Versatility and Differential Roles of Cysteine Residues in Human Prostacyclin Receptor Structure and Function*

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Prostacyclin plays important roles in vascular homeostasis, promoting vasodilatation and inhibiting platelet thrombus formation. Previous studies have shown that three of six cytoplasmic cysteines, particularly those within the C-terminal tail, serve as important lipidation sites and are differentially conjugated to palmitoyl and isoprenyl groups (Miggin, S. M., Lawler, O. A., and Kinsella, B. T. (2003) J. Biol. Chem. 278, 6947–6958). Here we report distinctive roles for extracellular- and transmembrane-located cysteine residues in human prostacyclin receptor structure-function. Within the extracellular domain, all cysteines (4 of 4) appear to be involved in disulfide bonding interactions (i.e. a highly conserved Cys-92–Cys-170 bond and a putative non-conserved Cys-5–Cys-165 bond), and within the transmembrane (TM) region there are several cysteines (3 of 8) that maintain critical hydrogen bonding interactions (Cys-118 (TMIII), Cys-251 (TMI), and Cys-202 (TMV)). This study highlights the necessity of sulfhydryl (SH) groups in maintaining the structural integrity of the human prostacyclin receptor, as 7 of 12 extracellular and transmembrane cysteine residues studied were found to be differentially indispensable for receptor binding, activation, and/or trafficking. Moreover, these results also demonstrate the versatility and reactivity of these cysteine residues within different receptor environments, that is, extracellular (disulfide bonds), transmembrane (H-bonds), and cytoplasmic (lipid conjugation).

Prostacyclin and its receptor play key roles in vascular smooth muscle relaxation and inhibition of platelet aggregation. A host of studies, including IP² receptor knock-out (IP⁻⁻⁻⁻) mice, has implicated dysfunctional IP activity in numerous cardiovascular abnormalities, including thrombosis, myocardial infarction, stroke, hypertension, and atherosclerosis (2–5). Despite significant progress in our understanding of G-protein coupled receptors (GPCRs) in general, the details of human prostacyclin receptor structure-function remains largely unknown.

Cysteine chemistry is both fascinating and intriguing. The sulfhydryl or thiol (SH) reactive groups of this amino acid are very susceptible to oxidation and can readily form stable dimers (i.e. disulfide S-S bridges), which play important roles in the organization and maintenance of protein tertiary structure. Somewhat analogous to hydroxyl groups (OH) found on serines, sulfhydryl (SH) side chains are also polar and can participate in hydrogen bonding interactions and can additionally coordinate trace metals (e.g. zinc) (6). Sulfur atoms are also quite nucleophilic and react readily with electrophilic molecules to form a variety of thiol-linked derivatives (e.g. thioethers, thioesters, and thioacetals). Thus, cysteine side chains are common sites for various biological coupling and conjugation reactions, including palmitoylation, isoprenylation, disulfide cross-linking, and thiol-disulfide exchange (7–9). In this study our goal was to assess the structural and functional contributions of cysteines within the human prostacyclin receptor (hIP) receptor. Early mutagenesis studies in bovine rhodopsin (using Cys→Ser mutations) revealed the importance of amino acids Cys-110 and Cys-187, which are essential in the formation of normal functional rhodopsin protein (10). It was later shown that these two highly conserved cysteine residues form a disulfide bond in the extracellular domain (11). Further mutagenesis and mass spectrometry confirmed the presence of this disulfide bond and its importance in stabilizing rhodopsin (12, 13). Because of the initial discovery of this imperative disulfide bond within rhodopsin, many other investigations have gone on to demonstrate the presence and importance of similar S-S bonds in other GPCRs (14–18). Mutagenesis of the thromboxane receptor (TP) at the conserved extracellular S-S cysteine positions (Cys-105 and Cys-183) revealed decreased binding affinity and low amplitude calcium signaling (19, 20). Interestingly, in addition to this conserved disulfide bridge, further studies on the β2-adrenergic receptor as well as the gonadotropin-releasing hormone receptor have suggested the presence of a second, non-conserved disulfide bond (8, 21).

The C-terminal tail of the human prostacyclin receptor contains six resident cysteines, which have been extensively studied. Five of these cysteine residues are located within CAAAX consensus motifs thought to comprise sites for lipid anchoring (e.g. palmitoylation or isoprenylation). Given that a good deal of evidence has already been presented for both palmitoylation as well as isoprenylation at these C-terminal cysteine sites on the human prostacyclin receptor (1, 7), we have opted to exclude...
this region from our current investigation. The remaining 12 cysteine residues within the extracellular and transmembrane domains of the hIP receptor were individually converted to alanine using site-directed PCR mutagenesis. We report that in addition to the highly conserved disulfide interaction between Cys-92 (top of transmembrane (TM) III) and Cys-170 (exoloop 2), an additional, putative non-conserved disulfide bridge may exist between Cys-5 (N terminus) and Cys-165 (exoloop 2), within the extracellular domain of the hIP, as well as other prostanoid receptors (hDP and hEP2). Moreover, this interaction seems to serve a critical, yet distinct purpose compared with its conserved counterpart. Furthermore, in the transmembrane domain Cys-118 (TMIII), Cys-202 (TMV), and Cys-251 (TMVI) were found to be necessary for preserving normal receptor binding affinity, activation capacity, and/or cell-surface expression through probable hydrogen bonding networks. Such observations provide further insights into the molecular functioning of the hIP receptor and contribute to the mechanistic understanding of how the hIP protein is stabilized during the continuum of conformational changes that occur upon agonist-induced activation.

EXPERIMENTAL PROCEDURES

Materials—Radiolabeled \(^{3}H\)iloprost (17.0 Ci/mmol) and nonradiolabeled iloprost were purchased from Amersham Biosciences. Oligonucleotide primers were purchased from Sigma-Genosys (The Woodlands, TX), whereas hIP cDNA was a generous gift from Dr. Mark Abramovitz (Merck Frosst, Quebec, Canada).

Cysteine-to-Alanine Mutations—Twelve (within the extracellular and transmembrane domains) of a total of 18 cysteine residues were individually mutated to alanine (Fig. 1A). The choice of amino acid substitution was critical as cysteine-to-serine (Cys→Ser) mutations in rhodopsin have been shown to have greater adverse effects on receptor conformation than cysteine-to-alanine (Cys→Ala) substitutions (10, 12). Additionally, the C-terminal residues were not included in this study because these have been extensively characterized (1). Human IP cDNA tagged at the C terminus with the 1D4 epitope tag (last 14 amino acids of rhodopsin) was cloned into the plasmid vector pMT4, and point mutations were generated using conventional methods of PCR mutagenesis as previously described (23). Ten microliters of the PCR product was used to transform competent DH5α Escherichia coli cells (−2×10⁹ 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 and Membrane Preparations—Transient transfections were performed on COS-1 cells as previously described (24). In brief, cells were incubated in DNA (10 or 20 µg/plate) in diethylaminoethyl dextran (DEAE-dextran; Sigma) (0.2 mg/ml Dulbecco’s modified Eagle’s medium) and harvested 72 h post-transfection. Transfected COS-1 cells were washed in phosphate-buffered saline and harvested. 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 was 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 EGTA, and 12.5 mM MgCl₂) followed by re-suspension in 1× HEM containing 10% glycerol and stored at −70°C (25). Either Bradford or BCA protein assays were performed to quantitate membrane proteins.

Ligand Binding Affinity—Ligand binding characteristics for the expressed receptors were initially determined through a series of competition binding assays using the radiolabeled ligand \(^{3}H\)iloprost. Analysis involved construction of reaction mixtures (in duplicate wells) containing 50 µg of membrane, HEM buffer, and 15 nM \(^{3}H\)iloprost along with 1 of 11 different concentrations of cold (nonradiolabeled) iloprost ranging from 10 µM to 0.1 nM. After 1.5 h of incubation at 4°C, reactions were stopped by the addition of ice-cold 10 mM Tris/HC1 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/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 nonradiolabeled iloprost. Data were analyzed using GraphPad Prism® software (GraphPad software, Inc., San Diego, CA). IC₅₀ values were converted to Kᵢ by the Cheng-Prusoff equation, and Kᵢ values were expressed as a mean ± S.E. For saturation binding experiments to determine Bₘₐₓ and Kᵢ, the concentration of \(^{3}H\)iloprost was varied from 1 to 100 nM. Nonspecific binding was determined by the addition of a 500-fold excess of nonradiolabeled iloprost. Data were analyzed using GraphPad Prism® software (GraphPad Software). Analysis of variance and Student’s t tests were used to determine significant differences (p < 0.05).

Receptor Activation cAMP Determination—The wild-type and cysteine mutant constructs were analyzed for signal transduction capabilities. COS-1 cells were transiently transfected with 2 µg of receptor DNA in 25-mm plates as described above. \(^{3}H\)cAMP was used in competition for a cAMP-binding protein against known concentrations of nonradiolabeled cAMP followed by determination of the unknowns. The reaction was allowed to proceed for 2 h at 4°C. Charcoal was used to remove excess unbound cAMP. Samples were counted in 5 ml of Ecoscint™ H (National Diagnostics). Results were analyzed with GraphPad Prism® software. For the dose response, a non-linear, curve-fitting program (GraphPad Prism®) was used, and the EC₅₀ (mean ± S.E.) was determined for wild-type hIP1D4 and mutant constructs. Analysis of variance and Student’s t tests were used to determine statistically significant differences (p < 0.05).

Confocal Immunofluorescence Microscopy—COS-1 cells were seeded into six-well tissue culture plates containing sterilized poly-L-lysine (Sigma)-treated glass coverslips and transiently transfected with 1.0 µg/ml wild-type or mutant (C5A, C92A, C165A, and C170A) hIP DNA according to the aforementioned transfection protocol. Cells were fixed and permeabilized 48 h post-transfection in ice-cold methanol. Prostacyclin receptors were labeled using anti-1D4 monoclonal antibody (C-terminal-
tagged hIP), 1:1000 dilution, for 60–90 min followed by goat anti-mouse IgG F(ab')2 AlexaFluor®568 fluorescent antibody (Molecular Probes, Inc.) 1:200 dilution, for 60–90 min. The endoplasmic reticulum was labeled using anti-calnexin polyclonal antibody (StressGen Biotechnologies), 1:400 dilution, for 60–90 min followed by goat anti-rabbit IgG AlexaFluor®488 fluorescent antibody (Molecular Probes), 1:200 dilution, for 60–90 min. Cells were post-fixed with 4% paraformaldehyde (Fluka) for 5 min, mounted with ProLong Gold with 4',6-diamidino-2-phenylindole, and examined via confocal microscopy using a Zeiss LSM 510 Meta Laser Scanning Confocal Microscope System (i.e. inverted Zeiss Axiovert 200 microscope with two conventional photomultipliers and one meta polychromatic multi-channel detector) (Carl Zeiss Microimaging, Inc., Thornwood, NY). Representative cells were selected, and images were captured at high resolution (63×) for cytoplasmic or plasma membrane localization.

Molecular Modeling of hIP Receptor—A theoretical, three-dimensional homology model of the seven transmembrane α-helices of the hIP was constructed using the internet-based protein-modeling server, SWISS-MODEL (GlaxoSmithKline) (26). The homology model was generated using the 2.8-Å-resolution x-ray crystallographic structure of bovine rhodopsin as the template (PDB code 1HZX). The transmembrane domains were energy-minimized, utilizing the Gromos96 force field to improve the stereochemistry of the model and remove unfavorable clashes (SWISS-MODEL). Polypeptide chains corresponding to the extracellular and cytoplasmic loop regions were manually constructed. The large C-terminal tail region of the hIP was excluded from this particular model. Initial torsion angles were derived from full polypeptide secondary structure prediction using the PRed multiple sequence alignment consensus server (27). The subsequent loop peptides were energy-minimized using the NAMD molecular dynamics simulator (28). Resulting structures were sequentially attached to the transmembrane homology model. Compiled structures were energy-minimized using NAMD, including constraints for either 1) no extracellular disulfide bonds, 2) a single, conserved extracellular disulfide bond (Cys-92–Cys-170), or 3) dual extracellular disulfide bonds (Cys-92–Cys-170 and Cys-5–Cys-165). This latter structure, with dual extracellular (disulfide) constraints, yielded the lowest energy conformation and was utilized as the preferred hIP receptor model for subsequent comparisons. Although our model was a useful first pass tool in providing preliminary insights and predictions of sulfhydryl hIP structure function, biochemical and molecular pharmacological techniques were necessary to confirm our hypothesis.

RESULTS

Our current knowledge of the hIP receptor is limited. Of particular interest has been the role of cysteine residues within the extracellular and TM domains of the protein. Cysteines contain a highly nucleophilic sulfhydryl or thiol (SH) side chain that is capable of acting as a nucleophilic catalyst. Moreover, with a pK_{a} of ~8, the chemical reactivity of cysteine sulfhydryls is easily modified by environmental conditions. Within a reducing environment, cysteine residues may be involved in bioconjugation reactions, whereas conversely, within an oxidizing environment, side chains may dimerize to form disulfide (S-S) linkages. These latter interactions play an invaluable role in intramolecular cross-linking of proteins that can increase molecular stability within harsh environments as well as confer resistance to proteolytic cleavage. We wished to elucidate the differential contributions of conserved and non-conserved cysteines within the transmembrane region of the hIP receptor as well as explore the potential existence of a second, putative non-conserved disulfide bond within the extracellular domain.

Extracellular Cysteines Are Required for Receptor Trafficking, Binding, and Activation—Site-directed mutagenesis was performed targeting all cysteine residues present within the hIP receptor, excluding those located in the C-terminal tail. Thus, 12 of the total 18 cysteine residues (Table 1, Fig. 1A) were individually mutated to alanine (Cys→Ala) and assessed for functional effects on agonist binding (affinity) and receptor activation (potency), as measured via cAMP production. Binding affinity (K_{i}) could not be detected using competition binding (15 nM [3H]iloprost and 1 μg of DNA/ml transfection solution) and dose-response (EC_{50}) characteristics for wild-type hIP and cysteine-to-alanine mutations

| Mutations      | K_{i} ± S.E. (nM) | EC_{50} ± S.E. (nM) | Expression pmol/mg of protein |
|---------------|------------------|---------------------|------------------------------|
| Wild type     | 7.9 ± 1.7 (9)    | 12 ± 0.1 (10)      | 2.1 ± 0.5                    |
| C5A           | ND (3)*          | ND (3)*             | 0.2 ± 0.1*                   |
| C92A          | ND (4)*          | ND (3)*             | 0.6 ± 0.2*                   |
| C118A         | ND (4)*          | ND (3)*             | 0.5 ± 0.2*                   |
| C135A         | 6.4 ± 1.1 (3)    | 0.9 ± 0.3 (3)      | 0.5 ± 0.5                    |
| C170A         | 5.3 ± 2.1 (3)    | 1.2 ± 0.3 (4)      | 2.1 ± 0.9                    |
| C151A         | 8.9 ± 2.3 (3)    | 1.9 ± 0.6 (4)      | 1.4 ± 0.6                    |
| C165A         | ND (4)*          | ND (3)*             | 0.5 ± 0.2*                   |
| C170A         | ND (4)*          | ND (3)*             | 0.8 ± 0.3*                   |
| C202A         | 4.4 ± 1.7 (3)    | 1.2 ± 0.4 (3)      | 0.4 ± 0.1*                   |
| C211A         | 9.4 ± 2.3 (3)    | 0.9 ± 0.1 (3)      | 1.4 ± 0.4                    |
| C251A         | ND (4)*          | 3.1 ± 0.2 (4)*     | 0.4 ± 0.2*                   |
| C259A         | 11.3 ± 2.4 (3)   | 0.8 ± 0.2 (3)      | 3.2 ± 0.3                    |

*p < 0.05.

TABLE 1

Functional comparison of binding affinity (K_{i} from competition binding using 15 nm [3H]iloprost and 1 μg of DNA/ml transfection solution) and dose-response (EC_{50}) characteristics for wild-type hIP and cysteine-to-alanine mutations

Bold indicates critical cysteine residues for hIP structure function. ND, not detectable.
Cys→Ala substitutions compared with wild-type hIP (Table 1). The additional use of confocal microscopy corroborated reduced cell surface expression and demonstrated defective trafficking with endoplasmic reticulum co-localization (calnexin) and retention (Fig. 2C). Wild-type hIP predominantly trafficked to the cell surface as observed with the red fluorescence surrounding the cell in the phase contrast micrograph overlay (Fig. 2C). Because of the nature of overexpression systems, some wild-type hIP was also detectable in the endoplasmic reticulum as observed with co-localization with the green fluorescence. Both C5A and C165A showed a marked reticular pattern co-localized predominantly to the endoplasmic reticulum, with reduced cell surface. C92A and C170A were also found predominantly in an endoplasmic reticulum location.
Extracellular and Transmembrane Cysteines in hIP

which shows that both residues are either exclusively present or exclusively absent within the various prostanoid receptors. One of the cysteines (Cys-5 or Cys-165) is always found in the presence of the other. Thus, at equivalent positions within the human IP, EP2, and DP receptors, both the Cys-5 and Cys-165 residues are found, whereas conversely, at equivalent positions within the human EP1, EP3, EP4, TP, and FP receptors, both cysteines are absent (Fig. 1B). In contrast, Cys-92 and Cys-170 are conserved at equivalent positions among all the prostanoid receptors, suggesting that Cys-92 interacts with Cys-170, and (when present) Cys-5 interacts with Cys-165.

If critical disulfide (S-S) bonds are present within the extracellular domain of the hIP receptor, then chemical reduction of the wild-type receptor should be associated with defects in structure (folding-conformation-stability) and function (binding and activation). Competition binding was performed in the presence of increasing concentrations of 1.4–140 mM β-mercaptoethanol (β-ME; Fig. 3A) and 1–100 mM 1,4-dithiothreitol (Fig. 3B), which break disulfide (S-S) bonds and maintain sulfhydryl (SH) groups in the reduced state. As can be viewed in Fig. 3, a sequential decrease in agonist binding was observed for wild-type hIP receptors treated with increasing concentrations of either 1,4-dithiothreitol or β-ME. The significant difference and decline in raw counts (specific binding) in response to increasingly higher concentrations of reducing (perinuclear), again with reduced cell surface expression. Thus, in conjunction with the findings from previous binding and activation studies, these results also show defects for the four extracellular cysteines, suggesting that there may be dual functional connections between the highly conserved Cys-92–Cys-170 and the less conserved Cys-5–Cys-165.

Sequence Alignments and Treatment with Reducing Agents Suggest Formation of Dual Extracellular Disulfide Bonds (Cys-5 to Cys-165 and Cys-92 to Cys-170)—In addition to our initial experimental findings, further supportive evidence that a second disulfide bond exists between the non-conserved extracellular cysteine residues Cys-5 and Cys-165 can be found in human prostanoid receptor sequence alignments (Fig. 1B), agent is indicative of an increasing pool of defective IP (i.e. reduced S-S bonds to SH). Clear sigmoidal competition binding curves suggest a population of receptors in which the disulfide bond remained intact. As shown, the IC_{50} values, measured in fold-log molar concentrations of iloprost agonist, log[iloprost](M), remained unchanged for wild-type hIP = −7.4 ± 0.2 log M (untreated), −7.4 ± 0.2 log M (1.4 mM β-ME), −7.6 ± 0.3 log M (14 mM β-ME), and −7.8 ± 0.4 log M (140 mM β-ME) (Fig. 3A). A corresponding decrease in cAMP signaling levels was also observed, demonstrating a parallel reduction in the levels of functioning receptor (Fig. 4B) with no significant change in EC_{50} = −9.3 ± 0.4 log M (wild-type hIP-untreated), −9.4 ± 0.8 log M (140 mM β-ME), and −9.5 ± 0.8 log M (14 mM β-ME).
Additional evidence, albeit indirect, for the presence of dual disulfide bonds can be gleaned from the observation that the low levels of specific binding for both the C92A and C170A mutations quickly dissipates upon the addition of \( \cdot \text{H}_{9252} \text{-mercapto-} \) ethanol (Fig. 5), implying further reduction of a secondary disulfide bridge. The culmination of these results in combination with saturation binding and confocal microscopy suggests that both putative disulfide bonds are important.

Rhodopsin-based Computer Modeling Supports the Formation of Dual Disulfide Bonds with Differential Function—In addition to experimental-functional analyses, computer-assisted modeling was used to assess whether such dual bonding was structurally feasible within an energy-minimized model of the hIP (see Fig. 7). Despite evident limitations in the manual construction and the addition of loop regions to our existing three-dimensional homology model, the composite, energy-minimized structure (i.e. TM domain plus N terminus, 3 exoloops, and 3 cytoloops) suggested that the presence of a secondary disulfide bond was structurally feasible. Energy minimization placed the freely rotating N terminus between exoloops 2 and 3, preserving the conserved disulfide bridge.

**FIGURE 2.** Effects of reducing agents on binding affinity for wild-type hIP. Panel A, \( \cdot \text{H}_{9252} \) dithiothreitol (DTT) treatment of membrane samples expressing wild-type protein. Shown are treatments with 1,4-dithiothreitol concentrations of 1.0 mM (\( \bigodot \)), 10 mM (\( \bigtriangledown \)), and 100 mM (\( \bigtriangleup \)). The gray bar highlights IC\(_{50}\) value for both curves. Shown are composite results from at least three separate experiments.

**TABLE 2**

| Receptor | \( K_d \) (nM) | S.E. (n) |
|----------|----------------|---------|
| Wild type| 13.4 ± 2.6 (10) |         |
| CS5A     | 121.5 ± 29.4 (4) |         |
| C92A     | 113.9 ± 31.4 (3) |         |
| C165A    | 142.4 ± 18.0 (6) |         |
| C170A    | 140.0 ± 14.2 (4) |         |

* \( p < 0.001. \)

**FIGURE 3.** Effects of reducing agents on binding affinity for wild-type hIP. Panel A, \( \cdot \text{H}_{9252} \) dithiothreitol (DTT) treatment of membrane samples expressing wild-type protein. Shown are treatments with 1,4-dithiothreitol concentrations of 1.0 mM (\( \bigodot \)), 10 mM (\( \bigtriangledown \)), and 100 mM (\( \bigtriangleup \)). The gray bar highlights IC\(_{50}\) value for both curves. Shown are composite results from at least three separate experiments.

**FIGURE 4.** Effects of reducing agent on agonist-induced cAMP generation for wild-type hIP. Dose-response curve showing cAMP production of wild-type hIP at zero (black square), 14 mM (filled circle), and 140 mM (gray square) \( \cdot \text{H}_{9252} \) concentrations. Shown are composite responses from at least three separate experiments.
between Cys-92 (TMIII) and Cys-170 (exoloop 2) and facilitating the formation of a second stable disulfide linkage between Cys-5 (N terminus) and Cys-165 (exoloop 2) (see Fig. 7A). Additional molecular dynamics and secondary structural analyses are required for further refinement of the composite structure. Nevertheless, this homology model provides a basis for additional structural insights and predictive capabilities. Validation of the composite model can be achieved through subsequent experimental testing (mutagenesis studies) of model-based predictions.

Compensatory Mutation Partially Restores Binding Affinity via Non-conserved Disulfide Bond Reformation—Based upon initial assessments of the putative Cys-5–Cys-165 disulfide bridge within our homology model, in the context of the C5A mutation, conversion of an adjacent residue to cysteine might also result in covalent formation of a disulfide bond with Cys-165 (Fig. 6A). Furthermore, restoration of receptor function would most likely only occur in the presence of a covalent S-S interaction, because hydrogen bonding (of SH) would be unlikely to support such drastic changes at both amino acid positions. Thus, the adjacent residue Arg-6 was chosen as the candidate amino acid to be converted to cysteine (C5A-R6C) in an attempt to reinstate the uncoupled S-S linkage resulting from the initial C5A mutation. Competition binding analysis revealed a return of binding affinity with the compensatory mutation (C5A-R6C, \( K_i = 8.2 \pm 4.5 \text{ nM}, n = 3 \); wild-type hIP \( K_i = 9.6 \pm 2.8 \text{ nM}, n = 4 \) in comparison to the undetectable \( K_i \) value from the original C5A mutant receptor (Fig. 6B). However, the maximal binding was reduced considerably, suggesting only a small population had regained a disulfide bond. Such results are suggestive of a stabilizing disulfide interaction between Cys-92 and Cys-165, which mimics (incompletely) the putative non-conserved Cys-5–Cys-165 disulfide bond found in the native hIP receptor. To further support the restoration of a disulfide bond, reduction of C5A-R6C with 140 mM \( \beta \)-ME was performed with resulting reduction and a shift to the right of the C5A-R6C graph. The highest concentration of cold ioprost addition was now at background vector control levels 31.9 \pm 2.2 raw counts (cpm) \( (n = 10) \).

**Cys-118 in the Highly Conserved ERX Motif Affects Binding and Activation**—In examining cysteines within the transmembrane domain of the hIP, Cys-118 is adjacent to the highly conserved endoplasmic reticulum motif located on the cytoplasmic side of TMIII. Sequence alignments for all 961 humanGPCRs identified in the GPCRDB (GPCR database) showed that Tyr (56%), Phe1%), and Cys (4%) were the most common amino acids at this position. The C118A mutant was severely defective with undetectable binding with competition binding and poorly detectable saturation binding in addition to undetectable activation values (Table 1). Receptor expression was also significantly diminished (estimated \( B_{\text{max}} \) C118A = 0.5 \pm 0.2 pmol/mg of membrane protein, \( p < 0.05 \)). Molecular modeling analysis indicated that Cys-118 is located at the beginning of exoloop 2 within a solvent-accessible cavity between TMIII and TMV. In certain conformations the sulfhydryl side chain may participate in a critical intra-\( \alpha \)-helical stabilizing H-bonding or thioester interaction with this residue (Fig. 7B).

**Cys-251 May Provide a Stabilizing Interaction with TMVII**—Cys-251 had adverse effects on hIP receptor binding and activation. It is completely conserved throughout all human prostanoid receptors (Fig. 1B). Although competition binding was unable to determine binding affinity (reduced affinity and expression), saturation binding detected a \( K_i \) of 44.5 \pm 6.5 nm (\( n = 3, p < 0.001 \)), consistent with the reduced potency (Table 1). Upon examination of the Cys-251 residue within our previously developed three-dimensional molecular homology model of the hIP binding domain (25), it appears that the Cys-251 residue may be tethered to key residues within the immediate ligand binding pocket of the hIP receptor. As shown in Fig. 7C, the reactive (SH) side chain of Cys-251 (TMVII) forms a hydrogen-bond network in close proximity to Phe-278 and the ligand binding pocket. In previous studies Phe-278 was shown to directly interact with receptor-bound ligand and inhibit ligand dissociation (29).

**C202A Exclusively Affects Expression**—Cys-202 is nearly conserved across all the human prostanoid receptors, except for the hTP (Fig. 1B). It is located at the bottom of TMV near the beginning of the third cytoplasmic loop (cytoloop 3) (Fig. 7D).
Although the C202A appeared to be wild-type-like in binding and activation, there was a significant decrease in plasma membrane receptor expression ($B_{\text{max}}$ Cys-$202 = 0.4 \pm 0.1, p < 0.05$) (Table 1). Within our homology model, the sulfhydryl (SH) group of Cys-$202$ appears to form a hydrogen-bonding network with Asn-$203$ and Ala-$198$ (Fig. 7D).

**DISCUSSION**

Prostacyclin (PGI$_2$) analogs are now widely used for the treatment of pulmonary hypertension (30–33). More recently it has also been suggested that prostacyclin may also be useful as a therapeutic agent in treating lung (34) and colon cancers (35, 36). Recent clinical studies showing increased cardiovascular events arising from selective cyclooxygenase-2 inhibition have also highlighted the important role of PGI$_2$ in cardiovascular protection (37, 38). Furthermore, in vivo studies of prostacyclin receptor knock-out (IP$^{-/-}$) mice have shown that PGI$_2$ is a key modulator of platelet-vascular interactions and may play a role in protection against atherosclerosis (2, 3). Thus, the culmination of these studies has lent a great deal of insight into the important roles of PGI$_2$ activity as 1) an effective pharmacological agent and 2) a critical physiological-homeostatic mediator. However, we are now only beginning to decipher the molecular structure and function of the G-protein-coupled receptor (i.e. hIP) that mediates these imperative functions of PGI$_2$.

**A Putative Second Disulfide Bond within the hIP Receptor—**The goal of this study was to determine the functional roles played by extracellular- and transmembrane-located cysteine residues within the hIP to improve our molecular understanding of this important cardiovascular protein. Strong evidence has supported the existence and necessity (for proper ligand binding) of the conserved disulfide bond in other prostaglandin receptors, such as the thromboxane receptor (19, 20). Interestingly, mutations of the Cys-$170$ equivalent cysteine in the EP3 suggested that it is not critical for agonist binding and activation (40). In our present study we look at the differential effects on binding, activation, and expression of these putative conserved and non-conserved disulfide bonds within the extracellular domain of the hIP receptor. We demonstrate that the conserved disulfide bond (Cys-$92$–Cys-$170$) is insufficient for maintaining proper ligand binding characteristics, and an additional extracellular bond (Cys-$5$–Cys-$165$) may be required for hIP stability. Support for this notion of a second disulfide bond within the extracellular domain of the hIP could be observed upon sequential treatment with β-ME. Furthermore, the lack of significant change in either IC$_{50}$ or EC$_{50}$ values (only a change in specific counts) upon treatment with reducing agents strongly supports a secondary disulfide requirement for stability. Potential explanations for such added structural stringency could be the unusual orientation of receptor-bound PGI$_2$ (25), which maintains dual pentagonal rings facing TM1 and TMII, as opposed to other similarly structured class A ligands (e.g. 11-cis-retinal and epinephrine), which face away from this region toward TMV and TMVI. Perhaps maintenance of this unique binding pocket requires an additional stabilizing bond extending from the N terminus (C5) to the second extracellular loop (Cys-$165$). The presence of putative dual disulfide bonds in the hIP has been suggested only recently (as a means of receptor oligomerization) in published work using differential tagging and Western analysis (41). Recent work has also demonstrated potential important roles for GPCR and hIP dimerization (42, 43). Rather than direct disulfide bonding, the non-conserved extracellular free sulfhydryl groups may be involved in critical homo- or heterodimerization. Alternatively, an added disulfide

**FIGURE 7. Molecular modeling of critical extracellular and transmembrane cysteines.** Panel A, shown is the three-dimensional rhodopsin-based homology model of the human prostacyclin receptor extracellular domain and upper transmembrane domains. N terminus and extracellular loops (exoloops 1–3) are shown in green. The computer-generated, energy-minimized structure has the N terminus cradled between extracellular loops 2 and 3 (exoloop 2 and 3) with stabilizing, covalent disulfide linkages (solid yellow bonds) between residues Cys-$5$ (N terminus) and Cys-$165$ (exoloop 2) as well as Cys-$92$ (top TMIII) and Cys-$170$ (exoloop 2). Panel B, shown are the lower regions of the transmembrane domain and cytoplasmic loops in green. Residue Cys-$118$ is positioned at the bottom of TMIII, at the beginning of cytoplotope 2. Green dashed lines represent H-bonding interactions between the sulfhydryl (SH) side chain (yellow) of Cys-$118$ and Ala-$114$, which also resides in TMIII. Panel C, shown is the human prostacyclin receptor ligand binding domain. Receptor-bound PGI$_2$, and immediate binding-pocket residues (Arg-$279$, Phe-$278$, Phe-$95$, and Tyr-$75$) are shown with TM α-helices removed for clarity. Residue Cys-$251$ (TMVI) is indirectly tethered to the Phe-$278$ (TMVI) binding-pocket residue via a potential H-bonding network with Tyr-$281$ (TMVII). Panel D, shown are the lower regions of the transmembrane domain and cytoplasmic loops in green. Residue Cys-$202$ is positioned at the bottom of TMV, at the beginning of cytoplotope 3. Green dashed lines represent the H-bonding network between Cys-$202$, Asn-$203$, and Ala-$198$. Supports for this notion of a second disulfide bond within the extracellular domain of the hIP could be observed upon sequential treatment with β-ME. Furthermore, the lack of significant change in either IC$_{50}$ or EC$_{50}$ values (only a change in specific counts) upon treatment with reducing agents strongly supports a secondary disulfide requirement for stability. Potential explanations for such added structural stringency could be the unusual orientation of receptor-bound PGI$_2$ (25), which maintains dual pentagonal rings facing TM1 and TMII, as opposed to other similarly structured class A ligands (e.g. 11-cis-retinal and epinephrine), which face away from this region toward TMV and TMVI. Perhaps maintenance of this unique binding pocket requires an additional stabilizing bond extending from the N terminus (C5) to the second extracellular loop (Cys-$165$). The presence of putative dual disulfide bonds in the hIP has been suggested only recently (as a means of receptor oligomerization) in published work using differential tagging and Western analysis (41). Recent work has also demonstrated potential important roles for GPCR and hIP dimerization (42, 43). Rather than direct disulfide bonding, the non-conserved extracellular free sulfhydryl groups may be involved in critical homo- or heterodimerization. Alternatively, an added disulfide
bridge may be necessary to allow specific conformational changes required for receptor coupling and signaling via the G<sub>c</sub>-cAMP pathway. Although the underlying mechanism remains unclear, it is interesting to note that the three prostanoid receptors containing the putative, dual-bonding extracellular cysteines, namely the IP, DP, and EP2 receptors, all couple predominately to G<sub>c</sub> and modulate cAMP, whereas four of the remaining five prostanoid receptors that do not harbor the second S-S bonding cysteines signal through G<sub>/o</sub>(EP1, FP, TP) or through G<sub>110</sub>(EP3). The last member of this prostanoid receptor group (EP4) does, however, signal through G<sub>c</sub>.

Cysteines in Critical Hydrogen Bonding Networks and Lipid-anchoring Bioconjugations (Palmitoylation-Isoprenylation)—The three critical transmembrane cysteines Cys-118, Cys-202, and Cys-251 are all highly conserved amino acids. Both Cys-202 and Cys-251 are 88 and 100% conserved across the prostanoid receptors, respectively. Residue Cys-118, although only mildly (38%) conserved across the prostanoid family of receptors, is located within the common ER(Y/C) motif, which is highly preserved in class A GPCRs. By virtue of the tightly packed α-helical environment, the requirement for membrane flexibility and conformational movement for receptor activation, disulfide (S-S) bonding, or cysteine bioconjugations are not commonly found within the transmembrane domain. As a result, hydrogen bonding networks are the most likely molecular interactions to form between cysteines and other amino acids within the transmembrane domain of the hIP. Our simple first pass homology model provides some support for such potential transmembrane H-bonding interactions. However, further detailed biophysical analysis would be required to definitively confirm such interactions.

Palmitoylation and isoprenylation are forms of post-translational modification in which hydrophobic carbon moieties (i.e. C-16 palmitic acid, or C-15, C-20 isoprenoids) are covalently attached to cysteine residues of membrane proteins. Five of the remaining six cysteines located along the C-terminal tail of the hIP are positioned within lipid-anchoring (palmitoylation-isoprenylation) CAAX consensus motifs. The first of these motifs begins at amino acid position 308 and contains three cysteines, CCLC (Cys-Cys-Leu-Cys) (Fig. 1A). This motif is highly conserved across class A GPCRs and provides a site for lipid anchoring, which tethers part of the C-terminal tail to the cytoplasmic membrane, forming a fourth intracellular loop. In the hIP, this site is thought to be predominately conjugated with palmitic acid (palmitoylation) at both Cys-308 and Cys-311. There also exists a second CAAX motif at the very end of the C-terminus, starting at residue 383, CSLC (Cys-Ser-Leu-Cys). This particular motif is thought to be predominately isoprenylated (at Cys-383) and has been purported to result in a fifth intracellular loop, which may modify G-protein-coupling capacity (1, 44). Although excluded from our particular investigation, the importance of these remaining six C-terminal cysteines on hIP structure and function should not be dismissed.

The culmination of this study highlights the importance and versatility of extracellular and transmembrane resident cysteines in hIP stability and function. As demonstrated, a number of highly conserved cysteines within the transmembrane domain (e.g. Cys-118, Cys-202, and Cys-251) of the hIP receptor were found to be critical for proper receptor function (i.e. binding, activation, and expression). Furthermore, all of the cysteine residues located within the extracellular domain (e.g. Cys-5, Cys-92, Cys-165, and Cys-170) were found to carry out significant and differential roles in the maintenance of hIP structural integrity and activity. Evidence from site-directed mutagenesis, functional binding activation assays, treatment with thiol-reducing agents, compensatory mutations, sequence homology comparisons, and computer-assisted modeling all support the notion of dual disulfide bridges within the extracellular domain of the hIP; that is, a conserved linkage between Cys-92 and Cys-170 and a putative non-conserved linkage between Cys-5 and Cys-165.

Considering the importance of the human prostacyclin receptor in cardiovascular disease along with the susceptibility to reductive-oxidative stressors of extracellular cysteines within the hIP receptor protein, one could speculate that the redox environment found within blood vessels may play a critical role in both the normal and pathophysiological structure-function of the human prostacyclin receptor. Oxidative stress has been implicated in numerous cardiovascular abnormalities, including hypertension, atherosclerosis, and restenosis after angioplasty (39, 45), and oxidation of hIP proteins within the vasculature could alter structure (e.g. inducing aberrant disulfide cross-linking) and inhibit function. Furthermore, naturally occurring single nucleotide polymorphisms to and from cysteine (Xaa→Cys and Cys→Xaa), especially within the extracellular domain of the receptor, could also have drastic effects given the importance of these amino acids in upholding conformational integrity. Currently none have been found in the hIP; however, mutations at equivalent sites in rhodopsin (Cys-110 and Cys-187) have been found to lead to the retinal degenerative disease retinitis pigmentosa (22).

This study identifies critical cysteine residues within the extracellular and transmembrane domains of the hIP that, by nature of their versatility, are necessary for proper receptor function. In addition to lipid bioconjugation, strong evidence supports the existence of dual disulfide bonds within the extracellular region of the hIP as well as critical transmembrane hydrogen bonding. All serve differential roles in maintaining proper receptor configuration for binding affinity, receptor activation, cell surface expression, and trafficking.

REFERENCES

1. Miggin, S. M., Lawler, O. A., and Kinsella, B. T. (2003) J. Biol. Chem. 278, 6947–6958
2. Cheng, Y., Austin, S. C., Rocca, B., Koller, B. H., Coffman, T. M., Grosser, T., Lawson, J. A., and Fitzgerald, G. A. (2002) Science 296, 539–541
3. Egan, K. M., Lawson, J. A., Fries, S., Koller, B., Rader, D. J., Smyth, E. M., and Fitzgerald, G. A. (2004) Science 306, 1954–1957
4. Murata, T., Ushikubi, F., Matsuo, T., Hirata, M., Yamasaki, A., Sugimoto, Y., Ichikawa, A., Aze, Y., Tanaka, T., Yoshida, N., Ueno, A., Oh-ishi, S., and Narumiya, S. (1997) Nature 388, 678–682
5. Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999) Physiol. Rev. 79, 1193–1226
6. Stojanovic, A., Stitham, J., and Hwa, I. (2004) J. Biol. Chem. 279, 35932–35941
7. Hayes, J. S., Lawler, O. A., Walsh, M.-T., and Kinsella, B. T. (1999) J. Biol. Chem. 274, 23707–23718
8. Cook, J. V. F., and Eidne, K. A. (1997) Endocrinology 138, 2800–2806
Extracellular and Transmembrane Cysteines in hIP

1. Perlman, J. H., Wang, W., Nussenzeig, D. R., and Gershengorn, M. C. (1995) J. Biol. Chem. 270, 24682–24685
2. Karnik, S. S., Sakmar, T. P., Chen, H. B., and Khorana, H. G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8459–8463
3. Karnik, S. S., and Khorana, H. G. (1990) J. Biol. Chem. 265, 17520–17524
4. Davidson, F. F., Loewen, P. C., and Khorana, H. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4029–4033
5. Hwa, J., Klein-Seetharaman, J., and Khorana, H. G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4872–4876
6. Fay, J. F., Dunham, T. D., and Farrens, D. L. (2005) Biochemistry 44, 8757–8769
7. Shi, L., and Javitch, J. A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 440–445
8. Zhang, P., Johnson, P. S., Zollner, C., Wang, W., Wang, Z., Montes, A. E., Seidleck, B. K., Blaschak, C. J., and Surratt, C. K. (1999) Mol. Brain Res. 72, 195–204
9. Tarnow, P., Schoneberg, T., Krude, H., Gruters, A., and Biebermann, H. (2003) J. Biol. Chem. 278, 48666–48673
10. Lisenbee, C. S., Dong, M., and Miller, L. J. (2005) J. Biol. Chem. 280, 12330–12338
11. D'Angelo, D. D., Eubank, J. J., Davis, M. G., and Dorn, G. W., II (1996) J. Biol. Chem. 271, 6233–6240
12. Chiang, N., Kan, W. M., and Tai, H.-H. (1996) Arch. Biochem. Biophys. 334, 9–17
13. Noda, K., Saad, Y., Graham, R., and Karnik, S. (1994) J. Biol. Chem. 269, 6743–6752
14. Stojanovic, A., and Hwa, J. (2002) Receptors Channels 8, 33–50
15. Stitham, J., Martin, K. A., and Hwa, J. (2002) Mol. Pharmacol. 61, 1202–1210
16. Stitham, J., Stojanovic, A., and Hwa, J. (2002) J. Biol. Chem. 277, 15439–15444
17. Stitham, J., Stojanovic, A., Merenick, B. L., O'Hara, K. A., and Hwa, J. (2003) J. Biol. Chem. 278, 4250–4257
18. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
19. Cuff, J. A., Siddiqui, A. S., and Barton, G. J. (1998) Bioinformatics 14, 892–893
20. Phillips, J. C., Beaus, R., Wang, W., Gumbar, T., Tajkhorshid, E., Ella, E., Chipot, C., Skeel, R. D., Kale, L., and Schulten, K. (2005) J. Comput. Chem. 26, 1781–1802
21. Phillips, J. C., Braun, R., Wang, W., Gumbart, J., Tajkhorshid, E., Villa, E., Chipot, C., Skeel, R. D., Kale, L., and Schulten, K. (2005) J. Comput. Chem. 26, 1781–1802
22. Stojanovic, A., Ross, L. A., Blount, A. C., and Hwa, J. (2004) Biochemistry 43, 8974–8986
23. Jones, R. L., Qian, Y., Wong, H. N., Chan, H., and Yim, A. P. (1997) Clin. Exp. Pharmacol. Physiol. 24, 969–972
24. Nagaya, N., Okamura, M., Okano, Y., Satoh, T., Kyotani, S., Sakamaki, F., Nakanishi, N., Miyatake, K., and Kunieda, T. (1999) J. Am. Coll. Cardiol. 34, 1188–1192
25. Olszewski, H., Ghoefrani, H. A., Schmehl, T., Winkler, J., Wilkens, H., Hoper, M. M., Behr, J., Kleber, F. X., and Seeger, W. (2000) Ann. Intern. Med. 132, 435–443
26. Schiffer, E. L., and Touyz, R. M. (1998) J. Cardiovasc. Pharmacol. 32, Suppl. 3, 2–13
27. Keith, R. L., Miller, Y. E., Hoshikawa, Y., Moore, M. D., Gesell, T. L., Gao, B., Malkinson, A. M., Golpon, H. A., Nemenoff, R. A., and Geraci, M. W. (2002) Cancer Res. 62, 734–740
28. Schneider, M. R., Schirmer, M., Lichtner, R. B., and Graf, H. (1996) Breast Cancer Res. Treat 38, 133–141
29. Watanabe, K., Kawamori, T., Nakatsugi, S., Ohta, T., Ohuchida, S., Yamamoto, H., Maruyama, T., Kondo, K., Ushikubi, F., Narumiya, S., Sugimura, T., and Wakabayashi, K. (1999) Cancer Res. 59, 5093–5096
30. Grosser, T., Fries, S., and Fitzgerald, G. A. (2006) J. Clin. Investig. 116, 4–15
31. Fitzgerald, G. A. (2004) N. Engl. J. Med. 351, 1709–1711
32. Heitzer, T., Schlinzig, T., Krohn, K., Meintz, T., and Munzel, T. (2001) Circulation 104, 2673–2678
33. Audoly, L., and Breyer, R. M. (1997) J. Biol. Chem. 272, 13475–13478
34. Giguere, V., Gallant, M. A., de Brum-Fernandes, A. J., and Parent, J. L. (2004) Eur. J. Pharmacol. 494, 11–22
35. Wilson, S. J., Roche, A. M., Kostetskaia, E., and Smyth, E. M. (2004) J. Biol. Chem. 279, 53036–53047
36. Fotiadis, D., Iastrzebska, B., Phililpsen, A., Muller, D. J., Palczewski, K., and Engel, A. (2006) Curr. Opin. Struct. Biol. 16, 252–259
37. Miggin, S. M., Lawler, O. A., and Kinsella, B. T. (2002) Eur. J. Biochem. 269, 4654–4664
38. Griendling, K. K., and FitzGerald, G. A. (2003) Circulation 108, 1912–1916

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