The Unique Ligand-binding Pocket for the Human Prostacyclin Receptor

SITE-DIRECTED MUTAGENESIS AND MOLECULAR MODELING*

Received for publication, July 24, 2002, and in revised form, November 18, 2002
Published, JBC Papers in Press, November 21, 2002, DOI 10.1074/jbc.M207420200

Jeremiah Stitham, Aleksandar Stojanovic, Bethany L. Merenick, Kimberly A. O’Hara, and John Hwa‡

From the Department of Pharmacology & Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755

The human prostacyclin receptor is a seven-transmembrane α-helical G-protein coupled receptor, which plays important roles in both vascular smooth muscle relaxation as well as prevention of blood coagulation. The position of the native ligand-binding pocket for prostacyclin as well as other derivatives of the 20-carbon eicosanoid, arachidonic acid, has yet to be determined. Through the use of prostaglandin receptor sequence alignments, site-directed mutagenesis, and the 2.8-A x-ray crystallographic structure of bovine rhodopsin, we have developed a three-dimensional model of the agonist-binding pocket within the seven-transmembrane (TM) domains of the human prostacyclin receptor. Upon mutation to alanine, 11 of 29 candidate residues within TM domains II, III, IV, V, and VII exhibited a marked decrease in agonist binding. Of this group, four amino acids, Arg-279 (TMVII), Phe-278 (TMVII), Tyr-75 (TMII), and Phe-95 (TMIII), were identified (via receptor amino acid sequence alignment, ligand structural comparison, and computer-assisted homology modeling) as having direct molecular interactions with ligand side-chain constituents. This binding pocket is distinct from that of the biogenic amine receptors and rhodopsin where the native ligands (also composed of a carbon ring and a carbon chain) are accommodated in an opposing direction. These findings should assist in the development of novel and highly specific ligands including selective antagonists for further molecular pharmacogenetic studies of the human prostacyclin receptor.

Vascular smooth muscle relaxation and inhibition of platelet aggregation are two key physiological processes mediated by human prostacyclin. Dysfunctional prostacyclin activity has been implicated in the development of a number of cardiovascular diseases including thrombosis, myocardial infarction, stroke, myocardial ischemia, atherosclerosis, and systemic and pulmonary hypertension (1). In contrast to other members of the prostanoid family, there are currently no high affinity selective antagonists for the prostacyclin receptor. This finding suggests that the prostacyclin receptor may possess a unique ligand-binding pocket.

Receptor activation is contingent upon ligand binding interactions, which initiate a conformational change in protein structure that is subsequently transmitted to the G-protein. Determining the exact nature and location of receptor-ligand binding interactions at the molecular level is essential for understanding the functions of prostanoid receptor physiology. Moreover, such insights would lend to the development of novel and highly specific modes of treatment for prostanoid-related disorders. Based upon the position of the chromophore (covalently bound 11-cis-retinal) within the binding pocket of rhodopsin along with the location of other ligands within similar rhodopsin-type GPCRs (2), the putative binding pocket for GPCRs with small nonpeptide ligands is believed to be located predominantly within the hydrophobic core of the transmembrane domain in close proximity to the extracellular boundary of the receptor. However, the crucial anchoring points that comprise the fundamental structure of the binding pocket, securing important receptor-ligand associations between prostacyclin and its receptor, have yet to be determined.

As is the case with all prostanoids, prostacyclin (PGI 2) is a derivative of the C20 unsaturated fatty acid arachidonic acid (5,8,11,14-eicosatetraenoic acid) (Fig. 1). The general structure of prostanoid molecules consists of a centralized cyclopentane ring (thromboxane has an oxane ring) flanked by two hydrocarbon chains, the α- and ω-chains, whose configuration and functional groups determine further classification (Fig. 1). In particular, the prostacyclin molecule contains an additional oxalone (cyclic ether) ring fused to the cyclopentane ring as well as two hydroxyl groups located at C11 and C15. A characteristic terminal carboxylate group is present at the C1 position as well as carbon-carbon double bonds linking C5 to C6 and C13 to C14. Similar molecular features can be seen in synthetic prostacyclin analogues such as iloprost, a stable high affinity agonist that substitutes a secondary cyclopentane ring in place of the PGI 2 oxalone ring, carries an additional C16-methyl group and a ω-chain triple bond (Fig. 1). Side chains of certain amino acids have been shown in receptors to interact directly with substituents of ligands, conferring binding affinity (3, 4) through major forces such as hydrogen bonding, hydrophobic interactions, and ionic interactions. Thus, structural similarities and differences between both prostanoid receptors and prostanoid ligands play an important role in determining sites of interaction between receptor and ligand. For example, conserved serine residues found in TMV of the adrenergic receptors have been shown to interact with the...
conserved hydroxy groups extending from the catechol ring of the biogenic amines (4).

Recent studies have begun to identify generalized regions within the prostacyclin receptor and other prostaglandin receptors that appear crucial for ligand-binding specificity and affinity. Studies using chimeric combinations of mouse prostaglandin D and prostaglandin I receptors have shown that protein segments within transmembrane domains VI and VII (TMVI and TMVII) are involved in distinct binding interactions with prostacyclin side chains, whereas TMVI along with a portion of the first extracellular loop confers broader binding functions, incorporating recognition and interaction with the cyclopentane ring (5, 6). Additionally, glycosylation at Asn-7 and Asn-78 (7) and proline residues within the transmembrane domains (8) have been shown to be essential for proper binding and activation. Although neither of the two recently identified naturally occurring polymorphisms (i.e. V25M and R212H) have revealed inherent effects on binding, R212H in the third intracellular loop has been shown to exclusively effect activation and exhibits defective binding only under acidic conditions (9).

Using site-directed mutagenesis, prostaglandin ligand and receptor comparisons, and a three-dimensional computer-generated homology model of the hIP receptor derived from the recently published crystal structure of bovine rhodopsin (10), four crucial points of interaction between prostacyclin and the upper perimeter of the transmembrane domain of the hIP were identified. These crucial points include Arg-279 (TMVII), Phe-278 (TMVII), Tyr-75 (TMII), and Phe-95 (TMIII), which interact with the C1-COOH, oxalane ring and α-tail, C11-OH, and ω-tail of prostacyclin, respectively. This agonist-binding pocket is quite distinct from that of the biogenic amine receptors and rhodopsin.

EXPERIMENTAL PROCEDURES

Materials—Iloprost ligands, radiolabeled [3H]Iloprost (17.0 Ci/mmol) and non-radiolabeled iloprost, were purchased from Amersham Biosciences. Oligonucleotide primers were purchased from Sigma, whereas the hIP cDNA was a generous gift from Dr. Mark Abramovitz (Merck Frosst, Quebec, Canada).

Approach to Elucidating the hIP-binding Pocket—Initially, criteria were established to identify potentially important residues within the upper half of the transmembrane domain (i.e. the proposed locale of the putative prostacyclin-binding pocket) whose side-chain functional groups may interact with prostacyclin via electrostatic interactions, hydrogen bonding, or hydrophobic associations. Thus, candidate hIP residues with probable implications on ligand binding were targeted for site-directed mutagenesis, initially changing each residue to alanine. A series of competition binding assays were performed using iloprost, a stable high affinity analogue of prostacyclin. For those mutations eliciting a notable change in binding affinity, further appropriate residue replacements (catering to more specific size and/or polarity changes) were made to determine the specific characteristics of the amino acid with adverse effects on ligand binding. The influence on binding affinity was postulated to be the result of: 1) direct molecular interactions among critical binding-related residues and ligand side chains within the immediate binding pocket; 2) indirect molecular interactions among receptor residues involved in local preservation of the proximal binding domain; or 3) outlying interactions among α-helix-maintaining amino acids. To further define the receptor amino acids that interacted with ligand, sequence alignments for all prostaglandin receptor transmembrane domains were obtained from GPCRDB Prostaglandin (www.gpcr.org). Forty-two prostaglandin receptor sequences including multiple PE21, PE22, PE23, PE24, PF2R, TA2R, PD2R, and PI2R from different species were aligned for all prostanoid receptor transmembrane residues along with the maintenance of certain functional groups on other prostaglandins suggested probable functional correlations and importance. Residues identified within the putative binding pocket and exhibiting substantial influence on ligand binding affinity were reconstructed on a computer-generated hIP model based upon the crystal structure of rhodopsin. A structural-based prostacyclin molecule was then inserted (binding) to the model receptor comparing multiple positions. Taking into account all of the aforementioned features, a theoretical three-dimensional model of the prostacyclin-binding pocket was developed.

Construction of Mutant Receptors—Human IP cDNA was cloned into the plasmid vector pMT4, and point mutations were generated using conventional methods of PCR mutagenesis. Complementary oligonucleotide primers were designed extending 10–12 nucleotides 3′ and 5′ from the desired mutation site. The PCR reaction mixture contained 1× Pfu reaction buffer, 200 ng of DNA construct, 150 ng of each primer (sense and antisense), 10 mM dNTPs, and 2.5 units of Pfu DNA polymerase (Stratagene, Austin, TX) and was heated and cooled at 95 °C for 30 s, 65 °C for 1 min, and 88 °C for 10 min for 16 cycles. The products were digested with DpnI (New England Biolabs, Beverly, MA) and T4 DNA polymerase (Promega, Madison, WI) for 3 h to remove any parental wild-type strands of DNA. Ten microliters of PCR product was used to transform competent DH5α Escherichia coli cells (2× 10^9 cells) followed by DNA extraction from selected clones. Large plasmid preparations were performed using Wizard® Plus Maxiprep kits (Promega), and all mutant constructs were confirmed via PCR DNA dideoxynucleotide chain termination sequencing (Molecular Biology Core Facility, Dartmouth Medical School, Hanover, NH).

Transfection of COS-1 Cells—Transient transfections were performed on COS-1 cells as follows: the initial wash of cells with Cellgro® Dulbecco’s modified Eagle’s medium (DMEM, Mediatech, Inc., Herndon, VA) was followed by incubation of mutant DNA (20 μg/plate) in diethylaminoethyl-Dextran (DEAE-Dextran, Sigma) and DMEM. Cells were incubated at 37 °C with 5% CO2 for 6 h after which 0.1 mM chloroquine solution was added. Cells were subsequently incubated for 1 h, and chloroquine was removed through washes with DMEM. Cells were harvested 72 h post-transfection.

Membrane Preparations—Preparations of COS-1 cell membranes were carried out as follows: cells were washed in phosphate-buffered saline and harvested using cell scrapers. Vortexing (providing shear forces) for 3 min in sucrose (0.25 M) was followed by low speed spin (×1260 × g) for 5 min, and the supernatant collected. After a high speed centrifugation (×30,000 × g for 15 min), the pellet was then washed twice in 1× HEM (20 mM Hepes, pH 7.4, 1.5 mM MgCl2, and 12.5 mM MgCl2) followed by resuspension in 1× HEM containing 10% glycerol and stored at −70 °C. A Bradford protein assay was performed to quantitate membrane proteins.

Ligand Binding—Ligand-binding characteristics for the expressed receptors were determined through a series of competition binding assays using the radiolabeled ligand [3H]Iloprost. An analysis involved the construction of reaction mixtures (in duplicate wells) containing 50 μg of membrane protein, HEM buffer, and 15 mM [3H]Iloprost along with 1 of 11 different concentrations of cold (non-radiolabeled) iloprost ranging from 10 μM to 0.1 mM. After 1.5 h of incubation at 4 °C, reactions were stopped by the addition of ice-cold 10 ml Tris/HC1 buffer, pH 7.4, and transferred onto Whatman glass-fiber filters (GFF) and a cell harvester. The filters were washed five times with ice-cold Tris/HC1 buffer, and radioactivity was measured in the presence of 5 ml of Ecoscint™ H scintillation fluid (National Diagnostics, Atlanta, GA). Nonspecific binding was determined by the addition of a 500-fold excess of non-radiolabeled iloprost. The concentration of [3H]Iloprost was varied from 1 to 100 nM for saturation binding studies. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). IC50 values were converted to KI, using the Cheng-Prusoff equation, and KI values were expressed as a mean ± S.E. An analysis of variance (ANOVA) and Student’s t tests were used to determine significance differences (p < 0.05).

Western Blot Analysis—The presence of mutant protein with low affinity to iloprost was determined through Western blot analysis using monoclonal antibodies targeting the 1D4 epitope tag (8). Thirty microliters of membrane preparation containing 30 μg of membrane protein was subjected to 10% SDS-polyacrylamide gel electrophoresis. This was transferred to a nitrocellulose membrane, immunoblotted using a 1D4 monoclonal antibody and peroxidase-conjugated rabbit anti-mouse secondary antibody, and detected with enhanced chemiluminescence reagents.

Modeling of Prostacyclin Ligand—Because of the instability of the native ligand (prostacyclin), a synthetic analogue (iloprost) (Fig. 1) was used in all experimental assays. However, seeing that both structures maintain structural identity and the same recognition pattern, a model that confers binding specificity with the hIP (e.g. C1-COOH, C11-OH, C15-OH, cyclohexane ring, α-chain, and ω-chain) in addition to identical binding affinities, it was our feeling that the endogenous prostacyclin molecule would be more appropriate and biologically relevant within our model.
Fig. 1. Structures of prostanoid precursor and selected prostanoid ligands. An illustration comparing the structural similarities and differences of some representative prostanoid ligands is shown. A, arachidonic acid (5,8,11,14-eicosatetraenoic acid) serves as the precursor molecule for all native prostaglandin and thromboxane ligands. B, three native prostanoids (PGI2, PGE1, and TXA2) showing common features: the C1-COOH, the α-chain; the C11-OH, the ω-chain; and the C15-OH. There are distinct variances within the centralized ring structure with prostacyclin having two rings. C, the structure of iloprost (stable, high affinity synthetic PGI2 analogue) is shown. It has the four common structural features described in B. However, a cyclopentane ring replaces the oxalane ring, there is a C18-methyl substitution, and there is a C18-triple bond.

ing system. Computer-assisted molecular modeling of the prostacyclin ligand (native agonist to the hIP receptor) was performed using Swiss PDB Viewer (GlaxoSmithKline, Geneva, Switzerland) (11). A three-dimensional PGI2 molecule was modeled based upon the known chemical structure as well as a previously predicted conformation of a receptor-associated PGI2 (12). Initial modeling constraints required the adjustment of individual atoms such that they would conform to the chemically acceptable limits of the ligand structure. This included the adjustment of all covalent bonds to agree with standard lengths and angles: sp2-hybridized C–C single bond = 1.54 Å and 109.5°; sp3-hybridized C=C double bond = 1.33 Å and 120.0°; sp3-hybridized C–O single bond = 1.40 Å and 108.0°; and sp2-hybridized C=O double bond = 1.20 Å and 120.0°. Because of substantial torsional strain, all interior angles for both five-membered rings were approximated at 108.0° with the exception for the C–O–C bond angle of the oxane ring, which was set closer to 112.0°. The root mean square deviation for all bonds and angles was calculated at 0.006 Å and 0.4°, respectively, compared with the standard numbers referenced above. Furthermore, the two rings were constructed to assume the common envelope configuration with the characteristic four co-planar atoms combined with a single bond. The structural conformation of our receptor-bound prostacyclin molecule is similar to that of previous investigations in modeling conformations of receptor-associated PGI2 (12) where the α-chain is maintained in a bent conformation (back upon the two centralized rings), whereas the long hydrophobic ω-chain is in an extended configuration. This configuration was independently confirmed using MacSpartan Pro software where the energy-minimized conformations (~7 to ~10 kcal/mol) all exhibited such a ring and α-chain conformation with the major variances being in position of the ω-chain (data not shown).

Modeling of hIP Receptor—A theoretical three-dimensional homology model of the seven-transmembrane α-helices of the hIP receptor was constructed using the internet-based protein-modeling server Swiss Model (GlaxoSmithKline) (11). Amino acid sequences from all prostanoid receptors were obtained from theGPCR data base (42 sequences in total, GPCRDB Prostainoid) and aligned with those of the bovine rhodopsin receptor. Based upon this alignment, seven distinct peptide segments (each containing 26 amino acids) corresponding to the seven-transmembrane domains of the hIP receptor were determined, and a homology model was generated using the 2.8 Å resolution x-ray crystallographic structure of the bovine rhodopsin receptor as the template (Protein Data Bank code 1HZX). Receptor residues were tethered by harmonic constraints to their corresponding rhodopsin transmembrane templates and assembled into helical conformations by successive manipulations of selected degrees of freedom (rigid body rotational/translational followed by torsional). Once assembled, the transmembrane domains were energy-minimized utilizing the Gromos96 force field to improve the stereochemistry of the model and remove unfavorable clashes (Swiss Model). Visualization and evaluation of the model as well as insertion of the prostacyclin ligand was performed using the Swiss PDB Viewer. Additional amino acids were added to the extracellular transmembrane domain region (α2d) and allowed to adopt an α-helical conformation (where appropriate) using the crystal structure of rhodopsin as a template. From the crystal structure of the rhodopsin, some of the transmembrane α-helices extend beyond 26 amino acids (e.g. TMIII). Furthermore, the binding of prostacyclin may extend into the extracellular domain. The complete interhelical loops were intentionally excluded from our model as they are known in rhodopsin to be flexible with areas missing from the crystal structure. In addition, they share no homology with the loops of the hIP. Thus, our study focuses exclusively on the putative binding pocket within the transmembrane domain of the hIP.

RESULTS AND DISCUSSION

The prostacyclin receptor serves important roles in vascular smooth muscle relaxation, platelet aggregation, and inflammation (13–16). Prostacyclin agonists are now widely used for the treatment of pulmonary hypertension (17–20). More recently, it has also been suggested that prostacyclin may also be useful as a therapeutic agent in treating lung (21) and colon cancers (22, 23). Our current knowledge of the hIP receptor is very limited with only a handful of studies addressing the structure-function characteristics of this important receptor. Currently, there are no commercially available high affinity antagonists and only a few stable high affinity agonists. An understanding of the particular residues that constitute the ligand-binding pocket would be useful in determining any unusual features this receptor may contain and assist in the development of more functionally specific hIP ligands, particularly selective antagonists.

11 of 29 Candidate Residues When Mutated to Alanine Had a Significant Decrease in Binding Affinity for Iloprost—Candidate residues were first individually mutated to alanine (alanine scanning) to determine those that had an affect on binding affinity (Table I). These amino acids were selected based upon their potential to interact with prostacyclin side chains. To avoid preconceived bias with regard to the binding pocket position within the seven-transmembrane domains, all prospective binding pocket residues such as charged, polar, and large side-chain amino acids (e.g. phenylalanines and tyrosines) in the upper half of the TM domain were mutated. The wild-type prostacyclin receptor expressed well (1.8 pmol/mg membrane protein) with a binding affinity (Kd) for iloprost of 7.9 ± 1.7 nM (n = 9) (Table I). The competition binding best fitted to a
**Human Prostacyclin Receptor Agonist-binding Pocket**

**TABLE I**

*Competition binding and saturation binding experiments for the initial 29 transmembrane mutations to alanine*

Shown are the mean $K_i \pm S.E.$ from at least three separate experiments (number of repetition indicated by $n$) performed in duplicate. Saturation binding results are the mean $\pm S.D.$ of at least two experiments (in picomole/milligram of membrane protein).

| TM   | Construct | $K_i$, iloprost $nM$ (n) | $B_{max}$, pmol/mg | Agonist interactions |
|------|-----------|--------------------------|--------------------|---------------------|
| I    | hIP1D4    | 7.9 $\pm$ 1.7 (9)        | 1.8                |                     |
|      | S20A      | 8.7 $\pm$ 2.3 (3)        | 1.6                |                     |
|      | F24A      | 4.9 $\pm$ 1.3 (3)        | 1.2                |                     |
|      | D60A      | $>$500 (5)$^a$           | 0.1                |                     |
|      | S65A      | 5.1 $\pm$ 1.7 (3)        | 2.2                |                     |
|      | F68A      | 12.4 $\pm$ 3.4 (4)       | 1.0                |                     |
|      | S68A      | 62.3 $\pm$ 28.5 (4)$^a$  | 1.2                |                     |
|      | F72A      | 7.0 $\pm$ 2.7 (3)        | 0.4                |                     |
|      | Y75A      | $>$500 (6)$^a$           | 0.5                | C11-OH, cyclopentane ring |
|      | D93A      | 4.7 $\pm$ 2.0 (3)        | 1.8                | $\alpha$-chain       |
|      | F95A      | 143.6 $\pm$ 98 (6)$^a$   | 0.8                |                     |
|      | F97A      | 80.3 $\pm$ 13.9 (3)$^a$  | 0.6                |                     |
|      | T100A     | 4.0 $\pm$ 1.1 (3)        | 0.8                |                     |
|      | F101A     | 9.5 $\pm$ 1.9 (4)        | 0.4                |                     |
|      | F102A     | 4.2 $\pm$ 1.1 (3)        | 0.2                |                     |
|      | S185A     | 7.1 $\pm$ 2.3 (3)        | 1.3                |                     |
|      | IV        | F150A      | $>$500 (4)$^a$       | 0.1                |                     |
|      | V         | F184A      | 13.2 $\pm$ 5.6 (3)   | 0.2                |                     |
|      | S185A     | 85.8 $\pm$ 22.5 (5)$^a$  | 0.2                |                     |
|      | Y188A     | $>$500 (5)$^a$           | 0.2                |                     |
|      | S232A     | 15.0 $\pm$ 6.0 (3)       | 0.6                |                     |
|      | R258A     | 8.3 $\pm$ 3.0 (3)        | 0.4                |                     |
|      | F260A     | 11.4 $\pm$ 4.7 (4)       | 1.4                |                     |
|      | VII       | E271A      | 18.7 $\pm$ 10.0 (3)  | 0.8                |                     |
|      | D274A     | $>$500 (4)$^a$           | 0.3                | oxalane ring, $\alpha$-chain |
|      | F278A     | 351.3 $\pm$ 88.9 (5)$^a$ | 0.8                |                     |
|      | R279A     | $>$500 (5)$^a$           | 0.5                | C1-COOH              |
|      | F280A     | 10.8 $\pm$ 4.3 (5)       | 0.9                |                     |
|      | Y281A     | 15.7 $\pm$ 8.1 (3)       | 2.1                |                     |
|      | F283A     | 6.2 $\pm$ 1.8 (4)        | 0.7                |                     |

$a$ $p < 0.001$.

$b$ $p < 0.01$.

$c$ $p < 0.05$.

One-site competition binding curve. In the absence of a high affinity antagonist, the labeled agonist iloprost was used for competition binding. Further studies were performed on the wild-type receptor in the presence of Gpp(NH)p, a non-hydrolyzable form of GTP, to effectively uncouple G-protein from the receptor. The presence of 100 nM Gpp(NH)p had no significant effect on binding affinity for wild-type protein ($7.9 \pm 0.1$ nM ($n = 3$)), establishing that this affinity was not the result of G-protein coupling. Of the 29 residues mutated, 11 had a significant effect on binding (Table I) (Fig. 2, squares). These were located in TMII (D60A, S68A, and Y75A), TMIII (F95A and F97A), TMIV (F150A), TMV (S185A and Y188A), and TMVII (D274A, F278A, and R279A). In the absence of a high affinity antagonist for ligand binding, only those mutant receptors with significant differences of 0.5–1.7 log in binding affinity (S68A, F95A, F97A, S185A, and F278A) compared with wild-type receptor were able to yield a competition binding curve (Fig. 3). For those remaining mutants with extremely low binding affinity (>$>$500 nM) (D60A, Y75A, F150A, Y188A, D274A, and R279A), little iloprost binding was observed despite adequate amounts of protein being present. Both Western analysis (Fig. 4) and saturation binding (Table I) showed detectable yet significantly reduced amounts of protein expression for these severely affected mutant receptors. Moreover, Western analysis also revealed the complex-glycosylated states (multiple bands) that are typically observed with these receptors (8).

**Evidence from Sequence Alignments, Ligand Comparisons, and Modeling Supporting Four Residues as Having Direct Agonist Receptor Interactions**—Receptor configuration and TM helices were based upon sequence homology and alignment with the crystal structure of the rhodopsin receptor. Highly conserved ligand substituents on prostaglandins are likely to...
expression was decreased greater than 3-fold in comparison with the wild-type construct (R279A $K_{i} = 0.5 \text{ pmol/mg membrane protein}$ versus hIP1D4 $K_{i} = 1.5 \text{ pmol/mg membrane protein}$). Previous mutagenesis studies performed on the EP3 (25, 26) and EP2 (27) receptors have highlighted the impact of this residue in both ligand binding as well as receptor activation. Moreover, it has been shown that this residue has the capacity to not only form an ionic bond with the C1-carboxylate group of various ligands but serves as a hydrogen donor for carbonyl groups as well. EP1 receptor studies have confirmed that the primary interaction between this residue and ligand constituents is ionic (electrostatic) rather than hydrogen bonding since modification to various esters resulted in a greatly reduced affinity and potency (28). Thus, the complete level of conservation of Arg-279 across all of the prostaglandin receptors, marked effect on ligand binding (when mutated to alanine), and complete conservation of the C1-carboxylate among all of the native prostaglandin agonists strongly supports a direct ionic interaction between Arg-279 and the C1-carboxylate of prostacyclin (Fig. 5).

**Phe-278 (TMVII) Provides Supplemental Hydrophobic Interaction with Oxolane Ring and $\alpha$-Chain**—Directly adjacent to the crucial Arg-279 residue is Phe-278, an exclusive residue found only in the IP receptor at this position. The distinctive-ness of this residue seems to directly correlate with unique structural features found on IP receptor ligands, in particular the additional oxolane (cyclic ether) ring found on the native prostacyclin ligand. Some synthetic IP ligands such as iloprost and carbacyclin contain secondary cyclopentane rings at this position, whereas other naturally occurring prostaglandins (e.g. PGE$_1$) do not (Fig. 1). A drastic reduction in binding affinity was observed in the F278A mutation with $K_{i} = 351.3 \pm 88.9 \text{ nM (n = 5)}$ iloprost ($p < 0.001$) (Table I). Expression was also diminished as indicated by the lowered $B_{\text{max}}$ value of 0.8 pmol/mg membrane protein. Unlike the positively charged Arg-279, the Phe-278 residue contains a completely non-polar side chain. Thus, the most likely association between this residue and prostacyclin involves a hydrophobic ring-ring interaction between the oxolane ring of prostacyclin (cyclopentane ring of iloprost) and the Phe-278 phenyl constituent. As observed with our model (Fig. 5), there may be an additional interaction with the $\alpha$-chain. It is our belief that both of these ring structures serve as unique yet corresponding elements that are crucial for proper ligand binding and help to supplement the main electrostatic bond at Arg-279. The combination of Phe-278 and Arg-279 facilitate key anchoring points at the C1-carboxylate chain and secondary oxolane ring of prostacyclin, accommodating the $\alpha$-chain such that it remains in a “bent” state (Fig. 5) as observed with the energy-minimized configurations of prostacyclin.

**Tyr-75 (TMIII) Exhibits Functional Duality with C11-Hydroxyl and Cyclopentane Ring**—The Tyr-75 residue on TMIII is conserved throughout the majority of the prostaglandin receptors with the exception of the thromboxane receptors, which contain a histidine residue. Interestingly, a C11-hydroxyl group is present on all native prostaglandins with the exception of thromboxane. In the prostaglandins, this hydroxyl is attached to C11, a member of a cyclopentane ring; however, in thromboxane, the equivalent oxygen is incorporated as part of a six-membered oxane ring (Fig. 1). Furthermore, from energy minimization, the C11-hydroxyl group on prostacyclin is in close proximity with the C1-carboxylate moiety. This is closely paralleled by the close proximity between Arg-279 and Tyr-75, adding further support for our model. Thus, our mutagenesis and modeling results predict a direct interaction between Tyr-75 and the C11-hydroxyl group as binding of agonist with the Y75A mu-

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**Experimental Procedures**

Western analysis was performed as described under “Experimental Procedures” using the high affinity 1D4 monoclonal antibody to the C-terminal epitope tag. Those mutations, D60A, Y75A, F95A, F278A, and F278A, and R279A, resulting in significant binding deficits were compared (all transfected in parallel using $20 \mu$g F150A, Y188A, D274A, F278A, and R279A) that had significantly reduced binding affinity as compared with wild-type hIP1D4. All of them show a significant and parallel shift to the right as compared with the wild-type curve.
tant revealed a greater than 50-fold decrease in affinity ($p < 0.001$) with little specific binding ($K_I > 500 \text{nM iloprost}$) (Table I) as compared with the hIP1D4 wild-type receptor ($K_I = 7.9 \pm 1.7 \text{nM iloprost}$). To determine the precise structural features involved in this residue-ligand interaction (i.e. the presence of a phenyl ring, a hydroxyl group, or both), further mutagenesis (Y75F and Y75S) was performed. Unexpectedly, no significant defects in binding were observed with either of the additional mutations with $K_I$ values comparable with wild type ($Y75F K_I = 10.5 \pm 4.1 \text{nM iloprost}$ ($n = 5$) and $Y75S K_I = 13.3 \pm 2.8 \text{nM iloprost}$ ($n = 4$)). Expression was decreased to roughly 30% for all three mutations with $B_{\text{max}}$ values of 0.5, 0.4, and 0.5 pmol/mg membrane protein for Y75A, Y75F, and Y75S, respectively. From our modeling, hydrogen bonding appears to be the major means for interaction (Fig. 5); however spatial flexibility (by angstroms) in either the ligand or the receptor may allow for the formation of hydrophobic ring-ring interactions between the central (non-oxolane) cyclopentane ring of prostacyclin and the Ty75-phenyl ring. Thus, either interaction alone (i.e. Y75F hydrophobic ring-ring association or Y75S hydrogen bond) is sufficient in maintaining efficient receptor-ligand affinity. Possessing a high level of conservation across all prostanoid receptors (apart from the thromboxane receptor), shared common structural C11-hydroxyl groups with ligands (apart from thromboxane), close proximity to the critical Arg-279 residue, and reduced affinity upon mutagenesis to alanine supports Ty75 as an anchoring point to the C11-hydroxyl of prostacyclin.

**Phe-95 (TMIII) Is a Key Element in Accommodating the ω-Chain**—The Phe-95 residue is part of a phenylalanine-rich area within TM domain III. According to our model and binding analyses, Phe-95 plays a crucial role in accommodating the highly hydrophobic ω-chain of prostacyclin. A cluster of hydrophobic residues is present in TMIII on all prostanoid receptors in the region of the putative binding pocket. The binding affinity for the F95A mutation differed significantly from the wild-type hIP1D4, revealing a considerable binding deficit ($F95A K_I = 143.6 \pm 98 \text{ nM iloprost}$ ($n = 6$); $p < 0.05$) (Table I). The $B_{\text{max}}$ value for F95A was 0.8 pmol/mg membrane protein. The Phe-95 side chain provides a planar hydrophobic “sidewall” that helps to secure the freely rotating ω-chain constituent (Fig. 5). Previous chimera studies have shown this entire TM region to be interchangeable between mouse prostaglandin I and mouse prostaglandin D receptors (6). Furthermore, TMIII is one of two critical regions required for GPCR activation (29–31). We have previously shown that this region is also likely to be similarly important in the hIP (8). Thus, with attachments for the ω-chain carboxylate group (Arg-279, TMVII) and both centralized rings (Phe-278 (TMVII) and Ty75 (TMIII)) accounted for, the large hydrophobic region of TMIII, which includes Phe-95, comprises the fourth fundamental point of attachment-interaction between receptor and ligand, accommodating the ω-chain.

**Residues That Affect Binding but Are Predicted to Have Indirect Ligand-Receptor Interactions**—Despite significant effects on binding affinity, amino acid residues Asp-60, Ser-68, Phe-97, Phe-150, Ser-185, Tyr-188, and Asp-274 are not predicted by our model to be directly involved in receptor-ligand binding (Table I). Asp-60 (TMIII), although highly conserved (100%) across all prostanoloid receptors, lies too far (12.3 Å) from our proposed ligand-binding pocket. Similarly, Ser-68 in TMII, although moderately conserved (~40%), is not predicted to be directly involved in binding. Phe-97 contributes to the largely hydrophobic region found in TMIII. Although it is very close to its Phe-95 counterpart, Phe-97 is oriented away from the receptor-bound prostacyclin molecule and thus has no direct impact on ligand binding. When mutated to alanine (F97A), a marked decrease in affinity suggests a potential role as a TMIII position stabilizer through interhelical interactions with the adjacent TMIV. Phe-150 is also an important binding-related residue as indicated by the marked reduction in binding affinity upon mutation to alanine (F97A) (Table I). Furthermore, contributions to ligand binding are indirect due to the increased distance (~8.5 Å) from the bound ligand as predicted by our model. Ser-185 in TMV is found in only 29% of all prostanoloid receptors and has been shown to moderately affect ligand-binding affinity upon mutation to alanine. According to our model, Ser-185 is in close proximity to both Phe-150 (TMIV) and Tyr-188 (TMV). The Tyr-188 position is conserved only in mass (across the prostanoloid receptors) with the majority of amino acids at this position being phenylalanine. Unlike Ty75, the Tyr-188 residue is not predicted to be directly involved in receptor-ligand interactions but rather may serve as a structural contributor, participating in potential hydrophobic (ring-ring) interactions with F146 (TMIV). Asp-274 in TMVII is conserved in ~40% of all prostanoloid receptors. Within our proposed model, no direct residue-ligand interaction was evident; however, the proximity to the ligand com-
with the wild-type protein with Phe-95 and Val-71 residues. Phe-95 interacts shows proximity of both Phe-95 (TMIII) and Val-71 (TMII) to the affinity. between Phe-95 and the hydrophobic interaction with the /H9275/H9253 combined with a /H9275 carbon carboxylate group (negative charge) sug-

With the receptor-bound prostacyclin, &emdash;chain area of prostacyclin. An additional residue, Val-71, was targeted as a possible compensatory mutation can-

in the presence of F95A, a co-mutation of V71F restores binding affinity by substituting for the lost hydrophobic interaction between Phe-95 and the &omega;-chain.

combined with an &epsilon;-chain of prostacyclin. The other points of ligand receptor interactions (i.e. Arg-279, Phe-278, and Tyr-75) have been removed for clarity. A, the wild-type protein with Phe-95 and Val-71 residues. Phe-95 interacts with the &epsilon;-chain of prostacyclin. B, mutating Phe-95 to alanine removes hydrophobic interaction with the &epsilon;-chain, leading to a drop in binding affinity. C, in the presence of F95A, a co-mutation of V71F restores binding affinity by substituting for the lost hydrophobic interaction between Phe-95 and the &omega;-chain.

bined with a &gamma;-carbon carboxylate group (negative charge) suggests that Asp-274 is an essential structural contributor near the binding domain, possibly through the formation of a salt bridge. Therefore, these indirect binding-related residues are important in supporting the fundamental binding pocket and anchoring points established for the receptor-bound prostacyclin model (i.e. Arg-279, Phe-278, Tyr-75, and Phe-95). Further model refinements were pursued.

**Model Based Identification of Additional Interactions and Compensatory Mutations**—With the receptor-bound prostacyclin molecule in place and the structure refined, the ability to probe for other receptor-ligand interactions was now possible using the Swiss PDB Viewer as an exploratory device. Two additional residues, namely Leu-67 (TMII) and Met-99 (TMIII), were initially identified as being potential binding pocket contrib-

**L67A and L67W (TMII)**—A probable binding pocket contrib-
arator, Leu-67, is found in only a small number of prostanoid receptors including the hIP. Normally, such a small and comparatively unreactive (non-polar) molecule would not be sought out as a direct contributor to ligand binding as was corrobo-
rated by an L67A mutation, which exhibited wild-type-like affinity ($K_i = 4.8 \pm 1.1 \text{ nM iloprost (n = 3)}$) (Fig. 5). However, when converted to a much larger amino acid (i.e. tryptophan, L67W), a significant decrease in binding affinity was observed ($K_i > 500 \text{ nM iloprost}; p < 0.001 (n = 3)$). A steric repulsion between the larger L67W side chain and the &omega;-chain of prostacyclin is predicted by our model (Fig. 5). Therefore, with accommodating position, size, and side-chain neutrality, Leu-67 seemingly complements other hydrophobic residues in containing the &omega;-chain of prostacyclin as it is bound to the hIP receptor.

**M99L (TMIII)**—Another prospective binding pocket residue with potential direct interaction with ligand constituents was Met-99 (TMIII), which is highly conserved and present in $\sim 88\%$ of all prostanoid receptors. In reviewing our model (Fig. 5), it was our belief that Met-99 may contribute to binding affinity through hydrogen bond formation with the C15-hydroxyl group of prostacyclin, which is a highly preserved feature in all prostaglandin ligands. However, a methionine-to-

troleucine change (M99L) at this position exhibited no significant change in binding affinity ($K_i = 2.7 \pm 0.7 \text{ nM iloprost (n = 3)}$) as compared with wild type. This is consistent with previous studies on the EP2, EP3, and EP4 receptor subtypes, which showed that the conserved C15-hydroxyl group may not play an important role in agonist affinity (28, 32).

**F95A (TMIII) in Combination with V71L or V71F (TMII)**—As both the above results added marginal support to our model, we directed our focus toward producing compensa-
tory mutations that might help counteract one of the more destructive binding pocket changes examined earlier, namely the important Phe-95 residue (TMIII) that (upon mutation to alanine) disrupted the hydrophobic interaction with the &omega;-chain. Upon examination of our model, Val-71 in TMII appeared to be a good candidate for compensation of the F95A-induced binding deficit when changed to phenylalanine (V71F)
Thus, we investigated both V71L and V71F mutations in conjunction with the original F95A mutation. The combined F95A/V71L mutation exhibited a very poor binding affinity ($K_i$ > 500 nM ($n=3$)) in comparison with F95A/V71F of 7.2 ± 3.0 nM ($n=3$). Despite this rescue in binding, no improvement in expression was observed (0.2 pmol/mg membrane protein). The validity of our model was supported by this compensatory mutation.

**Model-based Prediction of hIP Activation by Prostacyclin**—It should be noted here that our model is a static image of the initial binding of ligand to receptor. Such an interaction in reality is dynamic with significant changes in both receptor and ligand conformation. Being an agonist, prostacyclin and iloprost within the binding pocket would result in conformational changes initiating receptor activation. It has been observed for rhodopsin as well as other GPCRs including our hIP studies that poor receptor expression occurs upon mutation of residues critical for ligand binding (33, 34). This observation strongly suggests that, in addition to binding, these residues may also serve as important structural stabilizers in the empty state (no ligand). Moreover, these binding pocket residues may contribute to the constraining influence on receptors that when broken by ligand (e.g. salt bridges) leads to receptor activation (33). As a consequence of prostacyclin or iloprost binding, such stabilizing factors may be disrupted, leading to both ligand and receptor conformational changes. Given the position of prostacyclin in the binding pocket, we would predict that TMIII and TMVII would rotate and move apart upon agonist binding. There is precedence for such movements as biochemical and EPR assays on rhodopsin upon photosomerization of 11-cis-retinal to all-trans-retinal have shown movements in TMIII and TMVII (29, 35–38). These major changes are most probably induced by ligand chain movements (e.g. $\alpha$-chain moves TMVI and $\omega$-chain TMIII). Thus, the first piece of evidence substantiating this hypothesis has now been provided; however, further studies are required for definitive confirmation.

**The Unique IP Agonist-binding Pocket**—This study pinpoints specific residues that comprise the fundamental structure of the hIP-binding pocket, securing crucial receptor-ligand associations as well as those amino acids in close proximity to the general binding domain. Our findings support a structural model of receptor-bound prostacyclin in which four distinct anchoring sites (comprised by seven TM amino acids) link ligand to receptor. These observations were somewhat unexpected because they placed the prostacyclin-binding pocket at the same level but in an opposing direction to the ligands of rhodopsin and the biogenic amine receptors, both of which also have ligands consisting of a carbon ring with a hydrocarbon chain (catecholamine and 11-cis-retinal, respectively). For rhodopsin, the $\beta$-ionone ring of 11-cis-retinal (20 carbons in size) faces TMV and TMVI, and the carbon chain is covalently attached via a Schiff base to TMVII (10). With the biogenic amines (9 carbons), hydroxyl groups from the catechol rings interact with serines on TMV and the amine group with an acidic residue in TMIII (4, 39). Prostacyclin like 11-cis-retinal and the biogenic amines has similarly important interactions with TMVII and TMIII; however, the bicyclic rings face TMI and TMII rather than TMV and TMVI (Fig. 7). We hypothesize that this may be a unique feature of the prostacyclin receptor that has reduced the availability of high affinity selective ligands. This insight may assist in the development of unique and highly specialized agents including additional agonists and, more notably, selective antagonists for the treatment and study of prostanoid-related disorders.