The unique ligand-binding pocket for the human prostacyclin receptor: Site-directed mutagenesis and molecular modeling.

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SUMMARY

The human prostacyclin receptor (hIP) is a seven transmembrane α-helical G-protein coupled receptor (GPCR), 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 (PGI₂), as well as other derivatives of the 20-carbon eicosanoid, arachidonic acid, have yet to be determined. Through the use of prostanoid receptor sequence alignments, site-directed mutagenesis, and the 2.8Å 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 hIP 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, R279 (TMVII), F278 (TMVII), Y75 (TMII), and F95 (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.

Keywords: GPCR, G-protein coupled receptor, prostacyclin, PGI2, human prostacyclin receptor, ligand-binding pocket, iloprost
INTRODUCTION

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 rhodopsin-like GPCR subfamily, such as the adrenergic receptors or other members of the prostanoid family, there are currently no high-affinity selective antagonists for the prostacyclin receptor. This 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 (PGI₂) is a derivative of the C-20 unsaturated fatty acid arachidonic acid (5,8,11,14-eicosatetraenoic acid) (Figure 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
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classification (Figure 1). In particular, the prostacyclin molecule contains an additional oxolane (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), which substitutes a secondary cyclopentane ring (in place of the PGI2 oxolane ring), carries an additional C16-methyl group, and an ω-chain triple bond (Figure 1). Side-chains of certain amino acids have been shown in receptors to interact directly with substituents of ligands, conferring binding affinity (3,4) via 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 hydroxyl groups extending from the catechol ring of the biogenic amines (4).

Recent studies have begun to identify generalized regions within the IP and other prostanoid receptors that appear crucial for ligand-binding specificity and affinity. Studies using chimeric combinations of mouse prostaglandin D (mDP) and prostaglandin I (mIP) 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, while TMI, 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 N7 and N78 (7), and proline residues within the transmembrane domains (8) have also 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, prostanoid 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 include R279 (TMVII), F278 (TMVII), Y75 (TMII), and F95 (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 (Piscataway, NJ). Oligonucleotide primers were purchased from Sigma-Genosys (The Woodlands, TX), while 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. 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 between α-helix-maintaining amino acids. To further define the receptor amino acids that interacted with ligand, sequence alignments for all prostanoid receptor transmembrane domains were obtained (GPCR web database -GPCRDB Prostanoid --- www.gpcr.org). Forty-two prostanoid receptor sequences (including multiple PE21, PE22, PE23, PE24, PF2R, TA2R, PD2R and PI2R from different species) were internally compared to each other, as well as to rhodopsin. Similar principles were applied in comparing functional groups of prostacyclin (as well as its analogues) to other prostaglandin ligands. Conservation of amino acids at equivalent positions on corresponding receptors, along with maintenance of certain functional groups on other prostaglandins, suggested likely functional correlations and importance. Residues identified within the putative binding pocket, and exhibiting substantial influence on ligand binding affinity, were re-constructed on a computer-generated hIP model, based upon the crystal structure of rhodopsin. A structural-based prostacyclin molecule was then inserted (bound) onto the model receptor comparing multiple positions. Taking into account all 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 to 12 nucleotides 3' and 5' from the desired mutation site. The PCR reaction mixture contained 1x Pfu reaction buffer, 200ng of DNA construct, 150ng of each primer (sense and antisense), 10mM dNTPs, and 2.5 units of Pfu DNA polymerase (Stratagene --- Austin, TX), and was heated and cooled at 95 °C for 30 seconds, 55 °C for 1 minute and 68 °C for 10 minutes for 16 cycles. The products were then digested with DpnI restriction enzyme (Promega --- Madison, WI) for 3 hours to remove any
parental wild-type strands of DNA. Ten microliters of PCR product was used to transform competent DH5α E. coli cells (~2 x 10⁹ cells), followed by DNA extraction from selected clones. Large plasmid preparations were performed using Wizard® Plus Maxiprep kits (Promega --- Madison, WI), 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 Modification of Eagle’s Medium (DMEM; Mediatech, Inc. --- Herndon, VA) was followed by addition of mutant DNA (20 μg per plate) in Diethylaminoethyl-Dextran (DEAE-Dextran; Sigma --- St. Louis, MO) (0.2mg/ml DMEM). Cells were then incubated at 37 °C with 5% CO₂ for 6 hours, after which 0.1mM chloroquine solution was added. Cells were subsequently incubated for 1 hour and chloroquine was removed through washes with DMEM. Cells were harvested 72 hours post-transfection.

**Membrane preparations**

Preparations of COS-1 cell membranes were carried out as follows: cells were washed in phosphate buffered saline (PBS) and harvested using cell scrapers. Vortexing (providing shear forces) for 3 minutes in sucrose (0.25M) was followed by low-speed spin (~1260g) for 5 minutes and the supernatant collected. After a high-speed centrifugation (~30,000g for 15 minutes) the pellet was then washed twice in 1x HEM (20mM Hepes pH 7.4, 1.5mM EGTA, and 12.5mM MgCl₂), followed by re-suspension in 1x 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 $[^{3}\text{H}]$ iloprost. Analysis involved construction of reaction mixtures (in duplicate wells) containing 50µg of membrane, HEM buffer, and 15nM $[^{3}\text{H}]$ iloprost, along with one of 11 different concentrations of cold (non-radiolabeled) iloprost, ranging from 10µM to 0.1nM. After 1.5 hours of incubation at 4 °C, reactions were stopped by the addition of ice-cold 10mM Tris/HCl buffer (pH 7.4) and filtered onto Whatman® GF/C glass-fiber filters, using a Brandel® cell harvester. The filters were washed 5 times with ice-cold Tris/HCl buffer, and radioactivity measured in the presence of 5ml 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 $[^{3}\text{H}]$ iloprost was varied from 1 to 100nM for saturation binding studies. Data was analyzed using GraphPad Prism® software (GraphPad Software, Inc. --- San Diego, CA). IC$_{50}$ values were converted to $K_i$ using the Cheng-Prusoff equation, and $K_i$ values were expressed as a mean ± SE. Analysis of variance (ANOVA) and students $t$ tests were used to determine significant 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 30µg of membrane protein was subjected to 10% SDS-polyacrylamide gel electrophoresis. This was transferred to nitrocellulose membrane, immunoblotted using a 1D4 monoclonal antibody and horseradish peroxidase-conjugated anti-mouse secondary antibody, and detected with enhanced chemiluminescence reagents.
Due to the instability of the native ligand (prostacyclin), a synthetic analogue (iloprost) (Figure 1) was used in all experimental assays. However, seeing that both structures are virtually identical and share the same reactive side components that confer binding specificity with the hIP (e.g. C1-COOH, C11-OH, C15-OH, bicyclic 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 modeling system. Computer-assisted molecular modeling of the prostacyclin ligand (native agonist to the hIP receptor) was performed using Swiss PDB Viewer (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: \( sp^3 \) hybridized C-C single bond = 1.54Å and 109.5°, \( sp^2 \) hybridized C=C double bond = 1.33Å and 120.0°, \( sp^3 \) hybridized C-O single bond = 1.40Å and 108.0°, and \( sp^2 \) hybridized C=O double bond = 1.20Å and 120.0°. Due to substantial torsional strain, all interior angles for both five-membered rings were approximated at 108.0°, except for the C-O-C bond angle of the oxane ring, which was set closer to 112.0°. The \( \text{rmsd (root mean square deviation)} \) for all bonds and angles was calculated at 0.006Å and 0.4°, respectively, compared to 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 fifth member out of plane. 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 an bent conformation (back upon the two centralized rings), while 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 human prostacyclin (hIP) receptor was constructed using the internet-based protein-modeling server, SWISS-MODEL (GlaxoSmithKline --- Geneva, Switzerland)(11). Amino acid sequences from all prostanoid receptors were obtained from the GPCR database (42 sequences in total; GPCRDB Prostanoid --- www.gpcr.org) 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 were determined and a homology model was generated, using the 2.8Å-resolution X-ray crystallographic structure of the bovine rhodopsin receptor as the template (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 (GlaxoSmithKline). Additional amino acids were added to the extracellular transmembrane domain region and allowed to adopt an α-helical conformation (where appropriate) using the crystal structure of rhodopsin as a template. From rhodopsin's crystal structure some of the transmembrane α-helices extend beyond 26 amino acids (e.g. TMIII). Furthermore, 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
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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 & 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.

Eleven 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 1). These amino acids were selected based upon their potential to interact with prostacyclin side-chains. In order to avoid preconceived bias as 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, 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 ($K_i$) for iloprost of $7.9 \pm 1.7 \text{ nM (n = 9)}$ (Table 1). The competition binding best fit to a 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-hydrolysable form of GTP, to effectively uncouple G-protein from the receptor. The presence of 100µM Gpp(NH)p had no significant effect on binding affinity for wild-type protein (7.9 ± 0.1 nM, n = 3) establishing that this affinity was not due to G-protein coupling. Of the 29 residues mutated, 11 had a significant effect on binding (Table 1 and squares in Figure 2). These were located in TMII (D60A, S68A, Y75A), TMIII (F95A, F97A), TMIV (F150A), TMV (S185A, Y188A) and TMVII (D274A, F278A, R279A). In the absence of a high-affinity antagonist for ligand binding, only those mutant receptors with significant differences of 0.5 to 1.7 log in binding affinity (S68A, F95A, F97A, S185A, and F278A) compared to wild-type receptor were able to yield a competition binding curve (Figure 3). For those remaining mutants with extremely low binding affinity (>500nM) (D60A, Y75, F150A, Y188A, D274A, and R279A) little iloprost binding was observed despite adequate amounts of protein being present. Both Western analysis (Figure 4) and saturation binding (Table 1) 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 is typically observed with these receptors (8).

Evidence from sequence alignments, ligand comparisons and modeling, supporting 4 residues as having direct agonist receptor interactions.

Receptor configuration and transmembrane (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 interact with conserved amino acids on the prostaglandin receptor. This principle has been used and confirmed in the investigation of many GPCRs including the adrenergic receptors (4) and rhodopsin (24). Independently, the computer-generated homology model of the hIP transmembrane domain (minus ligand) was evaluated and visualized using the Swiss PDB Viewer program. Initial superimposition of the model with the crystal structure of rhodopsin yielded significant structural similarity. To further determine the validity of our hIP model, a Ramachandran plot analysis was employed. Of the 182 amino acids formulating the transmembrane helices, 180 (99%) were within
the allowable region for a right-handed $\alpha$-helix, while 171 (94%) were within the preferred region. This corresponded well with the expected seven transmembrane $\alpha$-helical secondary structure of the hIP receptor. It was also determined (via Swiss PDB) that none of the residues were involved in any intra- or intermolecular clashes with the protein backbone or other side-chain constituents, nor were there any signs of global inconsistencies. By inserting prostacyclin into the model of the hIP, identification of distinct receptor-ligand interactions was possible. The major determinant for the final receptor-bound position of prostacyclin was based upon the experimentally observed requirement for the C1-carboxylate to interact with R279.

**R279 (TMVII) forms ionic interaction with the C1-carboxylate group ---** The most significant binding-pocket amino acid is the highly conserved (100%) R279 found within the seventh transmembrane domain (TMVII) of the hIP receptor. Upon mutation to alanine (R279A) a significant decrease in agonist binding affinity was observed ($K_i > 500$ nM, iloprost, p<0.001) (Table 1), as compared to the hIP1D4 wild-type receptor ($K_i = 7.9 \pm 1.7$ nM, iloprost). In addition, R279A protein expression was decreased greater than 3-fold in comparison to the wild-type construct (R279A $B_{max} = 0.5$ pmol/mg membrane protein versus hIP1D4 $B_{max} = 1.8$ 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, as modification to various esters resulted in a greatly reduced affinity and potency (28). Thus, the complete level of conservation of R279 across all of the prostanoid receptors, marked effect on ligand binding (when mutated to alanine), and complete conservation of the C1-carboxylate amongst all native prostanoid agonists, strongly supports a direct ionic interaction between R279 and the C1-carboxylate of prostacyclin (Figure 5).
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F278 (TMVII) provides supplemental hydrophobic interaction with oxolane ring and α-chain --- Directly adjacent to the crucial R279 residue is F278, an exclusive residue found only in the IP receptor at this position. The distinctiveness 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., PGE₃) do not (Figure 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 1). Expression was also diminished, as indicated by the lowered \( B_{\text{max}} \) value of 0.8 pmol/mg membrane protein. Unlike the positively charged R279, the F278 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 F278 phenyl constituent. As observed with our model (Figure 5), there may be an additional interaction with the α-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 R279. The combination of F278 and R279 facilitate key anchoring points at the C1-carboxylate chain and secondary oxolane ring of prostacyclin, accommodating the α-chain such that it remains in a “bent” state (Figure 5) as observed with the energy minimized configurations of prostacyclin.

Y75 (TMII) exhibits functional duality with C11-hydroxyl and cyclopentane ring --- The Y75 residue on TMII is conserved throughout the majority of the prostanoid receptors, with the exception of the thromboxane (Tx) receptors, which contain a histidine residue. Interestingly, a C11-hydroxyl group is present on all native prostanoids, except 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 (Figure 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 R279 and Y75, adding further support for our model. Our mutagenesis and modeling results thus predicts a direct interaction between Y75 and the C11-hydroxyl group as binding of agonist with the Y75A mutant revealed a greater than 50-fold decrease in affinity (p<0.001), with little specific binding (K_i > 500 nM iloprost) (Table 1), as compared to the hIP1D4 wild-type receptor (K_i = 7.9 ± 1.7 nM iloprost). In order 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. Unexpectantly, no significant defects in binding were observed with either of the additional mutations, with K_i values comparable to wild-type (Y75F K_i = 10.5 ± 4.1 nM iloprost, n = 5, and Y75S K_i = 13.3 ± 2.8 nM iloprost, n = 4). Expression was decreased by roughly 30% for all three mutations, with B_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 (Figure 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 centralized (non-oxolane) cyclopentane ring of prostacyclin and the Y75-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 R279 residue, and reduced affinity upon mutagenesis to alanine, supports Y75 as an anchoring point to the C11-hydroxyl of prostacyclin.

F95 (TMIII) is a key element in accommodating the ω-chain. The F95 residue is part of a phenylalanine-rich area within TM domain III. According to our model and binding analyses, F95 plays a crucial role in accommodating the mainly hydrophobic ω-chain of prostacyclin. A cluster of hydrophobic residues are
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 $K_i = 143.6 \pm 98$ nM iloprost, $n = 6$; $p<0.05$ (Table 1). The $B_{\text{max}}$ value for F95A was 0.8 pmol/mg membrane protein. The F95 side-chain provides a planar, hydrophobic “sidewall” that helps to secure the freely rotating $\omega$-chain constituent (Figure 5). Previous chimera studies have shown this entire TM region to be interchangeable between mIP and mDP 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 secure binding-pocket attachments for the $\alpha$-chain carboxylate group (R279, TMVII) and both centralized rings (F278, TMVII, and Y75, TMII) accounted for, the large hydrophobic region of TMIII, which includes F95, comprises the fourth fundamental point of attachment-interaction between receptor and ligand, accommodating the $\omega$-chain.

*Residues that affect binding but are predicted to have indirect ligand-receptor interactions*

Although significantly affecting binding, amino acid residues D60, S68, F97, F150, S185, Y188 and D274, are not predicted by our model to be directly involved in receptor-ligand binding (Table 1). D60 (TMII) although highly conserved (100%) across all prostanoid receptors, lies too far (12.3Å) from our proposed ligand binding pocket. Similarly, S68 in TMII although moderately conserved (approximately 40%), is not predicted to be directly involved in binding. F97 contributes to the largely hydrophobic region found in TMIII. Although very close to its F95 counterpart, F97 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. F150 is also an important binding-related residue, as indicated by the marked reduction in binding affinity upon mutation to alanine ($K_i > 500$ nM iloprost, $n = 4$; $p<0.001$) (Table 1). However, contributions to ligand binding are indirect due to the increased distance (~ 8.5Å) from the bound ligand, as predicted by our model. S185 in TMV is found in
only 29% of all prostanoid receptors, and has been shown to moderately affect ligand-binding affinity upon mutation to alanine. According to our model, S185 is in close proximity to both F150 (TMIV) and Y188 (TMV). The Y188 position, is conserved only in mass (across the prostanoid receptors), with the majority of amino acids at this position being phenylalanine. Unlike Y75, the Y188 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). D274 in TMVII is conserved in approximately 40% of all prostanoid receptors. Within our proposed model, no direct residue-ligand interaction was evident, however, proximity to the ligand combined with a γ-carbon carboxylate group (negative charge), suggest that D274 is an essential structural contributor near the binding domain possibly through formation of a salt bridge. These indirect binding-related residues are therefore important in supporting the fundamental binding pocket and anchoring points established for the receptor-bound prostacyclin model (i.e., R279, F278, Y75, and F95). 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 L67 (TMII) and M99 (TMIII), were initially identified as being potential binding-pocket contributors, affording probable receptor-ligand associations within the ω-chain area of prostacyclin. A further residue, V71, was targeted as a possible compensatory mutation candidate for the binding deficit created by F95A. The prognostic capability and succeeding results achieved with our working model lend to both its internal validity and accuracy.

**L67A & L67W (TMII) ---** A probable binding-pocket contributor, L67, is found in only a small number of prostanoid receptors, including the hIP. Normally, such a small and comparatively un-reactive (non-polar)
molecule would not be sought out as a direct contributor to ligand binding, as was corroborated by an L67A mutation, which exhibited wild-type-like affinity ($K_i = 4.8 \pm 1.1$ nM iloprost, $n = 3$) (Figure 5). However, when converted to a much larger amino acid (i.e., tryptophan --- L67W), a significant decrease in binding affinity was observed ($K_i > 500$ 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 (Figure 5). Therefore, with accommodating position, size, and side-chain neutrality, L67 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 M99 (TMIII), which is highly conserved and present in approximately 88% of all prostanoid receptors. In reviewing our model (Figure 5), it was our belief that M99 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-leucine change (M99L) at this position exhibited no significant change in binding affinity ($K_i = 2.7 \pm 0.7$ nM iloprost, $n = 3$) as compared to 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 towards producing compensatory mutations that might help counteract one of the more destructive binding pocket changes examined earlier, namely the important F95 residue (TMIII) that (upon mutation to alanine) disrupted the hydrophobic interaction with the $\omega$-chain. Upon examination of our model, V71 in TMII appeared to be a good candidate for compensation of the F-95A-induced binding deficit, when changed to phenylalanine (V71F) (Figure 6). 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 (Ki > 500nM, n = 3) in comparison to 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 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 photoisomerization of 11-cis-retinal to all-trans-retinal, have shown movements in TMIII, and TMVII (29,35-38). These major changes are most likely induced by ligand chain movements (e.g. α-chain moves TMVII and ω-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, where four distinct anchoring sites (comprised by seven TM amino acids) link ligand to receptor. These observations were somewhat unexpected as it 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 β-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 (Figure 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.

ACKNOWLEDGEMENTS

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REFERENCES

Human prostacyclin receptor agonist binding pocket


32. Abramovitz, M., Adam, M., Boie, Y., Carriere, M., Denis, D., Godbout, C., Lamontagne, S., Rochette, C., Sawyer, N., Tremblay, N. M., Belley, M., Gallant, M., Dufresne, C., Gareau, Y.,
Human prostacyclin receptor agonist binding pocket


FIGURES

Figure 1

Structures of prostanoid precursor and selected prostanoid ligands.

An illustration comparing the structural similarities and differences of some representative prostanoid ligands. 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 C16-methyl substitution, and there is a C18-triple bond.

Figure 2

Mutation sites on secondary structure of the hIP receptor.

Diagram highlighting the positions for all 31 (29 original plus 2 latter mutations) putative binding-pocket residues within the upper half of the seven-transmembrane helical domains (shaded boxes). Shown in bold squares are the residues that showed a significant difference in iloprost binding when mutated to alanine, as compared to the wild-type hIP1D4 protein. The circles are the residues that are unaffected by such an alanine mutation. Extracellular, transmembrane and cytoplasmic regions are designated based on the structure of rhodopsin, with approximate membrane boundaries numbered. The conserved disulfide bond, found in the extracellular domain, is indicated with a dashed line, as are the two palmitoylation sites, located along the cytoplasmic domain. The two glycosylation sites are indicated by the small circles. The C-terminus has been tagged with a 1D4 epitope (bold). A shaded box highlights the region for the putative ligand-binding pocket.
Figure 3

Competition binding for hIP1D4, and mutations that exhibited a significant decrease in binding affinity.

Competition binding curves (15nM [3H] iloprost versus 12 different concentrations of non-radiolabeled iloprost 10µM to 0.1nM). Shown are the mutant constructs (S68A, F95A, F97A, S185A, F278A) that had significantly reduced binding affinity as compared to wild-type hIP1D4. All show a significant and parallel shift to the right as compared to the wild-type curve.

Figure 4

Western analysis for hIP1D4 and mutants with very low binding affinity.

Western analysis was performed as described in Experimental Procedures, using the high-affinity 1D4 monoclonal antibody to the C-terminal epitope tag. Those mutations, D60A, Y75A, F150A, Y188A, D274A, F278A, and R279A, resulting in significant binding deficits were compared (all transfected in parallel using 20µg of DNA per 15cm plate). Thirty micrograms of membrane-prepared protein was loaded per well, with the exception of WT (hIP1D4), which only contained 15µg membrane protein.

Figure 5

Molecular model of receptor-bound prostacyclin and important binding-pocket residues.

Homology-based model of hIP receptor transmembrane domains with bound prostacyclin ligand. An extracellular view of the critical contacts made between receptor residues within transmembrane domains II, III, and VII, and prostacyclin is shown. Green-dashed lines represent interactions, either hydrogen bonding or electrostatic attractions. The major sites of interactions are; electrostatic interaction (green-dashed lines) between R279 and the negatively charged C1-carboxylate group of prostacyclin; hydrophobic ring-ring interaction between F278 and the oxolane ring of prostacyclin; hydrogen bond
Human prostacyclin receptor agonist binding pocket

(green-dashed line) and hydrophobic interactions between Y75 and the C11-hydroxyl group of prostacyclin and hydrophobic stabilizations among F95, L67, and the ω-chain of prostacyclin. F97, although not directly interacting with the ligand forms an important interhelical interaction with TMIV.

**Figure 6**

**Compensatory mutation of F95A with V71F**

Model of hIP showing proximity of both F95 (TMIII) and V71 (TMII) to the ω-chain of prostacyclin. The other points of ligand receptor interactions (i.e. R279, F278 and Y75) have been removed for clarity. A) The wild-type protein with F95 and V71 residues. F95 interacts with the ω-chain of prostacyclin. B) Mutating F95 to alanine removes hydrophobic interaction with the ω-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 F95 and the ω-chain.

**Figure 7**

**Positions of three ligands; 11-cis-retinal, epinephrine and prostacyclin in the agonist binding pocket.**

Homology modeling was performed as described in Experimental Procedures, using the rhodopsin crystal structure as the template. Extracellular view of the homology-based prostacyclin receptor transmembrane α-helices with bound prostacyclin ligand (red). Also superimposed in the IP receptor is the configuration of 11-cis-retinal (green) in rhodopsin, and epinephrine (yellow) in the adrenergic receptors.
Table 1: Competition binding and saturation binding experiments for the initial 29 transmembrane mutations to alanine. Shown are the Mean $K_i \pm SE$ from at least three separate experiments (number of repetition indicated by "n") performed in duplicate. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Saturation binding results are the mean of at least two experiments (in pmol per mg of membrane protein).

<table>
<thead>
<tr>
<th>TM</th>
<th>Construct</th>
<th>$K_i$ (nM) iloprost (n)</th>
<th>Bmax (pmol/mg)</th>
<th>Agonist interactions</th>
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<tr>
<td>IiP1D4</td>
<td>7.9 ± 1.7 (9)</td>
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<tr>
<td>I</td>
<td>S20A</td>
<td>8.7 ± 2.3 (3)</td>
<td>1.6</td>
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<td>F24A</td>
<td>4.9 ± 1.3 (3)</td>
<td>1.2</td>
<td></td>
<td></td>
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<tr>
<td>II</td>
<td>D60A</td>
<td>&gt;500 *** (5)</td>
<td>0.1</td>
<td></td>
</tr>
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<td>S65A</td>
<td>5.1 ± 1.7 (3)</td>
<td>2.2</td>
<td></td>
<td></td>
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<tr>
<td>F66A</td>
<td>12.4 ± 3.4 (4)</td>
<td>1.0</td>
<td></td>
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</tr>
<tr>
<td>S68A</td>
<td>62.3 ± 28.5 ** (4)</td>
<td>1.2</td>
<td></td>
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<td>F72A</td>
<td>7.0 ± 2.7 (3)</td>
<td>0.4</td>
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<td>Y75A</td>
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<td>F95A</td>
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<td>F102A</td>
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<td>S106A</td>
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<tr>
<td>Y188A</td>
<td>&gt;500 *** (5)</td>
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<td>VI</td>
<td>S252A</td>
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<tr>
<td>R258A</td>
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<td>VII</td>
<td>E271A</td>
<td>18.7 ± 10.0 (3)</td>
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<tr>
<td>D274A</td>
<td>&gt;500 *** (4)</td>
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<tr>
<td>F278A</td>
<td>351.3 ± 88.9 *** (5)</td>
<td>0.8</td>
<td>oxalane ring, α-chain</td>
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<td>R279A</td>
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<tr>
<td>F280A</td>
<td>10.8 ± 4.3 (5)</td>
<td>0.9</td>
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<tr>
<td>Y281A</td>
<td>15.7 ± 8.1 (3)</td>
<td>2.1</td>
<td></td>
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<tr>
<td>F283A</td>
<td>6.2 ± 1.8 (4)</td>
<td>0.7</td>
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<td></td>
</tr>
</tbody>
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A) Prostanoid Precursor:

Arachidonic Acid
(5,8,11,14-eicosatetraenoic acid)

B) Some Native Prostanoids:

PGI₂

PGE₁

TXA₂

C) Synthetic PGI₂ Analogue:

Iloprost

Figure 1
Figure 2
Figure 3
WT * D60A  Y75A  F150A  Y188A  D274A  F278A  R279A

* half amount loaded

Figure 4
The unique ligand-binding pocket for the human prostacyclin receptor: Site-directed mutagenesis and molecular modeling
Jeremiah Stitham, Aleksandar Stojanovic, Bethany L. Merenick, Kimberley A. O'Hara and John Hwa

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