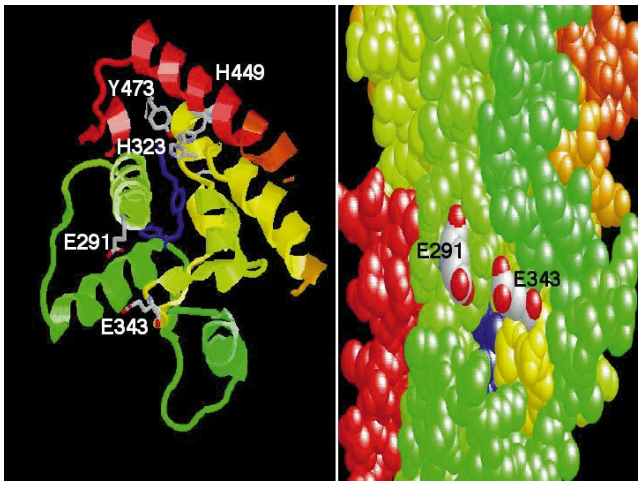

Fig. 6. Glu-291 and Glu-343 form a hydrophilic jaw around the novel ligand entry site proposed for PPARγ. The structure of the high affinity ligand, rosiglitazone, bound to hPPARγ was visualised using RASMAC version 2.6 from the coordinates supplied as 2PRG.pdb. The high affinity ligand, rosiglitazone, is shown in blue.

SB236636 (Fig. 5). Scatchard analysis of the binding of wild type hPPARγ to SB236636 revealed a K_d of 23 nM. Similar experiments using the mutant protein revealed a K_d of 100 nM. In both cases the receptor concentration (*x*-axis intercept) is very low compared to the amount of recombinant protein added. This is not due to inactive receptor in the preparations as we have confirmed the stoichiometry of binding of human PPARγ protein to CPA as being 1 molecule of CPA per monomer unit of PPARγ [23]. This underestimate of receptor concentration is therefore, presumably, due to the time-dependent dissociation of ligand during the filtration process. The Scatchard analysis revealed that the mutant receptor had a higher capacity for ligand than the wild type mutation (compare Fig. 5B and C). This again indicates that the mutant receptor is not defective in a non-specific manner. These results demonstrate that glutamic acid 291 is an important residue in the binding of high affinity drugs as well as for the binding of fatty acids.

4. Discussion

The involvement of a conserved glutamic acid in the ligand binding of PPARs has been confirmed using a fluorescent fatty acid ligand in both mPPARα and hPPARγ. This residue also influences the binding of the high affinity ligand, SB236636. It appears from the crystal structures of oestrogen receptor (ER), retinoic acid receptor (RAR) and thyroid hormone receptor (TR) that this region is involved in the binding of the carboxylic acid functions of their respective ligands. This is supported by the fact that his mutation does not affect the binding of the hydrophobic probe, 12-anthracene oleic acid. Our data would suggest that the glutamic acid may be directly involved in the coordination of fatty acid carboxylic acid groups. The role for glutamic acid in the coordination of carbonyl groups has recently been shown to be important in the binding of oestrogen to its nuclear receptor. In the ER, glutamic acid 353 is the corresponding residue to GLU291 PPARγ (Fig. 3). Glu-353 of ER cooperates with water and arginine 394 to form hydrogen bonds with the C3 carbonyl group of oestrogen [28]. However, this role for Glu-291 has not been observed in the recent crystal structures of hPPARγ containing the high affinity ligand BRL 49653 or the crystal structure of eicosapentanoic acid (EPA) bound to PPARδ [29,30]. In both cases hydrogen bonding of the compounds does not directly involve this residue. The structure proposed for the PPARs has shown that the carbonyl groups are hydrogen bonded to His-323 and His-449 (Fig. 6). These interactions are also utilised in the binding of EPA to PPARδ and appear to be required for the stabilisation of the 'charge clamp' that binds the co-activator proteins thus providing the molecular basis for ligand activated transcription.

In the binding of EPA to PPARδ, several helix 3 residues have hydrophobic interactions with the region of the acyl chain immediately preceding the carboxylate terminus. Most importantly, the acid-head group is held in place by a hydrophobic interaction with threonine 289 which is less than one helical turn from glutamic acid 291. It would therefore be possible that substitution of alanine for glutamic acid may interfere with this interaction. This may explain the differences observed in ligand binding and the lack of effect that this mutation has on the binding of 12-anthracene oleic acid. However, there is an alternative explanation for the role of this residue in ligand binding based on the observed orientation of Glu-291 in the crystal structures. In both *holo*- and *apo*-PPARγ, glutamic acid 291 appears to form an upper 'jaw' for the rather open space between H3 and the β-sheet which has been recently proposed to be the ligand entry site [29,30]. This 'jaw' has a glutamic acid 343 forming the lower portion and these two residues may provide a trapping mechanism for the acidic fatty acids. This evokes a 'lobster pot' type ligand entry model as opposed to the previously proposed 'trap door' model in which the AF2 helix would be pulled 'closed' towards helix 3 upon entry of a fatty acid. The lack of movement of the AF2 domain between *apo*- and *holo*-PPAR structures would also suggest that the fatty acid may enter from the other 'lobster pot' entry site. Further site-directed mutagenesis and biophysical analysis is required to determine the relevance of these two binding models.

In summary, glutamic acid 291 is a conserved amino acid between PPARs that is involved in the binding of ligands; however, this residue is not in itself a ligand contact residue. This study provides the first experimental evidence for the binding of ligands through a novel entry site that was first visualised by X-ray crystallography.

Acknowledgements: We would like to thank Dr Steve Smith and Dr Greg Murphy at SmithKline Beecham for their advice and for the radioligand, SB236636. This work was supported by a BBSRC ROPA Award No. MOLO4650

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