Identification of Residues Important for Agonist Recognition and Activation in GPR40*

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GPR40 was formerly an orphan G protein-coupled receptor whose endogenous ligands have recently been identified as free fatty acids (FFAs). The receptor, now named FFA receptor 1, has been implicated in the pathophysiology of type 2 diabetes and is a drug target because of its role in FFA-mediated enhancement of glucose-stimulated insulin release. Guided by molecular modeling, we investigated the molecular determinants contributing to binding of linoleic acid, a C18 polyunsaturated FFA, and GW9508, a synthetic small molecule agonist. Twelve residues within the putative GPR40-binding pocket including hydrophilic/positively charged, aromatic, and hydrophobic residues were identified and were subjected to site-directed mutagenesis. Our results suggest that linoleic acid and GW9508 are anchored on their carboxylate groups by Arg183, Asn244, and Arg258. More-
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be stronger with GW9508 than with linoleic acid. Importantly, GW9508 is uniquely able to make contact with His$^{137}$. Moreover, His$^{137}$ and particularly His$^{46}$ appear to interact with GW9508 in a pH-dependent manner. This study therefore provides an explanation for the more potent interaction found with this small molecule agonist than with linoleic acid and provides insight into the underlying mechanism of binding and activation for this receptor.

EXPERIMENTAL PROCEDURES

Materials—Linoleic acid was obtained in the form of a sodium salt from Spectrum (Gardena, CA). A 10 mM stock was prepared freshly with deionized water for each experiment. GW9508 was synthesized according to the previously reported procedure (3) and stored as 10 mM aliquots in Me$_2$SO. Ligand solutions of different concentrations were made in Hanks’ buffered saline solution. Me$_2$SO is included in the solutions to a final concentration of 0.2% in the assay well. The fluorescent calcium dye Calcium4 was obtained from Molecular Devices Corp. (Sunnyvale, CA). Anti-FLAG M2 antibody conjugated to fluorescein isothiocyanate was purchased from Sigma. Other chemicals were purchased from standard sources.

Residue Nomenclature—A residue in GPR40 is referred to by its amino acid identity in conjunction with its position along the amino acid sequence. Where comparison with other GPCRs would be informative, the nomenclature described by Balles-teros and Weinstein (20) is used alongside. In this nomenclature, the most conserved residue in a given transmembrane helix is assigned the index x.50 (where x is the transmembrane number), whereas the other residues are numbered relative to this position.

GPR40 Constructs and Site-directed Mutagenesis—Untagged, wild type GPR40 was a gift from Dr. Brian O’Dowd (1) and was subcloned into pcDNA3.1/hygro (+) (Invitrogen). Site-directed mutagenesis was performed using the QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA). To construct FLAG-tagged GPR40, a FLAG epitope was inserted at the N terminus of the receptor between the first methionine and second amino acid residue. A two step procedure was employed. In the first step, the full GPR40-encoding plasmid was amplified using standard PCRs with two 5’-phosphorylated primers. The forward primer was 5’-atggactacaagcagatgacaggaGAC-CTGCCCAGCGAGCTCTCCAG-3’ and the reverse primer was 5’-GCGTGCAGGCCAGGAGCAAGGCCC-3’ (lower-case letters indicated the FLAG sequence and the second codon in the untagged GPR40 is underlined). The PCR product was purified and ligated to obtain the tagged construct. To ensure proper expression, a Kozak (GGCCACC) sequence was placed before the first codon (21, 22) in the second step using the QuikChange II XL mutagenesis kit. Sequences on the promoter and the full insert in all constructs were verified by sequencing (MWG Biotech, High Point, NC).

Cell Culture and Transfection—Untagged or FLAG-tagged GPR40 was expressed in HEK-EM 293 cells. The conditions for cell culture were as reported previously (3). Transfection was carried out using Lipofectamine LTX and Plus reagent (Invitrogen) according to procedures recommended by the manufac-
turer. The cells were reseeded 1 day following transfection and then assayed the next day. For experiments using the fluorometric imaging plate reader, the cells were seeded onto 96-well plates at 60,000 cells/well. For flow cytometry, the cells were seeded onto 12-well plates at 600,000 cells/well.

Fluorometric Imaging Plate Reader Analysis—Receptor signaling assays were carried out as described by measuring calcium flux in response to the addition of agonist (3). The experiments were carried out in triplicate or quadruplicate for three or more times. Depending on the pH required, the Calcium4 dye was prepared in Hanks’ buffered saline solution supplemented with either 20 mM HEPES, pH 7.5, or 10 mM MES, pH 6.0. Unless stated otherwise, the experiments were carried out at physiological pH.

Analysis of Dose-Response Data—Agonist-stimulated responses in wild type and mutant receptors were determined as maximum minus minimum values after subtracting the baseline response in vector-transfected control cells. The dose-response relationships were analyzed by fitting sigmoidal curves to the data sets using GraphPad Prism 4 (San Diego, CA). All of the data sets from each receptor construct were assigned the same values of EC$_{50}$ and Hill coefficient. An F test was performed to determine whether the best fit was obtained with the Hill coefficient set to one or with the Hill coefficient floating as a parameter. Although we did not measure binding, we assume that changes in potency reflect changes in binding affinity with receptors that exhibit maximal responses similar to wild type.

Analysis of Surface Receptor Expression—The expression of FLAG-GPR40 and FLAG-tagged mutants in the plasma membrane was estimated by flow cytometry in a FACSCalibur (BD Biosciences, Franklin Lakes, NJ) using a procedure essentially the same as the one we described previously (23). The cells were harvested using 1 mM EDTA, 1 mM EGTA in phosphate-buffered saline, washed once with phosphate-buffered saline containing 0.1% fatty acid-free bovine serum albumin (binding buffer), and incubated for 2 h in the dark with anti-FLAG fluorescein isothiocyanate (10 μg/ml) in binding buffer. Subsequently, the cells were washed twice and fixed with 1% paraformaldehyde. Receptor expression in mutant receptors was compared with wild type FLAG-GPR40. Base-line fluorescence was determined using vector-transfected cells. The experiments were carried out in triplicate or quadruplicate for three or more times.

Molecular Modeling—The homology model for GPR40 was constructed based on the 2.65 Å resolution structure of rhodopsin (Protein Data Bank code 1gzm), and the binding site for GW9508 was experimentally verified by us as reported previously (3). The coordinates of the validated GPR40 complex with GW9508 are available online (3). Although, a low resolution x-ray structure of photoactivated rhodopsin has been published recently (24), it is not consistent with all of the changes predicted by biochemical and biophysical methods. Therefore, we used our GPR40 model obtained using the ground state of rhodopsin, which was optimized based on experimental information, for further characterization of the agonist-binding pocket. In silico mutation of the wild type receptor to L186F, H86F, and H137F and protonation of H137 and H86 were done using Schrödinger software (25, 26). Polak-Ribib Conjugate Gradi-
A convergence threshold on the gradient of 0.05 kJ/Å/mol was used for minimization of the obtained structures. Conformational searches for wild type receptor and mutant receptors were performed using the Monte Carlo multiple minimum method as implemented in MacroModel (25, 26). GW9508 and residues located within 6 Å of the ligand were subjected to extended torsional sampling. A shell of frozen atoms within 3 Å from the cavity was also included in the conformational search, whereas the remaining atoms were excluded. 1000 steps of the Monte Carlo multiple minimum method were performed, and the resulting structures were saved with a potential energy lower than 2000 kJ/mol. Merck molecular force field with correction for conjugated nitrogens (MMFFs) force field and the surface area-based version of the generalized born (GB/SA) model were used for treatment of solvent, and its dielectric constant was assigned a value of 1.

**FIGURE 1. Diagrams of the two-dimensional topology of GPR40 and the agonists used in this study.** In a, GPR40 is depicted in a snake-like plot adapted from the plot of rhodopsin as described by Palczewski et al. (29). The single mutations described in the present study are shown in gray with the letters of the amino acid residues enclosed in rectangles. Residues conserved within GPCRs are shown in black. In b, chemical structures of the agonists GW9508 and linoleic acid are shown.
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Comparison of pharmacological potency (EC$_{50}$) and maximal response (R$_{\text{max}}$) elicited by GW9508 or linoleic acid in wild type GPR40 and its indicated mutants

| Construct | GW9508 | Linoleic acid |
|-----------|--------|--------------|
|           | logEC$_{50}$ | R$_{\text{max}}$ | Δlog EC$_{50}$ | logEC$_{50}$ | R$_{\text{max}}$ | Δlog EC$_{50}$ |
| Wild type | -6.76 ± 0.03 | 100 | -4.91 ± 0.02 | 100 | -4.91 ± 0.02 | 100 |
| Y12A      | -6.16 ± 0.04 | 74 ± 6 | 0.60 | -4.49 ± 0.05 | 86 ± 6 | 0.42 |
| Y91A      | -4.84 ± 0.02 | 54 ± 7 | 1.72 | -3.99 ± 0.03 | 47 ± 12 | 0.92 |
| Y91F      | -6.62 ± 0.07 | 63 ± 7 | 0.13 | -4.84 ± 0.05 | 92 ± 14 | 0.08 |
| V240A     | -5.69 ± 0.05 | 82 ± 8 | 1.07 | -4.11 ± 0.04 | 65 ± 11 | 0.80 |
| V240F     | -6.36 ± 0.03 | 87 ± 11 | 0.40 | -4.59 ± 0.05 | 89 ± 16 | 0.32 |
| H86A      | -5.86 ± 0.02 | 78 ± 6 | 0.90 | -4.25 ± 0.03 | 81 ± 18 | 0.66 |
| H86F      | -6.32 ± 0.03 | 110 ± 21 | 0.44 | -4.66 ± 0.02 | 88 ± 3 | 0.25 |
| H137A     | -4.43 ± 0.13 | 53 ± 10 | 2.33 | -4.61 ± 0.03 | 59 ± 7 | 0.30 |
| H137F     | -5.67 ± 0.05 | 59 ± 3 | 1.10 | -4.66 ± 0.05 | 75 ± 14 | 0.25 |
| L186F     | -5.55 ± 0.05 | 48 ± 2 | 1.21 | -4.88 ± 0.03 | 60 ± 9 | 0.03 |
| L233F     | -6.92 ± 0.06 | 73 ± 6 | 0.16 | -4.95 ± 0.03 | 71 ± 4 | -0.03 |
| V237F     | -7.05 ± 0.04 | 64 ± 7 | -0.29 | -4.92 ± 0.03 | 82 ± 12 | -0.01 |
| R183A     | no response  | no response | no response | no response  | no response | no response |
| N244A     | -5.48 ± 0.03 | 79 ± 9 | 1.29 | -4.37 ± 0.03 | 53 ± 9 | 0.54 |
| R258A     | no response  | no response | no response | no response  | no response | no response |
| R259K     | ±2.08        | ±2.08    | ±2.08 | ±2.08        | ±2.08    | ±2.08 |
| K259A     | -6.58 ± 0.05 | 84 ± 12 | 0.18 | -4.88 ± 0.04 | 110 ± 18 | 0.03 |

*Response remained unsaturated with the highest concentration of the agonist.

Minimization was carried out using the same method as described above.

Linoleic acid was docked to the optimized GPR40 model after extraction of GW9508 (3). Automated flexible docking was performed using the Glide program with extra precision setting (27), while all other settings were kept in default. The docking box was determined based on the location of GW9508. The final orientation was selected based on the Glide scoring function. Binding surfaces for linoleic acid and GW9508 were represented using the MOLCAD surfaces module of Sybyl 7.2 (28) with a probe radius of 1.2 Å and colored based on the electrostatic potential. Energetic components for residues in contact with GW9508 or linoleic acid were calculated using the atom set (ASET) module of MacroModel, with (MMFFs) and the (GB/SA) model for implicit solvent.

RESULTS AND DISCUSSION

To predict the possible interactions of GPR40 with linoleic acid and GW9508, each ligand was docked individually into the receptor model. Twelve amino acids from the putative binding pocket were selected for site-directed mutagenesis, including residues that were identified to interact with GW9508 previously (Fig. 1a). In accordance with the chemical nature of the agonists (Fig. 1b), the chosen residues were either positively charged/hydrophilic (Arg$^{183}$ (5.39), Asn$^{244}$ (6.55), Arg$^{258}$ (7.35), or Lys$^{259}$ (7.36)), aromatic (Tyr$^{12}$ (1.39), Tyr$^{91}$ (3.37), Tyr$^{240}$ (6.51), His$^{86}$ (3.32), or His$^{137}$ (4.56), or hydrophobic residues (Leu$^{186}$ (5.42), Leu$^{233}$ (6.44), or Val$^{237}$ (6.48)). Positively charged/hydrophilic residues were neutralized by mutation to the small hydrophobic residue Ala, aromatic residues were mutated to Phe or Ala.
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Changes in the potency (EC<sub>50</sub>) and maximal response (R<sub>max</sub>) in relation to the levels of receptor expression

| Transfection (ratios of plasmids | FLIPR | Flow cytometry (relative fluorescent intensity) |
|----------------------------------|-------|-----------------------------------------------|
| of plasmids GPR40:pcDNA<sup>a</sup> | log EC<sub>50</sub> | Relative R<sub>max</sub> | % | % |
| 15.00:1 (100) | −6.87 ± 0.14 | 100 | 100 |
| 3.8:11.3 (25.0) | −6.78 ± 0.12 | 100 ± 10 | 82 ± 12 |
| 1.9:13.1 (12.5) | −6.39 ± 0.15 | 103 ± 13 | 70 ± 11 |
| 0.9:14.1 (6.25) | −6.23 ± 0.17 | 78 ± 2 | 57 ± 4 |
| 0.5:14.5 (3.13) | −6.34 ± 0.12 | 62 ± 5 | 47 ± 4 |

*The values in parentheses in the first column indicate the percentage of GPR40.

To examine possible hydrogen bonding and aromatic interactions, and hydrophobic residues were mutated to the bulkier Phe.

GPR40 mutants were examined in transfected HEK-EM 293 cells by measuring receptor-mediated calcium flux using the fluorometric imaging plate reader. As reported previously, neutralizing the positively charged/hydrophilic residues markedly affected the interaction with GW9508 (3). The response to GW9508 was completely abolished by R183A or R258A mutations, whereas the maximal response was 80% of wild type, but the potency was reduced by more than 10-fold in the N244A mutant (Table 1). These three residues were shown by modeling to act as an anchor for orienting the carboxylate moiety of the agonist (Fig. 2). By contrast, linoleic acid elicited responses with all three of these mutations, albeit at levels below that of wild type receptor (Table 1). The potency of linoleic acid was lowered by ~3-fold in N244A and R258A mutant receptors, whereas Arg<sup>183</sup> appeared to be more important because its mutation to Ala reduced the potency by 7-fold. The fact that R183A displayed 53% of the maximal response of wild type receptor upon stimulation by linoleic acid indicates that R183A was expressed, and Arg<sup>183</sup> is relatively more important for GW9508 signaling than for linoleic acid signaling. In addition, mutation of Arg<sup>258</sup> to Lys generated a receptor that did not respond well to either GW9508 or linoleic acid. In contrast, Lys<sup>259</sup>, which faces away from the binding pocket and is not predicted to interact with either ligand, did not affect potency of GW9508 or linoleic acid when mutated to Ala. By employing flow cytometry using FLAG-tagged receptors, we also showed that the expression of N244A and R258K on the plasma membrane was similar to the wild type level (Fig. 3).

From the docked GPR40 model, we predicted that aromatic residues within the binding pocket may interact with hydrophobic regions of agonists. Mutation of Tyr<sup>12</sup>, Tyr<sup>91</sup>, and Tyr<sup>240</sup> to Phe had little or no effect on the potency of GW9508 and linoleic acid (Table 1), suggesting the absence of hydrogen bond interactions with the three tyrosine hydroxyl groups. The Y12A mutant was inactive with either agonist, but flow cytometric analysis of the FLAG-tagged construct showed that Y12A was expressed at the plasma membrane at ~45% of the level of wild type GPR40 (Fig. 3). The observed loss of function therefore suggests that Tyr<sup>12</sup> is important for receptor activation. Because in our model this residue does not make direct contact with the ligand, it is likely that Tyr<sup>12</sup> is involved in maintaining a proper fold that is essential for receptor function. The Ala mutants of Tyr<sup>91</sup> and Tyr<sup>240</sup> were expressed at ~60% of the wild type level (Fig. 3), and the potencies of GW9508 and linoleic acid were reduced by 52- and 8-fold in Y91A, respectively, whereas in Y240A there was a decrease in potencies of 8-10-fold with both agonists. Although a part of the potency decrease could be caused by the lower receptor expression, it is unlikely to account fully for differences in the fold decreases with GW9508 and linoleic acid and, in particular, for the 52-fold reduction in potency of GW9508. To further address the issue of receptor expression, we adjusted the level of expression of wild type GPR40 and determined the effect on the EC<sub>50</sub> of GW9508. The EC<sub>50</sub> was found to be reduced by only 4-fold when the expression was lowered to 57% (Table 2). Therefore,
we suggest that the diminished potency of GW9508 for the
Y91A mutation reflects loss of an interaction of the agonist with
the phenyl group on Tyr91. Although Y91F is expressed at a
level similar to wild type receptor (Fig. 3), its efficacy in
response to GW9508 is only 63% of wild type (Table 1), suggest-
ing that this residue may play an important role in the ability of
this particular agonist to induce an active conformation of the
receptor.

We have shown previously a possible amino-aromatic inter-
action of His137 with GW9508 (3). Its mutation to Ala or Phe
significantly diminished the potency of GW9508. In contrast,
neither the Ala nor the Phe mutations affected the potency of
linoleic acid (Table 1). Both His137 mutants are expressed at
higher levels than wild type receptor. Nevertheless, both muta-
tions rendered the mutant receptors unable to become fully
activated, because there was a 40% or greater reduction in the
maximal response with either agonist. Mutation of His137 to Phe
had a small effect on the response to either agonist, whereas the
Ala mutation diminished potency by ~5–8-fold.

The hydrophobic residue Leu186 plays an important role in
the interaction with GW9508 but not with linoleic acid. This is
demonstrated by the strong effect of the Phe mutant on the
potency of GW9508 only and the absence of effect on the
potency of linoleic acid. This residue is located near His137 in
three-dimensional space. Therefore, the effect of mutation could be due to steric hindrance caused by the Phe moiety pre-
venting the interaction of the agonist with His137. To test this idea
further, we performed a conformational search on the GPR40 model
in complex with GW9508. In the L186F mutant, we observed the
presence of conformations in which the Phe side chain moved into
the space between the ligand and His137 and the imidazole moiety of His137
moved away from the ligand (Fig. 4b). This disorientation was not
observed in the wild type receptor model (Fig. 4a). Steric hindrance in
L186F is, therefore, supported by experimental data and in silico anal-
ysis. Importantly, the reduced responses with L186F and the
two His137 mutants for both agonists suggest that this region of
the receptor is crucial for activation. Moreover, the lack of
effect of L186F on the potency of linoleic acid appears to be in
agreement with the above observation that His137 does not interact
directly with linoleic acid.

In a chomogenomic analysis of 30 residues that line the puta-
tive binding pockets of a number of GPCRs, Surgand et al. (4)
pointed out that Leu233 and Val237 in GPR40 are substituted
with Phe at the corresponding positions in GPR41 and GPR43,
the receptors for short chain fatty acids. This led to their
hypothesis that Leu233 and Val237 are residues that determined
the ligand specificity between the two fatty acid receptors. We
therefore tested this hypothesis by examining the effects of Phe
mutations on the potency for linoleic acid and GW9508. In
contrast to the significant effects of L186F described above,
mutants L233F and V237F exhibited no change in potency with
GW9508 or linoleic acid (Table 1). Furthermore, neither
L233F, V237F, nor GPR40 responded to simulation by the short
chain fatty acids propionate or butyrate (data not shown).

Taking the data together, we conclude that GW9508 and
linoleic acid bind to GPR40 by interacting with similar residues.
It appears that the higher potency of the GW9508 over linoleic
acid is due to stronger interactions contributed by Tyr91, His137,
Arg183, Asn244, and Arg258. In silico calculation of energies of
interaction for individual residues (Table 3) has shown a more
significant contribution of these five residues in the binding
energy of GW9508 in comparison with linoleic acid. Although
the activation data obtained from intracellular calcium release
experiments depends not only on ligand affinity but also on the
efficiency of multiple intermediate coupling/amplification steps in the activation cascade, we found a correlation between
the observed changes in potency and the calculated binding
energies. Also, the energies of interaction for individual resi-
dues as well as their combined contributions were greater for
GW9508 than linoleic acid. Moreover, the calculated electro-
static potentials for the MOLCAD surface of the binding cavity
demonstrates that although the binding pocket is mainly
hydrophobic, there are hydrophilic regions formed by His137
and Tyr91 that may form interactions with donor acceptor parts
of GW9508 and account for the high potency for GW9508 (Fig.
2, c and d).
Our mutagenesis experiments are consistent with interactions of GW9508 with His\textsuperscript{137} and His\textsuperscript{86}; mutation of His\textsuperscript{137} caused a greater loss in potency than that of His\textsuperscript{86}. Because the imidazole moiety on His has a $pK_a$ value near physiological pH, we hypothesized that the protonation status of the His might play a role in the interaction with GW9508. Furthermore, our earlier docking study showed that GW9508 can interact with His\textsuperscript{137} and/or His\textsuperscript{86} (3). We therefore investigated the pharmacology of GPR40 at pH 7.5 and 6.0. The experiment was conducted using the wild type receptor in parallel with H86F and H137F mutants to determine which residue might contribute to any effect. As seen in Table 4, acidification resulted in an increase in potency of GW9508 in the wild type receptor. The increase in potency was smaller than wild type when His\textsuperscript{86} was mutated to Phe. In the H137F mutant, the potency at pH 6.0 was increased by 10-fold to a level similar to wild type at pH 7.5, suggesting that the interaction with the protonated His\textsuperscript{86} may be able to compensate for the lost interaction with His\textsuperscript{137}.

To determine whether our model can explain the pH-dependent effect from a structural point of view, we performed a conformational search of wild type, H86F, and H137F mutants of the GW9508-receptor complex, with the two His residues in protonated or unprotonated form. The results of this analysis showed that in the wild type receptor upon protonation of both His\textsuperscript{137} and His\textsuperscript{86}, the terminal phenoxy ring and the benzylamino ring of the ligand formed two possible cation-aromatic interactions with the positively charged His\textsuperscript{137} and His\textsuperscript{86} (Fig. 5, a and b). The substitution of two amino-aromatic interactions with two cation interactions is in agreement with the increase in potency of GW9508 upon acidification. The H86F mutation led to the loss of one amino-aromatic interaction but did not significantly change the binding mode of GW9508 with respect to the wild type receptor (Fig. 5c), which is consistent with the small influence of H86F on ligand potency. Protonation of His\textsuperscript{137} in the H86F mutant led to the substitution of only one amino-aromatic interaction with a cation-aromatic interaction and a consequent increase of the potency of the ligand smaller than that registered for the wild type (Fig. 5d). On the contrary, H137F mutant had a disruptive effect on the binding of GW9508 (Fig. 5e), resulting experimentally in a marked decrease in potency. Protonation of His\textsuperscript{86} in the H137F mutant led to rescue of the ligand binding mode by formation of a cation-aromatic interaction (Fig. 5f). In the H137F mutant, acidification resulted in a significant rescue of the potency of GW9508, which reached a level close to that.

### Table 4

| Construct   | log EC\textsubscript{50} pH 7.5 | log EC\textsubscript{50} pH 6.0 | $\Delta$ log EC\textsubscript{50} | $p$ values |
|-------------|---------------------------------|---------------------------------|----------------------------------|------------|
| Wild type   | $-6.73 \pm 0.16$               | $-7.33 \pm 0.11$               | 0.60                             | 0.0358     |
| H86F        | $-6.13 \pm 0.08$               | $-6.51 \pm 0.06$               | 0.38                             | 0.0162     |
| H137F       | $-5.29 \pm 0.12$               | $-6.35 \pm 0.16$               | 1.06                             | 0.0063     |

**FIGURE 5.** Conformational analysis of the unprotonated and protonated binding pocket and GW9508 for the wild type receptor (a and b), the H137F mutant (c and d), and the H86F mutant (e and f). Only the structure of GW9508 (highlighted in green), along with His\textsuperscript{137}/HIP137 or His\textsuperscript{86}/HIP86 residues are shown for the generated conformations.
shown with the H86F mutant at pH 6.0 (Table 4). However, because of the presence of just one cation-aromatic interaction instead of two, the potency of GW9508 with the two mutants at pH 6.0 remains lower than the potency with the wild type at the same pH. The analysis, therefore, is consistent with the hypothesis that pH may have an influence on the binding of GW9508 at GPR40, resulting in a pH-dependent interaction of the ligand with His86 and His137.

The model described here provides important insights into the structure-activity relationships of GW9508 analogs reported by Garrido et al. (17). Reduced potency was observed with analogs in which the two methylenes in GW9508 that connect the carboxylate head group to the phenyl rings are replaced with fewer or more methylene groups. This two-methylene connecting group appears to provide the length required to position the phenyl rings for proper interaction with Tyr91 and especially His137. Moreover, as our data suggest, a proper coordination of the head group by the anchor residues may be important not only for binding but also for activation. This may explain why modifications of the carboxylate head group to amides of different bulkiness reduced agonist efficacy but preserved potency.

We posit there is a specific role of His137 (4.56) for the fatty acid subfamily of rhodopsin-like GPCRs because this His is not found in other receptors in this class. Within the fatty acid subfamily, Leu186 (5.42) is present in the G4-coupled GPR40 and GPR43 or is substituted conservatively with Met in the G3-coupled GPR41, and it is possible that a small side chain at position 186 is necessary to preserve the function of His137 (5.42). Although Surgand et al. (4) suggested that Leu233 (6.44) and Val237 (6.48) may be the residues offering specificity for long chain fatty acids, our data do not support this role. In the docked receptor model, His86 (3.32) and Asn244 (6.55) are among the residues that differ between the short chain and long chain fatty acid receptors and are close to the ligand. These two residues may therefore contribute to ligand specificity through proper coordination and orientation of fatty acids.

In conclusion, we have further defined and characterized the ligand-binding pocket within our homology model of GPR40 (3). We have identified hydrophilic/positively charged residues that are important for anchoring the carboxylate head group on both fatty acids and small molecule agonists. Furthermore, we have shown that the binding cavity of the receptor also contains a number of hydrophobic residues. Several of these hydrophobic and hydrophilic/positively charged residues appear to make stronger contacts with GW9508 and allow this synthetic ligand to activate GPR40 with higher potency than cognate fatty acids. These findings may provide a basis for rational drug discovery.

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