Differential Mapping of the Amino Acids Mediating Agonist and Antagonist Coordination with the Human Thromboxane A\(_2\) Receptor Coordination Protein*

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Despite the well documented involvement of thromboxane A\(_2\) receptor (TPR) signaling in the pathogenesis of thrombotic diseases, there are currently no rationally designed antagonists available for clinical use. To a large extent, this derives from a lack of knowledge regarding the topography of the TPR ligand binding pocket. On this basis, the purpose of the current study was to identify the specific amino acid residues in the TPR protein that regulate ligand coordination and binding. The sites selected for mutation reside within or in close proximity to a region we previously defined as a TPR ligand binding region (i.e. the C terminus of the second extracellular loop and the leading edge of the fifth transmembrane domain). Mutation of these residues caused varying effects on the TPR-ligand coordination process. Specifically, the D193A, D193Q, and D193R mutants lost SQ29,548 (agonist) binding and exhibited a dramatically reduced calcium response, which could not be restored by elevated U46619 (agonist) doses. The F184Y mutant lost SQ29,548 binding and exhibited a reduced calcium response (which could be restored by elevated U46619); and the T186A and S191T mutants lost SQ29,548 binding and retained a normal U46619-induced calcium response. Furthermore, these last three mutants also revealed a divergence in the binding of two structurally different antagonists, SQ29,548 and BM13.505. Two separate mutants that exhibited SQ29,548 binding yielded either a normal (F196Y) or reduced (S201T) U46619 response. Finally, mutation of other residues directly adjacent to those described above (e.g. E190A and F200A) produced no detectable effects on either SQ29,548 binding or the U46619-induced response. In summary, these results identify key amino acids (in particular Asp\(^{193}\)) involved in TPR ligand coordination. These findings also demonstrate that TPR-specific ligands interact with different residues in the ligand-binding pocket.

Thromboxane A\(_2\) (TXA\(_2\)) is a labile lipid mediator that binds to its seven-transmembrane receptor (TPR) (1–5) and elicits a variety of biological effects, including platelet activation (6, 7), vascular smooth muscle contraction (8), cell proliferation, and cell survival (9, 10). The specific involvement of TPR signaling in both hemostasis and thrombosis has been extensively studied. In this regard, ample evidence exists for the direct participation of TPR activation in the prevention of blood loss upon vascular damage as well as its participation in occlusive vascular disease (11–15). Indeed, the rationale for the current use of aspirin in the prevention of recurrent myocardial infarction and occlusive stroke (16, 17) is the ability of aspirin to block TXA\(_2\) synthesis. On the other hand, aspirin therapy is also associated with certain undesirable effects unrelated to inhibition of TXA\(_2\) production, particularly inhibition of prostacyclin synthesis. Consequently, as an alternative pharmacological approach, research efforts have been directed at designing specific TPR antagonists to inhibit TPR ligand binding. However, one difficulty facing the development of such antagonists is that to date there has not been a comprehensive analysis of the actual TPR binding domains at the amino acid level. Nevertheless, previous studies have attempted to identify certain amino acid residues that may participate in ligand coordination to this receptor. Initially, these studies focused on the transmembrane regions of the TPR protein, since earlier findings with the G-protein-coupled receptor bovine rhodopsin revealed a putative binding domain in its seventh transmembrane region (TM7) (18). The results from these studies implicated all of the transmembrane regions in mediating TPR-ligand binding, with the exception of TM2 (19–23). Similarly, molecular modeling studies also proposed that residues interacting with TPR ligands are found mainly in the transmembrane regions (24).

On the other hand, separate studies have suggested the possible involvement of different TPR regions in ligand coordination. In this connection, our previous experiments employing a biotinylated TPR antagonist (25) suggested that ligands interact with extracellular binding regions of the TPR protein. Furthermore, later studies using photoaffinity labeling and site-specific antibodies demonstrated that TPR ligand binding sites reside within an 11-amino acid sequence contained in the C-terminal

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3 The abbreviations used are: TXA\(_2\), thromboxane A\(_2\); TPR, thromboxane A\(_2\) receptor; TM, transmembrane; C-EL2, C-terminal segment of the second extracellular loop; HEK, human embryonic kidney; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; WT, wild type; EC\(_{50}\), effective concentration 50.
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segment of the second extracellular loop (C-EL2) (26). Although these previous studies identified EL2 as being critical for ligand binding, they did not identify the specific amino acids that participate in this process. Based on this consideration, the present work elucidated the effects of point mutations in C-EL2 of the thromboxane A₂ receptor and its close proximal region, TM5, on ligand coordination and function. Depending on the location and the nature of the amino acid substitution at the target sites in C-EL2 or TM5, three major phenotypes were observed: 1) a mutant receptor that lost SQ29,548 binding and exhibited a substantially reduced calcium response (which could not be restored with elevated U46619 doses); 2) a mutant that lost SQ29,548 binding and exhibited a reduced calcium response (which could be restored with elevated levels of the U46619); and 3) a mutant that could differentiate not only between SQ29,548 (antagonist) binding and functional responses (U46619) but also between two distinct classes of TPR antagonists (i.e. SQ29,548 and BM13.505). On the other hand, a “control” mutation that lacked any detectable effects on TPR structure or function was also observed, indicating that changes in only certain amino acids within C-EL2 influence the biological phenotype of this receptor protein. The results also provide evidence that these amino acids play a differential role in regulating the ligand–receptor interaction process and suggest possible mechanisms by which this process occurs.

MATERIALS AND METHODS

Reagents

Human embryonic kidney (HEK) cells were purchased from ATCC. Wild-type TPR cDNA was a gift from Dr. Colin Funk (University of Pennsylvania). Fura2/AM dye was purchased from Molecular Probes, Inc. (Eugene, OR). [³H]SQ29,548 was purchased from PerkinElmer Life Sciences. pcDNA3.1 vector was from Invitrogen. U46619 and SQ29,548 were purchased from Cayman chemical (Ann Arbor, MI). BM13.505 was a generous gift from Dr. K. Stegmeier (Roche Applied Science). Effectene transfection reagent was purchased from Qiagen. Fura2/AM dye was purchased from Molecular Probes, Inc. (Eugene, OR). [³H]SQ29,548 was purchased from PerkinElmer Life Sciences. pcDNA3.1 vector was from Invitrogen. U46619 and SQ29,548 were purchased from Cayman chemical (Ann Arbor, MI). BM13.505 was a generous gift from Dr. K. Stegmeier (Roche Applied Science). Effectene transfection reagent was purchased from Qiagen (Valencia, CA). Polyclonal rabbit anti-TPR IgG was previously made and characterized in our laboratory. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and normal rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Cell culture supplies were purchased from Fisher.

Selection of Amino Acids for Mutagenesis

Molecular modeling studies (27, 28) have indicated that crucial ligand coordination sites are likely to mediate binding to the receptor through hydrogen bonding, ionic interactions, or hydrophobic bonds. Thus, mutations were conducted (in C-EL2 or TM5) at amino acid sites that are charged, are hydrophobic, or contain hydrogen donors (i.e. Phe184, Thr186, Gln190, Ser191, and Asp193 in C-EL2 and Phe196, Phe200, and Ser201 in TM5). Examples of the engineered mutations are F184Y, T186A, E190A, S191T, D193A, and S201T. It was reasoned that analysis of these mutations would serve two purposes: first to identify critical amino acids involved in TPR ligand coordination and second to provide insight into the nature of the molecular forces involved in these coordination processes. The human wild-type TPR cDNA was subcloned into pcDNA3.1 vector. The mutants were generated according to the standard protocol of QuikChange site-directed mutagenesis (Stratagene) and confirmed by automated sequencing. Transfections into HEK cells were performed according to the protocol for Effectene transfection (Qiagen) to generate stable cell lines. Cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. After 48 h, G418 was added for selection of stable cell line clones. HEK cells represented an excellent model for the transfection studies, since they possessed very low basal expression of TPR as revealed by fluorescence-activated cell sorting (FACS), radioligand binding and Ca²⁺ studies (not shown).

Flow Cytometry in Resuspended Cells

Cells were harvested with trypsin, and their number was adjusted to 2–5 × 10⁶ cells/ml in phosphate-buffered saline (PBS). Fifty µl of cell suspension was placed in a flow cytometry tube and incubated with either a rabbit polyclonal anti-TPR antibody (1:50, v/v) or normal rabbit IgG (control) (1:100, v/v) for 1 h at room temperature. Next, 0.5 ml of PBS was added, and cells were washed by centrifugation for 5 min at 1200 rpm. The supernatant was aspirated, and this procedure was repeated at least twice. Fifty µl of the secondary fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was added (1:50, v/v) and incubated with the cells in darkness for 1 h at room temperature. PBS (0.5 ml) was then added, and single-color analysis was performed on the samples using a BD Biosciences FACStar analyzer. A lower limit threshold was set for data acquisition, thereby eliminating background scatter.

Whole Cell Radioligand Binding Experiments

Saturation Binding—The cells were seeded on poly-l-lysine-coated 12-well plates. Upon confluence, the cells were washed twice with PBS and then incubated with various predefined concentrations of [³H]SQ29,548 for 40 min at room temperature with gentle shaking. Next, the cells were washed twice with PBS, and 500 µl of 0.3 N NaOH was added. The plates were shaken for 10 min at room temperature to detach the cells, and 100 µl of 3.0 N HCl was added to neutralize the pH. The solubilized cell solution was then transferred to vials containing 8 ml of scintillation fluid. To calculate the nonspecific binding, the same concentration of radioligand was competed against 1000-fold excess of unlabeled SQ29,548. In each case, the cells were plated in duplicates, and the experiment was repeated at least three times. Samples were counted in a Beckman LS 6500 liquid scintillation counter. Saturation binding isotherm curves were generated by applying nonlinear regression, GraphPad PRISM software (San Diego, CA), to determine the affinity (Kₐ). It is noteworthy that saturation isotherms are not presented for certain mutants. This is because these mutants displayed no concentration-dependent specific binding for [³H]SQ29,548, which thus prevented a Kₐ calculation.

Displacement Binding—These experiments were performed using a protocol similar to the saturation binding protocol with minor modifications. Briefly, cells were washed with PBS and then incubated with 1 nM [³H]SQ29,548 at room temperature.
for 20 min with gentle shaking. Increasing concentrations of U46619 (the displacing ligand) (2 nM to 30 μM) were then added, and the cells were shaken gently for an additional 20 min. Next, the cells were solubilized, the cell solution was transferred to vials containing 8 ml of scintillation fluid, and the samples were counted for \[^{[3]H}\]SQ29,548 activity. Displacement

FIGURE 1. Reconstitution of a stable wild-type TPR-expressing HEK cell line. Cells were evaluated using flow cytometry, radioligand binding, and calcium mobilization. A, flow cytometry analysis. Cells were probed with an antibody targeting an extracellular domain of the TPR protein. B, saturation binding isotherm using \[^{[3]H}\]SQ29,548. Cells were incubated with various concentrations of \[^{[3]H}\]SQ29,548 to generate the saturation binding isotherm. C, 1 μM U46619-induced Ca\(^{2+}\) mobilization (inset shows dose-response analysis of the U46619-induced Ca\(^{2+}\) mobilization). D, effect of SQ29,548 on U46619-induced Ca\(^{2+}\) mobilization (inset shows dose-dependent inhibition of Ca\(^{2+}\) mobilization). Cells were also treated with carbachol as a control. E, effect of BM13.505 on U46619-induced Ca\(^{2+}\) mobilization (inset shows dose-dependent inhibition of Ca\(^{2+}\) mobilization). Cells were also treated with carbachol as a control. Results are representative of at least three different experiments in each case. FITC, fluorescein isothiocyanate.
binding curves were generated by applying nonlinear regression analysis and GraphPad PRISM software to determine the U46619 $K_a$.

**Cytosolic Calcium ($Ca^{2+}$) Measurements**

HEK cells were grown on poly-l-lysine-coated 25-mm diameter glass coverslips and washed twice with Hank’s balanced salt solution, prior to loading with 3 $\mu M$ Fura2/AM for 25 min at 37 °C. After two additional washes with Hank’s balanced salt solution, intracellular calcium was quantified by Fura2 fluorescence imaging (29, 30) using an Attofluor ratio vision digital fluorescence microscopy system (Atto Instruments, Rockville, MD) attached to an inverted microscope (Zeiss Axiosvert S100) and a F-Fluar 40 oil immersion objective. This system excites cells at 334 and 380 nm and measures emission at 520 nm. Briefly, a field of cells was visualized, and 50–60 cells were randomly selected. The change in the 334/380 excitation ratio was measured every 5 s. The average absolute peak change in ratio 334/380 of the selected cells within this field was measured. The effective concentration 50 ($EC_{50}$) values were determined by applying nonlinear regression analysis to the dose-response curves generated for each mutant. However, no $EC_{50}$ is presented in the case of mutants where the maximum wild-type functional response could not be restored by excessive agonist concentrations. Next, the average and S.E. of at least three separate experiments was calculated. This procedure was repeated for the wild type and each of the mutants. Statistical analysis was then performed to determine which mutants exhibited a response that was statistically different from that of the wild-type. For the inhibitor studies, Fura2-loaded cells were preincubated with either 0.05–1 $\mu M$ SQ29,548 or 0.05–5 $\mu M$ SQ29,548 and BM13.505 at room temperature for ~10 min prior to stimulation with agonist.

**Analysis of Data**

All experiments were performed at least three times. Data were analyzed using GraphPad PRISM statistical software (San Diego, CA) and presented as mean ± S.E. Saturation binding isotherms and displacement binding curves were generated using nonlinear regression analysis. Results from the wild type and the different mutants were compared using unpaired two-tailed Student’s t test, with $p < 0.05$ considered to be statistically significant.

**RESULTS**

**Reconstitution of a Stable Human Wild-type TPR-expressing HEK Cell Line**—Initially, HEK cells with stable expression of the wild-type human TPR were generated in order to serve as a control and allow comparison with the cells expressing point mutations in the TPR sequence. This stable cell line was characterized using three separate procedures: 1) fluorescence-activated cell sorting to determine and quantify cell surface receptor expression; 2) antagonist radioligand binding to evaluate the receptor binding affinity (calculate $K_a$); and 3) calcium mobilization experiments to assess agonist-induced functional activity and generate $EC_{50}$ values. FACS analysis revealed that these cells expressed TPR on their plasma membrane, as indicated by the rightward shift in the mean fluorescence intensity of the wild-type TPR cells compared with the control nontransfected cells (Fig. 1A and Table 1). The saturation binding isotherm generated using the radiolabeled TPR antagonist ([3H]SQ29,548) yielded a dissociation constant ($K_d$) of 7 ± 1 nM (Fig. 1B and Table 4), which is similar to the $K_a$ value previously reported for this ligand in human platelets (2, 31).

Calcium mobilization studies were performed using the TPR agonist U46619. The results demonstrated that U46619 stimulated substantial $Ca^{2+}$ mobilization in the transfected cells within a few seconds of its addition (Fig. 1C and Table 2). Dose-response analysis using 0.25–5 $\mu M$ U46619 indicated an $EC_{50}$ of 430 ± 20 nM, with a maximal calcium mobilization at 1 $\mu M$ (Table 3 and Fig. 1C, inset). Dose-response inhibition analysis was then performed for two TPR antagonists, SQ29,548 and BM13.505. It was found that complete inhibition of 1 $\mu M$ U46619-induced $Ca^{2+}$ mobilization required 1 $\mu M$ SQ29,548 (Fig. 1D) and 5 $\mu M$ BM13.505 (Fig. 1E). Moreover, the $Ca^{2+}$ response to carbachol was not blocked by either of these antagonists (Fig. 1, D and E), indicating that the U46619-induced $Ca^{2+}$ mobilization was specifically TPR-mediated. Collectively, these data demonstrate the successful reconstitution of wild-type human TPRs in HEK cells. These TPRs were stably expressed on the surface, possessed a binding $K_a$ comparable with human platelets, and exhibited TPR-mediated functional activity.

The effects of specific mutations (within C-EL2 or TM5 of TPR) on ligand-receptor binding and function were next evaluated, again using the three procedures described above. Nineteen mutants were engineered and stably expressed; D193A, D193E, D193N, D193Q, D193R, S191A, S191T, E190A, E190D, T186A, T186S, F184A, and F184Y were within C-EL2, whereas S201T, S201A, F200Y, F200A, F196Y, and F196A were within TM5 (Fig. 2). Only the clones that possessed TPR surface expression levels comparable with the wild type (Table 1) were utilized for further experimentation. This is an important consideration based on our previous findings that TPR ligand affinity can be affected by its surface expression levels (32).

Aspartic Acid 193 Mutations: D193A, D193E, D193N, D193Q, and D193R—The first amino acid residue selected for mutagenesis was aspartic acid (Asp), which is a charged residue that can interact with ligands through ionic or hydrogen bonding. This residue was first mutated to alanine, which does not possess the side chain of the original amino acid and therefore can no longer engage in such interactions. FACS analysis of this cell line revealed a rightward shift in the mean fluorescence intensity as compared with the control nontransfected HEK cells (data not shown, and see Table 1), indicating surface expression of TPRs. The effects of this mutation were first evaluated by radioligand binding experiments. It was found that the D193A mutation rendered the receptor incapable of binding to [3H]SQ29,548 (Fig. 3A), even at a radioligand concentration that normally saturates wild-type TPR binding (50 nM).

The functional consequence of this mutation was a loss of $Ca^{2+}$ mobilization in response to 1 $\mu M$ U46619 (Fig. 3B and Table 2). Moreover, raising the dose of U46619 by as much as 10-fold (i.e. to 10 $\mu M$) still only resulted in a 40% restoration of the wild-type response (Table 2). Thus, the $Ca^{2+}$ response in this mutant remained attenuated even at suprapharmacologic...
TABLE 1
MF1 ratio of the different stable cell lines
The MF1 ratio of the control (wild-type TPR) or various mutant TPRs was calculated. Results are representative of at least three different experiments in each case (p < 0.05, n = 3). MF1, mean fluorescence intensity.

| Cell line | MF1 ratio | Cell line | MF1 ratio |
|-----------|-----------|-----------|-----------|
| WT        | 3.01 ± 0.37 | D193A     | 3.08 ± 0.62 |
| F184A     | 3.11 ± 0.26 | D193E     | 3.10 ± 0.45 |
| F184Y     | 3.11 ± 0.05 | D193Q     | 2.85 ± 0.27 |
| T186A     | 3.10 ± 0.46 | D193R     | 3.07 ± 0.30 |
| T186S     | 2.90 ± 0.30 | F196A     | 2.76 ± 0.42 |
| E190A     | 3.00 ± 0.60 | F196Y     | 2.56 ± 0.08 |
| S191A     | 2.67 ± 0.23 | F200A     | 2.44 ± 0.10 |
| S191T     | 3.10 ± 0.34 | F200Y     | 2.86 ± 0.33 |
| D193N     | 3.10 ± 0.34 | S201A     | 3.14 ± 0.57 |
|           |           | S201T     | 2.75 ± 0.08 |

p < 0.05.

TABLE 2
The average absolute peak change in ratio 334/380 nm of the different stable cell lines
Shown is the change in the ratio 334/380 nm of the wild-type or various mutant TPRs. Data represent stimulation with 1 μM U46619, unless otherwise indicated. Results are representative of at least three different experiments in each case (p < 0.05, n = 3).

| Cell line | Ratio 334/380 | Cell line | Ratio 334/380 |
|-----------|---------------|-----------|---------------|
| WT        | 0.55 ± 0.05   | F200A     | 0.47 ± 0.08   |
| F184A*    | 0.12 ± 0.02   | F200Y     | 0.53 ± 0.07   |
| F184A (5 μM) | 0.52 ± 0.02 | S201A*    | 0.12 ± 0.01   |
| F184Y    | 0.13 ± 0.02   | S201A (5 μM) | 0.45 ± 0.03 |
| F184Y (5 μM) | 0.41 ± 0.01 | S201T*    | 0.45 ± 0.05   |
| T186A     | 0.46 ± 0.02   | D193E     | 0.51 ± 0.05   |
| T186S     | 0.52 ± 0.04   | D193N     | 0.41 ± 0.03   |
| E190A     | 0.57 ± 0.04   | D193A*    | 0.00          |
| E190D     | 0.45 ± 0.03   | D193A* (10 μM) | 0.21 ± 0.03 |
| S191A     | 0.43 ± 0.02   | D193Q*    | 0.00          |
| S191T     | 0.57 ± 0.05   | D193Q* (10 μM) | 0.19 ± 0.05 |
| F196A     | 0.48 ± 0.05   | D193R*    | 0.00          |
| F196Y     | 0.43 ± 0.05   | D193R* (10 μM) | 0.72 ± 0.04 |

p < 0.05.

TABLE 3
A summary of the EC50 values for the wild-type and mutant TPR-expressing cell lines
Results are representative of at least three different experiments in each case (p < 0.05, n = 3). NA, not applicable; mutants with no EC50 presented, since a wild-type calcium response could not be restored.

| Cell line | EC50 (nM) | Cell line | EC50 (nm) |
|-----------|-----------|-----------|-----------|
| WT        | 430 ± 20  | D193A     | NA        |
| F184Y     | 2850 ± 50*| D193E     | 430 ± 10  |
| F184Y     | 2425 ± 75*| D193Q     | NA        |
| T186A     | 460 ± 10  | D193R     | NA        |
| T186S     | 450 ± 30  | F196A     | 460 ± 30  |
| E190A     | 470 ± 10  | F196Y     | 455 ± 35  |
| E190D     | 425 ± 25  | F200A     | 420 ± 20  |
| S191A     | 435 ± 25  | F200Y     | 470 ± 20  |
| S191T     | 480 ± 10  | S201A     | 2235 ± 35*|
| D193N     | 435 ± 35  | S201T     | 2575 ± 25*|

p < 0.05.

Amino Acids Mediating TPR Ligand Interaction

To further define the interaction requirements at 193, the D193N mutant was studied. This mutant can engage in hydrogen bonding but not ionic interactions. It was found that the D193N mutant possessed surface protein levels similar to the wild type (Table 1) and exhibited a higher affinity for [3H]SQ29,548 (Kd) compared to the wild type (Table 1). Interestingly, although this mutant possessed a lower Kd for SQ29,548, it exhibited a 1 μM U46619-mediated calcium response that was not different from the wild type. Therefore, this mutation appears to selectively increase SQ29,548 binding. Furthermore, the observed calcium mobilization was completely inhibited with either 1 μM SQ29,548 or 5 μM BM13.505 (not shown). Thus, it appears that hydrogen bonding at position 193 is sufficient to support ligand binding/functional activity and that a negatively charged side chain is not critical for such activity.

On this basis, it might be predicted that a different amino acid capable of hydrogen bonding may also support TPR ligand binding and function. This was next explored by using the D193Q mutant. However, it was found that D193Q, which had expression levels comparable with the wild type (Table 1), neither bound [3H]SQ29,548 (Fig. 3G and Table 4) nor mobilized calcium in response to 1 μM U46619 (Fig. 3H, Table 2). Consequently, it appears that optimal ligand interaction at this position not only requires hydrogen bonding but also involves additional conformational and chemical characteristics of the amino acid side chain.

Finally, the effects of reversing the charge and modifying the side chain characteristics at position 193 were investigated. In this case, the negative charge of aspartic acid was replaced with positively charged arginine (D193R). Whereas this substituted amino acid can also engage in hydrogen bonding, such bonding activity was not sufficient to fully support either ligand binding or function. Specifically, although this mutant TPR was expressed on the plasma membrane at the same levels as the wild type (Table 1), there was a total loss of [3H]SQ29,548 binding (Fig. 3I) and a loss of the calcium response to 1 μM U46619 (Fig. 3J and Table 2). Therefore, the phenotype of this mutant is the same as that for the D193A and D193Q.

logical concentrations of U46619, and because of this, a meaningful EC50 value could not be obtained. On the other hand, the D193A mutant retained its ability to mobilize Ca2+ in response to a muscarinic receptor agonist (i.e. carbachol) (Fig. 3B), indicating that the reduced response to U46619 was not due to a general loss of calcium-mobilizing capability. Taken together, these data indicate that the D193A phenotype is represented by a loss of SQ29,548 binding and an inhibited functional response to U46619. Consequently, Asp193 appears to play a critical role in the TPR-ligand binding process, and hydrogen and/or ionic bonding at 193 is essential for this interaction.
It was found that functional effect, a higher concentration of U46619 was tested. In order to investigate the basis of this reduced effect was significantly reduced, it was nevertheless also suggest a loss of its antagonistic activity. This was indeed partial functional activity to U46619.

Due to the hydrophobicity of Phe184, the anticipated ligands at the 193-position of TPRs depends on a combination of several factors including hydrogen bonding (through the carboxyl group), the length/conformation of the amino acid side chain, and the charge characteristics of this side chain.

Phenylalanine 184 Mutations: F184Y and F184A—The second residue selected was phenylalanine (Phe184), which was mutated to a polar residue with a similar structure (i.e. tyrosine). Due to the hydrophobicity of Phe184, the anticipated coordination forces would be hydrophobic in nature. FACS analysis showed that the F184Y mutant was stably expressed on the plasma membrane of HEK cells (Table 1). Analysis using radioligand binding revealed that the F184Y mutant did not significantly bind [3H]SQ29,548, even at 50 nM (Fig. 4A). Surprisingly, this mutant cell line still mobilized Ca2+ in response to 1 μM U46619 (Fig. 4B and Table 2). Whereas the magnitude of this effect was significantly reduced, it was nevertheless reproducible. In order to investigate the basis of this reduced functional effect, a higher concentration of U46619 was tested. It was found that 5 μM U46619 restored the Ca2+ response (Fig. 4C and Table 2) to a level comparable with that seen with the wild-type TPR cells. This decreased sensitivity to U46619 was reflected in a much higher EC50 (2425 ± 75 nM) (Table 3) for this mutant, suggesting a decreased agonist affinity. This mutant therefore lost binding activity to SQ29,548 but retained partial functional activity to U46619.

The inability of SQ29,548 to bind to the F184Y mutant might also suggest a loss of its antagonistic activity. This was indeed found to be the case, since 1 μM SQ29,548 had no effect on 1 μM U46619-induced Ca2+ mobilization (Fig. 4D). Thus, a concentration of SQ29,548 that completely blocks the U46619-induced Ca2+ response in the wild-type TPR (Fig. 1D) was without effect in the F184Y mutant. The next experiments evaluated whether a structurally different class of TPR antagonists was also incapable of blocking this Ca2+ response. It was found, however, that the aromatic sulfonamide BM13.505 (5 μM) was completely effective in abolishing U46619-mediated function (Fig. 4E) even at 5 μM of agonist concentration (not shown). Consequently, it would seem that the F184Y mutant differentiates between two separate classes of TPR antagonists, presumably due to the significant differences in their chemical structures. A phenotype identical to the F184Y was observed with the F184A mutation. Specifically, this mutant lost [3H]SQ29,548 binding and antagonistic activity (Table 4 and data not shown), had an elevated EC50 for calcium mobilization (2850 ± 50 nM; Table 3), and its response to U46619 was blocked by BM13.505 (data not shown). Thus, it appears that hydrophobic interactions with specific structural requirements in the phenylalanine side chain are necessary for SQ29,548 and to a limited extent for U46619 coordination.

Threonine 186 Mutations: T186A and T186S—The next amino acid selected was threonine (Thr186), which was mutated to alanine in order to eliminate the side chain of the parent amino acid and thereby prevent coordination through hydrophobic bonding. As was the case for the previous mutants, FACS results demonstrated that the T186A receptor was stably expressed on the cell surface (Table 1). Radioligand binding studies revealed that this mutant was also incapable of binding [3H]SQ29,548 (Fig. 5A), which is consistent with a previous report of an amino acid substitution at this position (33). In contrast to the F184Y mutant, which exhibited a reduced functional response, the T186A cells responded normally to U46619 (Fig. 5B) at 10 nM; Table 3). Furthermore, this response was not blocked by 1 μM SQ29,548 (Fig. 5C) but was blocked by 5 μM BM13.505 (Fig. 5D). Consequently, the T186A phenotype is represented by a clear divergence between agonist (U46619) and antagonist (SQ29,548) binding. The T186A phenotype also represents a clear divergence between two different classes of TPR antagonists, since the same concentration of SQ29,548 (1 μM) that produces complete inhibition in the wild type is ineffective in the T186A mutant, whereas the same concentration of BM13.505 (5 μM) that produces inhibition in the wild type is equally effective in this mutant. These results therefore suggest that hydrogen bonding at this position is required for SQ29,548
but not for BM13.505 or U46619 coordination. Interestingly, the T186S mutation possessed a phenotype identical to that of the T186A (data not shown and Tables 1–4). Since serine can also engage in hydrogen bonding, this latter finding indicates that SQ29,548 coordination with the 186-position also requires a specific conformational orientation.

Serine 191 Mutations: S191T and S191A—Mutation of serine (Ser191) to either threonine or alanine (Tables 1, 2, and 4) yielded a phenotype indistinguishable from the T186A and T186S cells. FACS analysis showed that the surface expression of both receptor mutants was at levels comparable with the wild type (Table 1). Furthermore, these mutants were

FIGURE 3. Aspartic acid 193 mutations: D193A, D193E, D193N, D193Q, and D193R. Cells were evaluated using radioligand binding and calcium mobilization. A, binding of [3H]SQ29,548 to D193A cells. The bar graph represents binding of 50 nM [3H]SQ29,548 (a concentration that saturates wild-type TPR) (p < 0.05, n = 3). B, 1 μM U46619-induced Ca2+ mobilization. D193A cells were also treated with carbachol as a control. C, saturation binding isotherm of [3H]SQ29,548 to D193E. Cells were incubated with various concentrations of [3H]SQ29,548 to generate the saturation binding isotherm (p < 0.05, n = 3). D, 1 μM U46619-induced Ca2+ mobilization (p < 0.05, n = 3) for D193E. E, saturation binding isotherm of [3H]SQ29,548 to D193N. Cells were incubated with various concentrations of [3H]SQ29,548 to generate the saturation binding isotherm (p < 0.05, n = 3). F, 1 μM U46619-induced Ca2+ mobilization (p < 0.05, n = 3) for D193N. G, binding of [3H]SQ29,548 to D193Q cells. The bar graph represents binding of 50 nM [3H]SQ29,548 (p < 0.05, n = 3). H, 1 μM U46619-induced Ca2+ mobilization (p < 0.05, n = 3) for D193Q. Cells were also treated with carbachol as a control. I, binding of [3H]SQ29,548 to D193R cells. The bar graph represents binding of 50 nM [3H]SQ29,548 (p < 0.05, n = 3). J, 1 μM U46619-induced Ca2+ mobilization (p < 0.05, n = 3). D193R cells were also treated with carbachol as a control. Results are representative of at least three different experiments in each case.
incapable of binding [3H]SQ29,548 (Fig. 6A), retained their functional response to U46619 with an EC50 close to the wild type (Fig. 6B, Table 2, and Table 3), were not sensitive to SQ29,548 functional inhibition (Fig. 6C), but were sensitive to BM13.505 functional inhibition (Fig. 6D). Thus, it appears that similar to Thr186, Ser191 may contribute hydrogen bonding forces with some conformational constraints that are required for coordination with SQ29,548 but not required for coordination with either U46619 or BM13.505.

**Glutamic Acid 190 Mutations: E190A and E190D**—The last amino acid selected within C-EL2 was glutamic acid (Glu190), which was mutated to alanine in order to eliminate its side chain and hence its potential for interaction with ligands via ionic or hydrogen bonds. FACS analysis demonstrated that this mutant receptor was expressed on the plasma membrane (Table 1). The saturation binding isotherm generated from [3H]SQ29,548 established a $K_d$ value of 6 ± 1 nM (Table 4), which is very close to that of the wild-type TPR. Moreover, Ca2+ mobilization experiments revealed an EC50 of 470 ± 10 nM (Table 3) and intact response to 1 mM U46619 (Table 2), which was blocked by 5 mM BM13.505 (not shown). Therefore, substitution of Glu190, which is located between Thr186 and Asp193, appears to affect neither SQ29,548 interaction with this mutant TPR nor the ability of U46619 to elicit a Ca2+ response. Consequently, Glu190 does not seem to play a significant role in TPR-ligand coordination. A similar phenotype was observed with a different substitution at this position, i.e. E190D (data not shown, Tables 1–4). Based on these results, it appears that neither ionic nor hydrogen bonding at this position is required for TPR coordination with either agonists or antagonists.

**Serine 201 Mutations: S201T and S201A**—The next set of experiments were performed on stable cell lines expressing point mutations within the TM5 region of TPRs. This region was selected due to its close proximity to the C-EL2 domain and the lipophilic nature of TPR ligands, which may insert into nearby membrane regions. The serine residue (Ser201) was the first to be evaluated due to its ability to form hydrogen bonds via its hydroxyl moiety. This residue was mutated to threonine, which can also participate in hydrogen bonding. FACS analysis demonstrated that the S201T cells stably expressed the receptor protein on their plasma membrane (Table 1). Radioligand binding studies with [3H]SQ29,548 demonstrated a $K_d$ of 4 ± 0.3 nM (Fig. 7A and Table 4), which was not statistically different from that of the wild-type TPR. Furthermore, this mutant also revealed a significantly lower but reproducible Ca2+ mobilization response to 1 mM U46619 (Fig. 7B and Table 2). This functional response was also inhibited by SQ29,548 (1 μM; not shown) and by BM13.505 (5 μM; not shown). To investigate the basis of this decreased functional effect, a higher concentration of U46619 was tested. It was found that 5 μM U46619 restored the Ca2+ response (Fig. 7C and Table 2) to a level comparable with that of the wild-type TPR. This lower sensitivity to U46619 was reflected in an EC50 value (2235 ± 35 nM; Table 3) that was substantially higher than that of the wild-type TPR, suggesting that the S201T mutation resulted in a compromised agonist affinity. To further confirm this notion, displacement binding studies were performed using [3H]SQ29,548 and U46619 as the displacing ligand. Consistent with a lower affinity for U46619, there was a rightward shift in the S201T displacement curve relative to the wild-type TPR (Fig. 7D). Analysis of these data revealed a U46619 $K_i$ of 62 ± 1 nM for the S201T as compared with a $K_i$ of 14 ± 1 nM for the wild-type TPR. Hence, there is more than a 4-fold decrease in the U46619 affinity and functional responsiveness as a result of this mutation. These results suggest that Ser201 is not involved in the coordination of SQ29,548 but is involved in the coordination of U46619.

Next, analysis was performed on a stable cell line expressing the S201A TPR. The results for SQ29,548 and U46619 were identical to those found with the S201T mutant (Tables 1–4 and data not shown) (i.e. normal SQ29,548 binding and an elevated EC50 for U46619-induced Ca2+ mobilization). Taken together, the S201A mutant data suggest that the hydroxyl moiety of Ser201 may be necessary for hydrogen bond formation with U46619. Furthermore, if this is indeed correct, the S201T data indicate that there are also conformational requirements for bonding interactions with this hydroxyl group.

**Phenylalanine 196 Mutations: F196Y and F196A**—The second site selected was phenylalanine (Phe196). Since this residue is hydrophobic in nature, it has the capability to engage in hydrophobic coordination forces. The Phe196 was mutated to tyrosine, which is a polar amino acid possessing a similar structure. The F196Y mutant receptor was found to be stably expressed on the cell surface (Table 1). Radioligand binding studies indicated a higher affinity for [3H]SQ29,548 with a $K_d$ =

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**TABLE 4**

| A summary of the binding ($K_d$ value) and Ca2+ data for the wild-type and mutant TPR-expressing cell lines |
|--------------------------------------------------|
| Results are representative of at least three different experiments in each case ($p < 0.05$, $n = 3$). NA, not applicable. |

| Cell line | SQ binding $K_d$ | Ca2+ mobilization |
|-----------|-----------------|------------------|
| WT        | 7 ± 1           | ++++++           |
| F184A     | NA              | ++++*            |
| F184Y     | NA              | ++++*            |
| T186A     | NA              | ++++             |
| T186S     | NA              | ++++             |
| E190A     | 6 ± 1.0         | ++++             |
| E190D     | 5 ± 0.3         | ++++             |
| S191A     | NA              | ++++             |
| S191T     | NA              | ++++             |
| D193N     | 2 ± 0.3         | ++++             |

| Cell line | SQ binding $K_d$ | Ca2+ mobilization |
|-----------|-----------------|------------------|
| D193A     | NA              | +*               |
| D193Q     | NA              | +*               |
| D193R     | NA              | +*               |
| D193E     | 5 ± 0.2         | ++++             |
| F196A     | 4 ± 1.0         | ++++             |
| F196Y     | 3 ± 0.2         | ++++             |
| F200A     | 7 ± 2.0         | ++++             |
| F200Y     | 5 ± 1.0         | ++++             |
| S201A     | 5 ± 1.0         | ++++             |
| S201T     | 4 ± 0.3         | ++++             |

* $p < 0.05$.
3 ± 0.2 nM (Fig. 8A and Table 4), which was significantly lower than that of the wild-type TPR.

The next experiments investigated the functional effects of the F196Y mutation on Ca\(^{2+}\) mobilization. The results demonstrated a 1 μM U46619 response and an EC\(_{50}\) (455 ± 35 nM; Table 3) that were not different from the wild-type TPR (Fig. 8B and Table 2). Furthermore, this functional response was sensitive to inhibition by 5 μM BM13.505 (not shown). Therefore, similar to the D193N mutant, substitution of Phe\(^{196}\), which is more proximal to the extracellular surface than Ser\(^{201}\), appears...
to enhance SQ29,548 affinity for TPRs but has no effect on the U46619-elicited functional response. Regarding the F196A mutant, the expression level (Table 1), the $[^3]H$SQ29,548 $K_d$ value (4 ± 1 nM) (Table 4), the Ca$^{2+}$ mobilization response (Table 2), and the EC$_{50}$ (460 ± 30 nM) (Table 3) of this response were not found to be statistically different from the wild-type TPR. Also, similar to the F196Y mutation, the Ca$^{2+}$ response in this mutant was found to be blocked by 5 μM BM13.505 (not shown). Thus, the only deviation from the wild-type phenotype resulted from tyrosine substitution, which led to an enhanced affinity for SQ29,548.

Phenylalanine 200 Mutations: F200A and F200Y—Another hydrophobic residue adjacent to Ser$^{201}$ (i.e. phenylalanine (Phe$^{200}$)) was also mutated. Analysis was first performed on a stable cell line expressing its alanine substitution (F200A) on the cell surface (Table 1). Similar to alanine substitution at Phe$^{196}$, there was no effect on either $[^3]H$SQ29,548 binding ($K_d$ = 7 ± 2 nM; Table 4), 1 μM U46619-mediated Ca$^{2+}$ mobilization (Table 2), or its EC$_{50}$ (Table 3). As before, the functional response in these cells was inhibited by 5 μM BM13.505 (not shown). The results obtained with the F200Y mutant were indistinguishable from those of the F200A mutant (Table 1), i.e. a wild-type $K_d$ for $[^3]H$SQ29,548 (5 ± 1 nM) (Table 4), an intact U46619-induced functional response (Table 2), and a wild-type EC$_{50}$ (Table 3). These data suggest that Phe$^{200}$ does not play a critical role in the TPR-ligand interaction process for SQ29,548, BM13.505, or U46619.

In summary, mutations at Phe$^{184}$, Thr$^{186}$, Ser$^{191}$, Asp$^{193}$, Phe$^{196}$, and Ser$^{201}$ all produced effects on TPR ligand interaction and/or function. In contrast, the E190A, E190D, F200A, and F200Y substitutions yielded no detectable phenotype and, hence, can be considered as a “control phenotype.” Thus, although Glu$^{190}$ and Phe$^{200}$ are directly adjacent to amino acids that interact with TPR ligands (e.g. Ser$^{191}$ and Ser$^{201}$), they do not themselves play a critical role in the coordination processes to either SQ29,548, U46619, or BM13.505.

**DISCUSSION**

It is generally accepted that small, nonpeptide ligands, such as biogenic amines and nucleotides, bind to the transmembrane regions of their respective receptors (34). On the other hand,
peptide receptors seem to utilize residues within their extracellular loops for ligand coordination (35). Since prostanoids are small ligands, they were initially thought to coordinate with transmembrane regions of their corresponding receptors. However, recently there is accumulating evidence for a role of the extracellular loops in binding with small nonpeptide ligands (36). In addition, many studies on other G-protein-coupled receptors also suggested that residues within EL2 or EL3 can form part of the ligand binding pocket (34, 37–41). The same considerations seem to apply to TPRs, since results using a biotinylated antagonist previously suggested ligand interaction with TPR extracellular regions (25). Furthermore, subsequent experiments demonstrated that C-EL2 represents a crucial site for ligand docking and function (26), and the importance of this receptor region in such processes was later confirmed by a separate study on this receptor protein (33). However, despite these recent findings, information concerning the specific amino acids that participate in the TPR-ligand interaction process is still unavailable. On this basis, the current study used mutagenic analysis to investigate the involvement of single amino acids within the C-EL2 of the TPR in ligand binding and function. In addition, certain residues within TM5 were also evaluated due to the lipophilic nature of TPR ligands that may insert into the membrane regions near C-EL2.

FACS results demonstrated that all of the stable cell line clones selected for experimentation expressed the mutated receptor protein on their surface at levels comparable with the wild-type TPR-expressing cells. The data obtained with radioligand binding analysis and Ca^{2+}/H11001 mobilization experiments revealed a control phenotype as well as two minor and three major mutant phenotypes. The nature of each phenotype appeared to depend both on the location of the amino acid residue and the nature of the specific substitution. A summary of the binding and Ca^{2+}/H11001 data is provided in Table 4 for comparison.

Analysis of data obtained from point mutations within TM5 revealed two minor phenotypes. First, both the S201T- and S201A-expressing cells exhibited normal SQ29,548 binding but a reduced Ca^{2+} response to 1 μM U46619 and an increased EC_{50}. This attenuated response was found to be a consequence of a lowered agonist affinity for the mutant TPR, as shown by both the functional and ligand displacement studies. These data suggest a role for the hydroxyl moiety of Ser^{201} in forming a hydrogen bond with U46619 but
not with SQ29,548. These binding results are in agreement with earlier studies performed on the S201A recombinant protein, which also showed normal SQ29,548 binding and reduced agonist ([125I]-3-((S)-(1E,3R)-5-[(3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-en-7-yl)-5-heptenoic acid) binding (23). The second minor phenotype was revealed by the F196Y mutation. This mutation resulted in an increased receptor affinity for SQ29,548 but a wild-type Ca\(^{2+}\) response to U46619. While the molecular basis for this increased affinity is unclear, it may derive from the introduction of a hydroxyl group at position 196, which could potentially form a new coordination site for SQ29,548.

The first major phenotype was observed with two separate mutations. In particular, results from the F184Y and F184A mutants demonstrated a complete loss of SQ29,548 binding activity, a reduction in U46619-stimulated Ca\(^{2+}\) mobilization, and an elevated EC\(_{50}\). This attenuated response presumably resulted from a lowered agonist affinity, since increasing the U46619 concentration restored a wild-type Ca\(^{2+}\) response. Moreover, this U46619-induced effect was blocked by the antagonist BM13.505 but not by SQ29,548. Taken together, these data demonstrate that Phe\(^{184}\) is involved in ligand coordination with both SQ29,548 and U46619 but not with BM13.505. Similar to the present results, a phenylalanine to tyrosine mutation in the gonadotropin-releasing hormone receptor was also found to affect ligand binding, possibly due to the presence of a hydrophilic phenolic side chain of the tyrosine as opposed to the hydrophobic side chain of phenylalanine (42).

The second major phenotype, represented by a set of mutations (T186S, T186A, S191T, and S191A), is both interesting and complex. One aspect of this phenotype is a clear differentiation between antagonist binding and functional responses. Specifically, the mutants were devoid of SQ29,548 binding activity, but they nevertheless exhibited an intact response to the agonist U46619 with an EC\(_{50}\) value comparable with that of the wild-type TPR. A similar phenotype has also been documented for other receptors, such as the vasopressin receptor (43) and the gonadotropin-releasing hormone receptor (42). The second aspect of this phenotype is a clear differentiation between two structurally distinct classes of TPR antagonists (i.e. SQ29,548 and BM13.505). Since the T186S, T186A, S191T, and S191A mutants did not bind SQ29,548, it is not surprising that this antagonist also did not block their Ca\(^{2+}\) response to

FIGURE 7. S201T mutation. Cells were evaluated using radioligand binding and calcium mobilization. A, saturation binding isotherm using [3H]SQ29,548. Cells were incubated with various concentrations of [3H]SQ29,548 to generate the saturation binding isotherm (p < 0.05, n = 3). B, 1 μM U46619-induced Ca\(^{2+}\) mobilization (p < 0.05, n = 3). C, calcium mobilization using 5 μM U46619 (p > 0.05, n = 3). D, binding displacement of 1 nM [3H]SQ29,548 with increasing concentrations of U46619 (2 nM to 30 μM) (p < 0.05, n = 3). Results are representative of at least three different experiments in each case.
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U46619. Interestingly, however, this response was completely inhibited by the sulfonamide BM13.505. Therefore, these mutations appear to result in a receptor that does not have the ability to recognize structural differences between U46619 and BM13.505 but can recognize structural differences between the latter two ligands and SQ29,548.

Based on these considerations, it appears that both Thr$^{186}$ and Ser$^{191}$ are not critical for ligand coordination with either U46619 or BM13.505 but are required for binding interactions to SQ29,548. Previous molecular modeling studies have suggested that certain moieties in the $\omega$-chain of SQ29,548 participate in hydrogen bonding to the receptor. It is noteworthy that both Thr and Ser can participate in such bonding interactions. Therefore, if Thr$^{186}$ and Ser$^{191}$ are indeed coordination sites for SQ29,548, the observed loss of binding activity with the Thr to Ala or Ser to Ala substitutions would be expected. It might also be expected that the Thr to Ser or Ser to Thr substitutions may not interfere with SQ29,548 binding, since each contains a hydroxyl group capable of hydrogen bond formation. However, this was not found to be the case. Consequently, it appears that SQ29,548 interaction with Thr$^{186}$ and Ser$^{191}$ is very sensitive to even minor structural changes, such that removing or adding a methylene group interferes with the conformational requirements for hydrogen bonding at these two positions. This conclusion is consistent with a report on the human A$_2A$ adenosine receptor, where a threonine to serine substitution in TM3 also resulted in impairment of ligand recognition (44).

The D193A mutant represented the third major phenotype, which was defined by a complete loss of SQ29,548 binding as well as a dramatically reduced U46619 Ca$^{2+}$ response. This response could not be restored even by elevating the U46619 dose by an order of magnitude, indicating that this mutation causes a substantially lower affinity of the receptor for U46619. Therefore, the Asp$^{193}$ represents a critical coordination site for both SQ29,548 and U46619.

In contrast, the D193E mutation was found to bind and signal in a manner similar to that of the wild type. One reason for these divergent activities may derive from ion pairing or hydrogen bonding considerations. Thus, since alanine cannot mediate ionic or hydrogen bonding interactions, the D193A mutation would not be capable of supporting either agonist or antagonist binding. On the other hand, this limitation does not apply to the D193E mutant, which retains the potential for such interactions. This suggestion is consistent with previous studies on the muscarinic acetylcholine receptor (45, 46). Specifically, these latter results suggested that mutating an aspartic acid to glutamic acid maintained the receptor's ability to bind ligands, whereas mutation to multiple residues that do not interact using these coordination forces led to a loss of ligand binding. Therefore, it appears that an acidic amino acid at the 193-position can support both antagonist and agonist coordination. Also, one could assume that the ion or hydrogen bonding with amino acids at the 193 site is somewhat flexible relative to side chain length, since the addition of one methylene group to aspartic acid (D193E) had no influence on receptor binding and activation.

In subsequent experiments, the Asp$^{193}$ was subjected to additional substitutions in order to further define the nature of coordination forces with this amino acid. For example, to determine the importance of ionic binding at 193, the D193N mutant was evaluated. This mutation changes the parent residue into an isosteric amide (asparagine). Even though asparagine has a side chain length equal to aspartic acid, its carbonyl group is linked to NH$_2$ and therefore cannot engage in ionic bonding. The results demonstrated that although this mutant receptor bound SQ29,548 with a higher affinity, it exhibited a functional response to U46619 that was not different from that for the wild-type TPR. These results therefore indicate that hydrogen bonding mediated by the asparagine substitution at position 193 differentially increases TPR affinity for SQ29,548 relative to U46619. Thus, the spatial conformation of Asn$^{193}$ promotes a stronger interaction with SQ29,548 than that which occurs with either Asp$^{193}$ (wild type) or Gln$^{193}$. These data further suggest that Asp$^{193}$ may also be involved in similar bonding forces and that its charge is not necessary for the binding process.

As mentioned above, the extension of the Asp$^{193}$ side chain by one carbon (D193E) did not interfere with ligand binding. Furthermore, ligand interaction was also supported by replacement of the carboxylate with a carboxamide (D193N). When the side chain was extended by one carbon and the carboxylate was replaced with a carboxamide (D193Q), there was a loss of SQ29,548 coordination and a significantly reduced U46619-mediated functional response. An aspartic acid to glutamine mutation produced similar effects on the binding of certain ligands to the human gonadotropin-releasing hormone receptor (47). Hence, to maintain TPR binding properties, the extended alkyl side chain at 193 appears to require preservation of the carboxylic group. These findings further demonstrate that although D193Q shares certain characteristics with D193E and D193N, subtle changes at this site can lead to substantial changes in SQ29,548 and/or U46619 TPR interaction.

Finally, we analyzed the D193R mutant. Whereas this substitution is capable of hydrogen bonding, it might not be expected.
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to support normal ligand coordination with TPRs. This is because its extended side chain not only lacks a carboxylate group, but it also possesses the reverse charge characteristics of the wild-type amino acid (Asp). The results demonstrated that this notion was indeed correct, since the D193R lacked SQ29,548 binding and exhibited a severely impaired U46619 calcium response.

Collectively, our data indicate that the requirements for ligand coordination at position 193 of TPRs appear to depend on a combination of several factors. In the wild type (Asp), hydrogen bonding would seem to be sufficient for ligand coordination. This notion is supported by the Asn mutation, which also maintained ligand binding. On the other hand, the capacity to engage in hydrogen bonding is not sufficient if the side chain is lengthened by one carbon (Gln). Finally, ligand binding cannot be supported by a charge reversal (Arg) at this position.

These data also point to significant differences between the amino acid coordination sites for the ligands tested. In this regard, one important distinction is that the binding requirements for SQ29,548 appear to be highly rigid, whereas those for both U46619 and BM13.505 are considerably more flexible. For example, mutation of Phe<sup>184</sup>, Thr<sup>186</sup>, Ser<sup>191</sup>, and Asp<sup>193</sup> all produced dramatic effects on SQ29,548 binding. On the other hand, these same mutations produced either no effect on U46619 activity (Ser<sup>191</sup> and Thr<sup>186</sup>), a modest effect (Phe<sup>184</sup>), or a dramatic effect (Asp<sup>193</sup>). While the reasons for these particularly stringent binding requirements of SQ29,548 are unknown, they may in part derive from the different structural classes to which each of these ligands belong. Specifically, SQ29,548 is a 7-oxabicycloheptane derivative that seemingly has chemical characteristics that resemble the natural ligand TXA<sub>2</sub> more than either U46619 (a prostaglandin H<sub>2</sub> endoperoxide analog) or BM13.505 (a structurally unrelated sulfonamide). On this basis, SQ29,548 may achieve higher binding affinity at the expense of flexibility in its binding interactions. In this connection, it has been proposed that SQ29,548 possesses at least three critical pharmacophores (recognition sites) at a spatial orientation that is optimal for high affinity interactions with the TPR ligand binding pocket (27). Furthermore, it has also been suggested that two of these recognition sites are present in the relatively rigid ω-chain (27). Consequently, it is reasonable that SQ29,548 would exhibit a high degree of sensitivity to changes in the individual amino acids actively engaged in coordination with its pharmacophores.

Based on previous modeling studies (27, 28) of the SQ29,548 hairpin configuration and the present data, certain tentative amino acid assignments can be made. On this basis, we propose that 1) Phe<sup>184</sup> interacts with the ω-chain benzene ring by hydrophobic interactions, 2) Thr<sup>186</sup> interacts with the ω-chain carboxyl/carboxyl group by hydrogen bonding, 3) Ser<sup>191</sup> interacts with the ω-chain carboxyl group by hydrogen bonding, and 4) Asp<sup>193</sup> interacts with the ω-chain 14-position nitrogen also by hydrogen bonding. If these assignments are indeed correct, the 7-oxabicycloheptane ring would presumably fit a hydrophobic pocket of the TPR transmembrane region.

The present study also identifies three putative coordination sites for U46619 (i.e. Phe<sup>184</sup>, Asp<sup>193</sup>, and Ser<sup>201</sup>). Interestingly, there appear to be both shared and unshared residues for U46619 and SQ29,548 binding, which might be predicted based on the differences in their biological activity. In this regard, it is tempting to speculate that the commonly shared residues (Phe<sup>184</sup> and Asp<sup>193</sup>) may represent ligand recognition sites, whereas the unshared residues (Thr<sup>186</sup>, Ser<sup>191</sup>, and Ser<sup>201</sup>) may distinguish between agonist and antagonist activity. Clearly, additional studies will be required to further define these subtle distinctions in ligand recognition/efficacy. Nevertheless, the present results demonstrate that amino acids within the TPR binding domain play differential roles in ligand coordination depending upon the particular ligand that is complexed to the receptor protein.

In summary, the present studies have provided the first comprehensive investigation of ligand coordination with the C-EL2 region of human TPRs. The results have identified five key amino acids within this region (Phe<sup>184</sup>, Thr<sup>186</sup>, Ser<sup>191</sup>, Asp<sup>193</sup>, and Ser<sup>201</sup>) that participate in TPR ligand binding and function. In addition, these studies also revealed differential coordination profiles for three separate classes of TPR ligands (i.e. SQ29,548, U46619, and BM13.505). Finally, the results from these studies have provided insight into the mechanism (i.e. the molecular forces) which appear to be involved in these ligand-amino acid interactions. Collectively, these findings should be useful in more accurately defining the chemical requirements for TPR ligand coordination and, in turn, facilitate the development of rationally designed TPR antagonists for therapeutic purposes.

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REFERENCES

1. Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S., and Narumiya, S. (1991) Nature 349, 617–620
2. Kim, S. O., Lim, C. T., Lam, S. C., Hall, S. E., Komiotis, D., Venton, D. L., and Le Breton, G. C. (1992) Biochem. Pharmacol. 43, 313–322
3. Halushka, P. V., Mais, D. E., Mayeux, P. R., and Morinelli, T. A. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 213–239
4. Hung, S. C., Ghali, N. I., Venton, D. L., and Le Breton, G. C. (1983) Biochim. Biophys. Acta 728, 171–178
5. Le Breton, G. C., Venton, D. L., Enke, S. E., and Halushka, P. V. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4097–4101
6. Svensson, J., Hamberg, M., and Samuelsson, B. (1976) Acta. Physiol. Scand. 98, 285–294
7. Okuma, M., Hirata, T., Ushikubi, F., Kakizuka, A., and Narumiya, S. (1996) Pol. J. Pharmacol. 48, 77–82
8. Svensson, J., Strandberg, K., Tuimeo, T., and Hamberg, M. (1977) Prostaglandins 14, 425–436
9. Lin, X., Ramamurthy, S. K., and Le Breton, G. C. (2005) J. Neurochem. 93, 257–268
10. Ushikubi, F., Aiba, Y., Nakamura, K., Namba, T., Hirata, M., Mazda, O., Katsuya, Y., and Narumiya, S. (1993) J. Exp. Med. 178, 1825–1830
11. (1994) Br. Med. J. 308, 81–106
12. Patrono, C., Ciabattoni, G., and Davi, G. (1990) Stroke 21, IV130–IV133
13. Oates, J. A., FitzGerald, G. A., Branch, R. A., Jackson, E. K., Knapp, H. R., and Roberts, L. J., II (1988) N. Engl. J. Med. 319, 689–698
14. Ogletree, M. L. (1987) Fed. Proc. 46, 133–138
15. FitzGerald, G. A., Healy, C., and Daugherty, J. (1987) Fed. Proc. 46,
Amino Acids Mediating TPR Ligand Interaction

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154–158
16. Patrono, C. (1994) N. Engl. J. Med. 330, 1287–1294
17. Clarke, R. J., Mayo, G., Price, P., and FitzGerald, G. A. (1991) N. Engl. J. Med. 325, 1137–1141
18. Findlay, J. B., and Pappin, D. J. (1986) Biochem. J. 238, 625–642
19. Narumiya, S., Hirata, N., Namba, T., Hayashi, Y., Ushikubi, F., Sugimoto, Y., Negishi, M., and Ichikawa, A. (1993) J. Lipid. Mediat. 6, 155–161
20. Funk, C. D., Furci, L., Moran, N., and Fitzgerald, G. A. (1993) Mol. Pharmacol. 44, 934–939
21. Dorn, G. W., II, Davis, M. G., and D’Angelo, D. D. (1997) J. Biol. Chem. 272, 12399–12405
22. D’Angelo, D. D., Eubank, J. J., Davis, M. G., and Dorn, G. W., II (1996) J. Biol. Chem. 271, 6233–6240
23. Chiang, N., Kan, W. M., and Tai, H. H. (1996) Arch. Biochem. Biophys. 334, 9–17
24. Yamamoto, Y., Kamiya, K., and Terao, S. (1993) J. Med. Chem. 36, 820–825
25. Komiotis, D., Wencel-Drape, J. D., Dieter, J. P., Lim, C. T., and Le Breton, G. C. (1996) Biochem. Pharmacol. 52, 763–770
26. Turek, J. W., Halmos, T., Sullivan, N. L., Antonakis, K., and Le Breton, G. C. (2000) J. Biol. Chem. 277, 16791–16797
27. Jin, B., and Hopfinger, A. J. (1994) J. Chem. Inf. Comput. Sci. 34, 1014–1021
28. Taylor, D. M., Halushka, P. V., and Meier, G. P. (2003) Eur. J. Med. Chem. 38, 1015–1024
29. Tiruppathi, C., Yan, W., Sandoval, R., Naqvi, T., Pronin, A. N., Benovic, J. L., and Malik, A. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7440–7445
30. Brandoli, C., Sanna, A., De Bernardi, M. A., Follesa, P., Brooker, G., and Mocchetti, I. (1998) J. Neurosci. 18, 7953–7961
31. Armstrong, R. A., Humphrey, P. P., and Lumley, P. (1993) Br. J. Pharmacol. 110, 548–552
32. Huang, J. S., Dong, L., and Le Breton, G. C. (2006) Cell. Signal. 18, 564–576
33. So, S. P., Wu, J., Huang, G., Huang, A., Li, D., and Ruan, K. H. (2003) J. Biol. Chem. 278, 10922–10927
34. Hoffmann, C., Moro, S., Nicholas, R. A., Harden, T. K., and Jacobson, K. A. (1999) J. Biol. Chem. 274, 14639–14647
35. Gether, U. (2000) Endocr. Rev. 21, 90–113
36. Liapakis, G., Ballesteros, J. A., Papachristou, S., Chan, W. C., Chen, X., and Javitch, J. A. (2000) J. Biol. Chem. 275, 37779–37788
37. Olah, M. E., Jacobson, K. A., and Stiles, G. L. (1994) J. Biol. Chem. 269, 24692–24698
38. Stillman, B. A., Audoly, L., and Breyer, R. M. (1998) Eur. J. Pharmacol. 357, 73–82
39. abu Alla, S., Quitterer, U., Grigoriev, S., Maidhof, A., Haasemann, M., Jarnagin, K., and Muller-Esterl, W. (1996) J. Biol. Chem. 271, 1748–1755
40. Audoly, L., and Breyer, R. M. (1997) J. Biol. Chem. 272, 13475–13478
41. Shi, L., and Javitch, J. A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 440–445
42. Hoffmann, S. H., ter Laak, T., Kuhne, R., Reilander, H., and Beckers, T. (2000) Mol. Endocrinol. 14, 1099–1115
43. Cotte, N., Balestre, M. N., Aumelas, A., Mahe, E., Phalipou, S., Morin, D., Hibert, M., Manning, M., Durroux, T., Barberis, C., and Mouillac, B. (2000) Eur. J. Biochem. 267, 4253–4263
44. Townsend-Nicholson, A., and Schofield, P. R. (1994) J. Biol. Chem. 269, 2373–2376
45. Page, K. M., Curtis, C. A., Jones, P. G., and Hulme, E. C. (1995) Eur. J. Pharmacol. 289, 429–437
46. Fraser, C. M., Wang, C. D., Robinson, D. A., Gocayne, J. D., and Venter, J. C. (1989) Mol. Pharmacol. 36, 840–847
47. Betz, S. F., Reinhart, G. J., Lio, F. M., Chen, C., and Struthers, R. S. (2006) J. Med. Chem. 49, 637–647