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A profile of the residues in the second extracellular loop that are critical for ligand recognition of human prostacyclin receptor

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Abstract

The residues in the second extracellular loop (eLP2) of the prostanoid receptors, which are important for specific ligand recognition, were previously predicted in our earlier studies of the thromboxane A_2 receptor (TP) using a combination of NMR spectroscopy and recombinant protein approaches. To further test this hypothesis, another prostanoid receptor, the prostacyclin receptor (IP), which has opposite biological characteristics to that of TP, was used as a model for these studies. A set of recombinant human IPs with site-directed mutations at the nonconserved eLP2 residues were constructed using an Ala-scanning approach, and then expressed in HEK293 and COS-7 cells. The expression levels of the recombinant receptors were six-fold higher in HEK293 cells than in COS-7 cells. The residues important for ligand recognition and binding within the N-terminal segment (G159, Q162, and C165) and the C-terminal segment (L172, R173, M174, and P179) of IP eLP2 were identified by mutagenesis analyses. The molecular mechanisms for the specific ligand recognition of IP were further demonstrated by specific site-directed mutagenesis using different amino acid residues with unique chemical properties for the key residues Q162, L172, R173, and M174. A comparison with the corresponding functional residues identified in TP eLP2 revealed that three (Q162, R173, and M174) of the four residues are nonconserved, and these are proposed to be involved in specific ligand recognition. We discuss the importance of G159 and P179 in ligand recognition through configuration of the loop conformation is discussed. These studies have further indicated that characterization of the residues in the eLP2 regions for all eight prostanoid receptors could be an effective approach for uncovering the molecular mechanisms of the ligand selectivities of the G-protein-coupled receptors.

Keywords

G-protein-coupling receptor; prostacyclin; prostacyclin receptor; prostaglandin I_2; prostanoid receptor

Prostacyclin [prostaglandin I_2 (PGI_2)] is a prostanoid that has antithrombotic and vasodilatory functions and is therefore an important vascular protector [1–5]. Also, PGI_2 is a key molecule that can neutralize the thrombotic and vasoconstrictive effects of thromboxane A_2 (TXA_2), one of the major promoters of strokes, heart attacks, and other vascular diseases [6,7]. Both PGI_2 and TXA_2 are synthesized through the cyclooxy-genase pathway from the

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same precursor, arachidonic acid. The biological functions of PGI_2 and TXA_2 are mediated by the PGI_2 receptor (IP) and TXA_2 receptor (TP), respectively – two of the eight specific prostanoid receptors in the G-protein-coupled receptor (GPCR) family, which is made up of seven transmembrane (TM) domains coupled to different G-proteins. TP was first purified from platelets in 1989, and in 1991 the cDNA was cloned from the placenta [8,9]. The human IP cDNA was cloned in 1994 [10]. For more than a decade, many attempts have been made to understand the molecular basis of the receptors that mediate PGI_2 and TXA_2 functions. However, owing to the difficulty in crystallizing the membrane proteins of GPCRs, the only crystal structure that has been successfully determined is that of rhodopsin [11–14], which offers a structural template for the molecular modeling of the TM domains of the prostanoid receptors, including IP [15,16] and TP [17]. Homology modeling-based mutagenesis of the TM domains of the prostanoid receptors suggested that the conserved regions of the third and seventh TM domains are involved in binding the common structures of the prostanoids, which include a carboxylic acid, a hydroxyl group at position 15, and two aliphatic side chains [18–20]. However, these common features between IP and TP could not sufficiently explain the receptors' selectivities in ligand recognition. To understand the different pathophysiological actions of PGI_2 and TXA_2, it is important to understand the molecular mechanism by which IP and TP selectively recognize ligand molecules on the extracellular side of their membrane, transfer them into the membrane domain, and finally trigger the different types of G-protein binding on their intracellular membrane side. Structural characterization of the receptors' extra-cellular domains is a key step in revealing their mechanisms of action at the molecular level.

To overcome the difficulty of characterizing the structures for the extracellular loop (eLP) domains of the prostanoid receptors without access to their crystal structures, we explored a strategy for generating an experimental 3D structure model for human TP. The 3D structure was completed through assembly of the NMR structure of the computation-guided constrained peptides that mimicked the TP eLPs and were connected to the conserved seven-TM model generated from homology modeling using the rhodopsin crystal structure [21]. The NMR structure-based model reveals the structural features of the TP eLPs [17,22], in which the key residues V176, L185, T186 and L187 in the second eLP (eLP2) demonstrated possible direct contact with a molecule from the TP antagonist [23]. The results of the NMR studies were further confirmed by recombinant TP studies using site-directed mutagenesis [21–23]. The key residues from eLP2 of TP believed to be involved in forming specific ligand recognition pockets have been proposed [21]. A similar molecular mechanism could also be applied to other prostanoid receptors, such as IP. In this work, we performed a mapping of the residues that are important for receptor–ligand recognition in the eLP2 region of human IP. A profile of the IP eLP2 residues involved in the receptor agonist recognition is revealed.

Results

Design of the site-directed mutagenesis for mapping of the residues important in ligand recognition for IP eLP2 using structural and sequence alignment

Recently, the residues in the eLP2 region of human TP that are involved in ligand recognition and binding have been demonstrated by our group [17,21–23]. These findings have provided fundamental information suggesting that eLP2s in other prostanoid receptors could also be involved in specific ligand recognition. On the basis of the IP model created by homology modeling using the templates of the crystal TM structure of rhodopsin and the NMR fragment structure of the TP eLPs, IP eLP2 is likely to form a disulfide bond with the first eLP of IP and become exposed on the surface of the protein [16] (Fig. 1A). The eLP2 regions between human TP and IP share approximately 40% identity (Fig. 1B). The identical residues, QY-PGSWCFL, in the eLP2 regions are centrally located within the eLP (Fig. 1B).

The previously identified key residues, V176, T186 and L187, in TP eLP2 [21] are not conserved in IP eLP2 (Fig. 1B). This indicates that the corresponding residues of IP eLP2 could be involved in specific ligand recognition. This hypothesis has led us to design a set of IP mutations using the Ala-scanning approach to characterize the function of the nonconserved residues in IP eLP2. Two previously identified mutants, C165A and P179A [24,25], were also included as positive controls. This mutation should provide information for the individual residues that are important in IP ligand recognition as well as show any differences from that of TP.

Expression of wild-type IP in COS-7 and HEK293 cells

The introduction of the microplate-based scintillation counter (MicroBeta or TopCount) for receptor binding assays has dramatically improved and increased our screening capacities. It is particularly convenient and reliable for determining GPCR activities on live cell membranes, because it allows us to directly culture the cells on the same plates that will later be used for the assays. However, one of the limitations of the micro-plate-based binding assay is that it is only suitable for a small amount of cells in 96-well or 24-well plates expressing a relatively high amount of targeted receptor. Thus, establishing a high-level expression system for the recombinant prostanoid receptor in mammalian cells is a key step towards obtaining reliable data using the microplate-based binding assay. The expression efficiencies of recombinant IP in HEK293 and COS-7 cells cultured in 24-well plate were compared. With 3.1 nm (4000 c.p.m.) [^{3}H]iloprost per well, the maximum binding for COS-7 cells expressing wild-type IP (IP-wt) was approximately 2% of the total c.p.m. added, whereas the binding of HEK293 cells expressing the same receptor reached up to 10% (Fig. 2Ab). These data indicated that the expression level of recombinant IP is approximately six-fold higher in HEK293 cells than in COS-7 cells (Fig. 2Ab). A similar result was obtained in the western blot analysis (Fig. 2Aa).

Functional residues in the less conserved N-terminal segment of IP eLP2

From the sequence alignment of the eLP2s between IP and TP, it was noted that IP eLP2 could be further subdivided into three segments. The first is the less conserved N-terminal segment from residues G159 to C165; the second is the conserved central segment from P166 to F171; and the third is the less conserved C-terminal segment from L172 to G180. TP and IP have very specific ligand-binding properties. The less conserved regions may play key roles in the determination of the receptor specificities. Ala-scanning mutagenesis was performed for the first, less conserved segment, and the results of this profile are shown in Fig. 2Bb. The expression levels of the IP mutants in HEK293 cells are similar to those of IP-wt, as confirmed by western blot (Fig. 2Ba). However, the mutants G159A, Q162A and C165A lost approximately 70%, 90%, and 100%, respectively, of their [^{3}H]iloprost-binding activities (Fig. 2Bb). It should be made clear that the C165A mutant still showed a high expression level in the western blot, even though it lost almost all of its ligand-binding activity. In contrast, the Q160A, H161A and Y164A mutants retained full activity upon binding to [^{3}H]iloprost (Fig. 2Bb); therefore, they served as an internal negative control for the experiments. To further test these results, all of the recombinant IPs were also overexpressed in the COS-7 cell line, where identical results were observed (Fig. 3). Furthermore, a dose-response assay was used to show that the G159A, Q162A and C165A mutants lost [^{3}H]iloprost-binding activity (Fig. 4). Very little binding activity was observed for each of the three mutants in the assay, even when increasing amounts of [^{3}H]iloprost were used. A combination of the results obtained from the two cell lines and the titration assays demonstrated the involvement of the N-terminal nonconserved residues G159, Q162 and C165 in the agonist recognition of IP.

Functions of the conserved segment (second segment) of the eLP2 region

Less specific effects on receptor recognition for the different ligands have been predicted, because the residues from P166 to F171 (PGSWCF) of the conserved segment in IP eLP2 are identical to those of TP eLP2 (Fig. 1B). However, it has been noted that mutation of the conserved residues in the segments, such as Cys183 in TP [26], destroys its ligand-binding activity, which indicates that the conserved residue is also nonspecifically involved in forming a general ligand recognition pocket. In our previous NMR structural studies of TP eLP2, it was noted that the conserved residues play an important role by constraining the segment to form a loop structure as well as the ligand recognition pocket [21]. This is particularly important for the Cys residue in the conserved segment, which could form a disulfide bond with another Cys in eLP1. However, the nature of the conservation in the eLP2 region between TP and IP has led to the prediction that the residues in the segment are involved in the configuration of a general ligand recognition pocket for the receptors, but the residues are less important for determining the receptor selectivities in binding to the different prostanoids. This hypothesis is further supported by our additional mutagenesis studies, including a western blot (Fig. 5A) and a ligand-binding assay (Fig. 5B), in which one mutant, S168A, completely lost its ligand-binding activity, and another mutant, S168T, recovered its full ligand-binding activity following binding to [^{3}H]iloprost. This finding also indicated that the conserved S168 residue's involvement in the configuration of the ligand recognition pocket most likely takes place through its hydrophilic and hydrogen-bonding side chain(s). When the –OH group in the S168A mutant was replaced by a hydrophobic –CH_3 group, the general ligand recognition property of the receptor was disturbed. However, the ligand recognition pocket could be constructed by replacing the S168 with a Thr residue, which has a similar hydrophilic and hydrogen-bonding side chain. In addition, the activity could be fully recovered (Fig. 5B). The side chain of the Thr residue is bigger than that of the Ser, and has no effect on the ligand binding. Therefore, this implies that S168 is not involved in specific ligand recognition.

Functional residues in the C-terminal segment of eLP2

Like the N-terminal segment, the C-terminal segment of IP eLP2 is also a nonconserved segment. The idea that some residues in the segment are probably involved in specific ligand recognition is supported by the following four considerations: (a) the residue types for TP and IP are highly variable in amount in the segments of the prostanoid receptors (Fig. 1B); (b) the three residues L172, R173 and M174 from IP eLP2 correspond to L185, T186 and L186 of TP, which have been identified as important residues in the C-terminal segment involved in ligand recognition/binding for human TP [15,23]; (c) our previous studies have shown that after replacement of R173 with an Ala residue (R173A), IP loses ligand-binding activity [16]; and (d) it has also been reported that the mutant with replacement of P179 by Ala (P179A) has significantly reduced iloprost-binding activity [25]. On the basis of this information, the profile of the residue functions of the C-terminal segment is important for understanding the ligand selectivity of IP determined by the eLP2 region. The entire C-terminal segment was subjected to Ala-scanning mutagenesis, with the exception of A177. The previously reported R173A and P179A mutants were used as positive controls to compare with other mutants. The expression levels of all the mutants in the HEK293 cells were similar to that of IP-wt, as confirmed by western blot analysis (Fig. 6Aa). However, the L172A, R173A, M174A and P179A mutants had significantly reduced [^{3}H]iloprost-binding activity (Fig. 6Ab). In contrast, the R175A, W176A, Q178A, G180A and G181A mutants retained their [^{3}H]iloprost-binding activity (Fig. 6Ab). These data show that the newly identified L172 and M174 mutants, which are very similar to the previously identified R173 and P179 mutants with reduced ligand-binding activities, are important for receptor–agonist recognition and binding.

Functional analysis of the residues in IP eLP2 that are important for ligand recognition

Residue Q162 in the N-terminal segment of IP eLP2—In the N-terminal segment, the residues important for the ligand recognition described above include G159, Q162, and C165 (Fig. 2). It has been proposed that C165 might play a role in forming a disulfide bridge with the C5 residue in the N-terminal domain of IP [24]. As for G159, as it has no side chains, it is not likely to interact with any other residues or ligands. However, Q162 – an identified functional residue in the studies – might be of more interest. To investigate the chemical properties of the side chains of the amino acids important for ligand-binding activity in receptor–ligand recognition, two additional mutants (Q162E and Q162L) were designed. The side chains of Leu and Glu are similar in size to that of Gln. Replacement of the hydrophilic side chain of Gln with the hydrophobic side chain of Leu allowed the Q162L mutant to be used to test the potential hydrophilic contact with the ligand or other residues, and the Q162E mutant was used to test the important uncharged polar property. The Q162L mutant, which is similar to the Q162A mutant, completely lost the ability to bind to iloprost, suggesting that the hydrophilic property is important for the function of Q162 in ligand recognition (Fig. 6Bb). After replacement of the uncharged hydrophilic side chain of Gln with the negatively charged hydrophilic side chain of Glu, the ligand-binding activity of the Q162E mutant was significantly reduced, to 40%. This further suggests that the uncharged hydrophilic property is preferred for the ligand interaction (Fig. 6B).

Residues L172, R173 and M174 in the C-terminal segment of IP eLP2—The three residues L172, R173 and M174 in the C-terminal segment of IP eLP2, which are important for ligand recognition (Fig. 6Ab), correspond to the three active residues L185, T186 and L187 in TP eLP2 [21] (Fig. 1B). This suggests that the ligand recognition sites are probably in the same position for IP and TP. Further mutations were made to characterize the properties of the side chains of the three key residues for IP. For instance, the L172 residue is highly constrained in the ligand recognition site, because the L172A mutant completely lost binding activity (Fig. 6Ab); however, the L172I mutant retained most of its activity (Fig. 6Bb). The function of the R173 residue in the ligand recognition site is possibly to provide a noncharged hydrophilic or hydrogen bond, because the R173A mutant retained only 50% of its activity (Fig. 6Ab), but the R173T mutant retained full activity (Fig. 6Bb). Additionally, it was also confirmed that the side chain of M174 may contribute to a hydrophobic contact with the exception of the side chain size because the M174A mutant lost 60% of its activity (Fig. 6Ab), but the M174L mutant retained full activity (Fig. 6Bb).

Residue A177—To complete the scanning mutation in the C-terminal segment of IP eLP2, the remaining residue, A177, was also replaced with a polar residue, Arg (A177R). The mutant did not lose any iloprost-binding activity, even after this major change from an aliphatic side chain to a charged side chain (Fig. 6Bb). This could serve as a negative control for the mutagenesis experiments, as it indicates that the side chain of nonconserved A177 is not involved in the ligand recognition pocket.

Discussion

These comparisons between the COS-7 and HEK293 expression levels of IP-wt and mutants have clearly demonstrated that, with use of the pcDNA3.1 vector, the expression level that could be achieved in HEK293 cells was much higher than that in COS-7 cells. It should be noted that the expression levels of the receptor shown in the western blots could be the result of higher DNA transfection efficiencies or better protein expression from the cells, or perhaps both. This information could be useful in designing an expression system for other prostanoid receptors. The superior overexpression levels of the prostanoid receptor in HEK293 cells should provide more sensitive and reliable information regarding the

mutagenesis studies. For example, our previous conclusion that IP C165 forms a disulfide bond with C5 in the N-terminal domain was derived from the low expression levels of the C165A mutant in COS-7 cells when compared to that of IP-wt [24]. However, this phenomenon was not observed in the HEK293 cells expressing the C165A mutant (Fig. 2B). The identical expression levels between the C165A mutant and IP-wt (Fig. 2B) have initiated a discussion that calls for further evidence to identify the disulfide bridge between C165 and C5 in IP.

The residues G159 and P179, which have been identified as functional residues affecting ligand binding, are widely separated within the amino acid sequence. However, from the 3D structural model generated by homology modeling [16], it is clear that the two residues, located near both ends of IP eLP2, are in very close proximity (Fig. 7). There are several speculations regarding the involvement of G159 and P179 in ligand binding. Pro, with either a *cis* or a *trans* conformation, is a critical residue for the configuration of the secondary structure of the protein. The replacement of P179 with Ala could alter the distance between the N-terminus and the C-terminus of eLP2, which could then result in a conformational change of the ligand recognition site within the eLP2 region. Another hypothesis is that the main chain distance between G159 and P179 is approximately within 10–12 Å [16], which leads to the high possibility that it could be involved in the formation of the path of the ligand's movement into the TM's ligand pocket. Therefore, replacement of G159 with an Ala containing side chain methyl groups could block the ligand's access to the TM domain. It is also possible that the hydrophobic side chain of Ala might directly block the polar head of the iloprost moving into the TM pocket. From an analysis of the sequence alignment, it was determined that G159 and P179 are nonconserved in most prostanoid receptors (Fig. 1B). This leads to the possibility that the two active residues could be unique to IP. For further confirmation, all of these possibilities must be further tested experimentally.

We are the first group to have identified the three consecutive residues L185, T186 and L187 in TP eLP2, as well as their importance in receptor–ligand recognition [21]. Thus, we have predicted that the corresponding residues in other prostanoid receptors might also follow the TP pattern and have a similar involvement in the formation of their specific ligand recognition pockets. The three identified residues L172, R173 and M174 in IP eLP2 match very well their corresponding residues L185, T186 and L187 in TP eLP2 [21] (Fig. 1). This observation has provided the first experimental evidence to support the prediction resulting from the TP studies. To further confirm the hypothesis, the active residues in the eLP2 segments must also be screened for other prostanoid receptors.

Q162 of IP eLP2 corresponds to V176 of TP V176, which has been identified as an important residue involved in ligand recognition for the TP antagonist [21]. In addition, it is a nonconserved residue in the N-terminal segment of IP eLP2. The fact that all of the mutants (Q162A, Q162L, and Q162E) lost iloprost-binding activity (Figs 2Ab and 6Bb) shows the importance of the identity of the residue for ligand recognition, and furthermore indicates that the residues could determine specific ligand recognition of IP. A similar hypothesis could also be suggested for V176 in TP.

Finally, some important considerations that will have an impact on our future investigations should be pointed out. The construction of amino acid chimeras for eLP2 between TP and IP and the double mutants for their nonconserved residues in the L T L (TP) or L R M (IP) segment has been proposed, to provide further specific ligand-binding information. The ligand-binding data for some mutants, such as W176A and L172I, showed an increase that was clearly beyond the standard deviation. The substitutions of the hydrophobic residues may be contributing to the significant changes in ligand-binding activity. In addition, the fact that S168T retained its ligand-binding activity cannot exclude the possibility that S168

in IP and S181 in TP lead to differences from other prostanoids containing Thr as their standard amino acid at this position (Fig. 1). This could mean that the Ser in IP and TP may discriminate PGI_2/TXA_2 from the remaining prostaglandins/prostacyclins, or that it may be involved in general class discrimination. Also, the importance of the residues tested for receptor activity has been limited to ligand-binding activity. The possibility that the mutants may affect signaling without affecting ligand binding has not been completely excluded.

In conclusion, a profile of the residue functions in human IP eLP2 was obtained by Ala scanning mutagenesis. G159, Q162 and C165 within the N-terminal segment of IP eLP2, and L172, R173, M174 and P179 within the C-terminal segment, were identified as key residues important for agonist recognition (Fig. 7). Four (Q162, L172, R173, and M174) of these seven residues correspond to V176, L185, M186 and L187 identified in TP eLP2, which were involved in antagonist recognition. This finding has provided a direction for possibly revealing the ligand recognition residues in the eLP2s of other prostanoid receptors.

Experimental procedures

Materials

COS-7 and HEK293 cell lines were purchased from ATCC (Manassas, VA, USA). All of the media for culturing the cells were purchased from Invitrogen (Carlsbad, CA, USA). [^{3}H]Iloprost and unlabeled iloprost were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). DNA polymerase and *Dpn*I endonuclease were obtained from Stratagene (La Jolla, CA, USA). Rabbit anti-(human IP) serum was purchased from Cayman Chemical (Ann Arbor, MI, USA).

PCR cloning of human IP

PCR cloning was used to isolate the full-length cDNA of IP from the human lung cDNA library obtained from Invitrogen. PCR primers were designed on the basis of human IP cDNA with specific modifications. The primer sequences used were 5′-ATTCTCGAGATGGCGGATTCGTGCAGGAAC-3′ (forward) and 5′-AAGAATTCACAGGGTCAGCTTGAAATGTCAG-3′ (reverse), with *XhoI* and *Eco*RI sites on the ends. The full-length cDNA of IP was obtained from standard PCR amplification, which was performed in a 50 μL reaction mixture containing 1 unit of polymerase (New England Biolabs, Beverly, MA, USA), 0.4 μm each primer, and 2 μL of human lung cDNA, for 30 cycles of 98 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The amplified products were isolated from agarose gel and subcloned into the *Xho*I–*Eco*RI sites of the pAcSG transfer vector (Pharmingen, San Diego, CA, USA). The correct cDNA sequence of the receptor was confirmed by restriction enzyme digestions and DNA sequencing analysis using the Sanger dideoxy chain termination method [27].

Site-directed mutagenesis

A pAcSG–IP-wt cDNA cloned by our laboratory was first subcloned into the *Eco*RI–*Xho*I sites of the pcDNA3.1(−) expression vector to generate the plasmid pcDNA:hIP. The IP receptor mutations were then constructed by standard PCR using the pcDNA3.1(−) vector with IP-wt as a template and two synthetic oligonucleotide primers containing the desired point mutations. The primers, which were complementary to the opposite strands of the template, were extended during the temperature cycling (95 °C for 30 s, 53 °C for 1 min 30 s, and 68 °C for 13 min) for a total of 25 cycles, with an additional extension cycle of 68 °C for 10 min, using *Pfu* DNA polymerase from Stratagene. The mutation products were treated with *Dpn*I endonuclease for digestion of the parental DNA template, and confirmed by DNA sequencing. The plasmids were then prepared using a Midiprep kit from Qiagen (Valencia, CA, USA) for transfection into HEK293 or COS-7 cells.

Expression of recombinant IP in HEK293 or COS-7 cells

HEK293 or COS-7 cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in high-glucose DMEM containing 10% fetal bovine serum, antibiotics, and antimycotics. The cells were placed on 10 cm plates or 24-well plates, cultured overnight, and then transfected with the purified cDNA of pcDNA3.1(−)/IP-wt or mutant mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Approximately 48 h after transfection, the cells were used for binding assays and western blot analysis.

Ligand-binding assays

The cells transfected with the recombinant IP cDNA on the 24-well culture plates were washed twice with ice-cold binding buffer (40 mm Tris, 10 mm $MgCl_2$, 5 mm EDTA, pH 7.4) and then incubated with [^{3}H]iloprost in the presence or absence of unlabeled iloprost in a 0.2 mL reaction volume. After 30 min at room temperature, the cells (still on the plate) were washed three times with ice-cold binding buffer, solubilized with 5% Triton X-100, and then transferred to another 24-well plate with solid walls from PerkinElmer (Waltham, MA, USA). Following the addition of 0.5 mL per well of Optiphase superMix (PerkinElmer), the radioactivity of [^{3}H]iloprost (bound to the receptor in the cells) was counted using a MicroBeta 1450 Trilux Counter (PerkinElmer).

Western blot

The transfected HEK293 or COS-7 cells were scraped from the 10 cm or 24-well plates into ice-cold NaCl/P_i (pH 7.4), and collected by centrifugation at 3000 ***g***. After being washed three times, the pellet was resuspended in a small volume of the same buffer. Each protein sample (20 μg) was separated by 10% PAGE under denaturing conditions, and then transferred to a nitrocellulose membrane. A band recognized by antibody to human IP was visualized by a second antibody linked to horseradish peroxidase as described previously [28].

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Abbreviations

eLP	extracellular loop
eLP2	second extracellular loop of prostanoid receptors
GPCR	G-protein-coupled receptor
IP	prostaglandin I_2 receptor
IP-wt	wild-type prostaglandin I_2 receptor
PGI_2	prostacyclin or prostaglandin I_2
TM	transmembrane
TP	thromboxane A_2 receptor
TXA_2	thromboxane A_2

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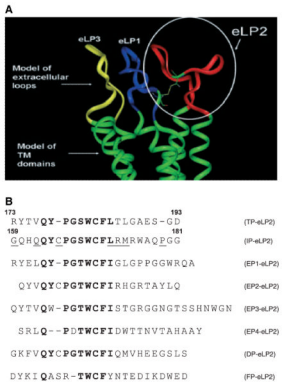

Fig. 1.

(A) A model of human IP with seven TM domains and extracellular loops created by homology modeling [16]. The eLP2 domain is indicated by a circle. (B) Sequence alignment of the eLP2 regions from the eight human prostanoid receptors. Identical residues among the eLP2 regions are shown in bold. The residues important for ligand recognition of IP are underlined.

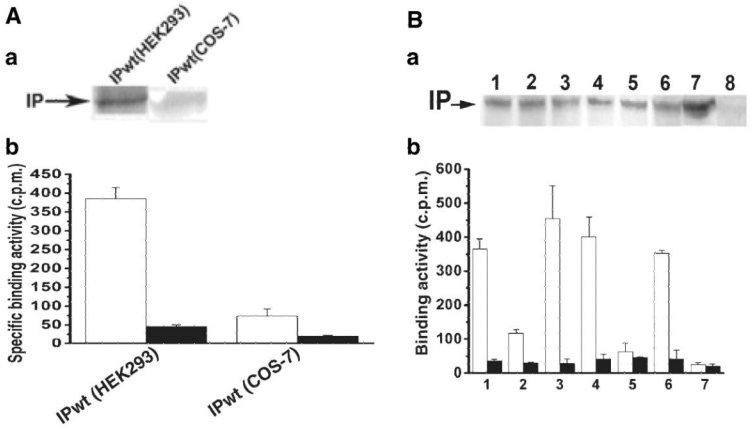

Fig. 2.

(A) (a) Western blot analysis of the recombinant IPs expressed in HEK293 or COS-7 cells. Fifty micrograms of cells transfected with the IP-wt cDNA were subjected to SDS/PAGE and transferred onto a nitrocellulose membrane, which was probed with rabbit anti-(IP peptide). The position of the IP protein is indicated on the left. (b) Binding of [^{3}H]iloprost to human IP-wt expressed in HEK293 and COS-7 cell lines. The cells were cultured on a 24-well plate and then transfected with 0.8 μg per well of the IP-wt cDNA mixed with Lipofectamine 2000. The binding of the IP agonist to the cells was measured with a MicroBeta 1450 Trilux counter using 3.1 nM [^{3}H]iloprost without (open bars) or with (closed bars) previous incubation with unlabeled iloprost (5 μM). The results presented in the figure are representative data from three assays (n = 3) and are shown as the mean ± SE. (B) (a) Western blot analysis of recombinant IP expressed in HEK293 cells. The cells were cultured on a 24-well plate, transfected with the cDNAs of IP-wt (1), or that of the mutants G159A (2), Q160A (3), H161A (4), Q162A (5), Y164A (6), or C165A (7), or no mutants (HEK293 cells, 8), and then analyzed by western blot as described in (Aa). The position of IP is indicated on the left by an arrow. (b) Binding of [^{3}H]iloprost to recombinant IP expressed in HEK293 cells. The binding of the IP agonist to the HEK293 cells transfected with the cDNA of IP-wt or the mutants described above was measured using the same procedures as those described in (Ab).

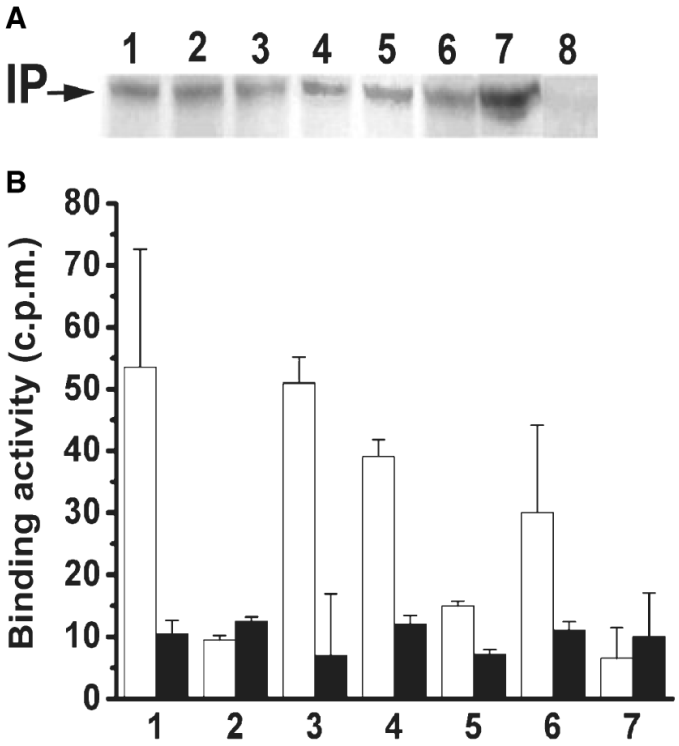

Fig. 3.

(A) Western blot analysis of recombinant IP expressed in COS-7 cells. The cells were transfected with the cDNAs of IP-wt (1), or that of the mutants, G159A (2), Q160A (3), H161A (4), Q162A (5), Y164A (6), or C165A (7), or no mutants (COS-7 cells, 8) as in Fig. 2Ba, and were treated according to the method described in Fig. 2Aa. (B) Binding of [^{3}H]iloprost to recombinant IP expressed in COS-7 cells. The binding of the IP agonist to the COS-7 cells transfected with the cDNA of IP-wt or the mutants described above was measured using the same procedures as described in Fig. 2Ab.

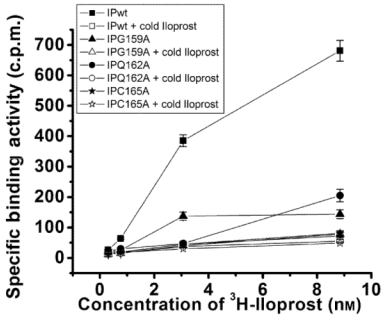

Fig. 4.

Dose–response properties of [^{3}H]iloprost binding to the recombinant IPs expressed in HEK293 cells. The cells expressing recombinant IP, as shown, were incubated with increasing amounts of [^{3}H]iloprost without (closed symbols) or with (open symbols) previous incubation with unlabeled iloprost, and were then detected with a MicroBeta 1450 Trilux counter as described in Figs 2 and 3. The results are presented as the mean ± SE and are representative data from three assays (n = 3).

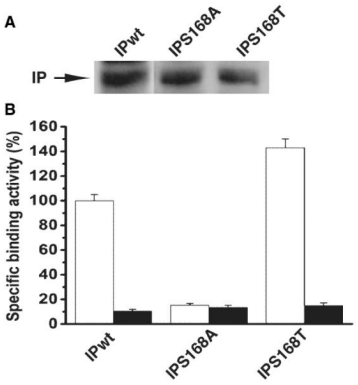

Fig. 5.

Western blot analysis (A) and binding assays (B) for the mutants in the conserved segment of IP eLP2. HEK293 cells transfected with cDNA of IP-wt (1), S168A (2) or S168T (3) were analyzed by western blot as well as by the agonist-binding assays (n = 3), using [^{3}H]iloprost as described in Fig. 2.

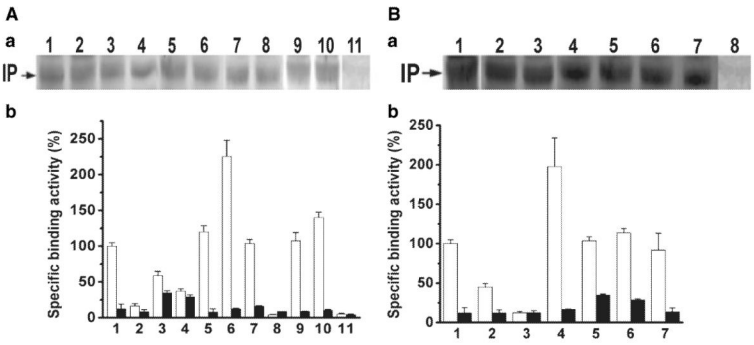

Fig. 6.

(A) Western blot analysis (a) and binding assay (b) for IP eLP2s with mutations in the C-terminal segment. HEK293 cells were cultured on a 24-well plate, and then transfected with cDNA of IP-wt (1), L172A (2), R173A (3), M174A (4), R175A (5), W176A (6), Q178A (7), P179A (8), G180A (9), G181A (10), or vector (11). The overexpressed recombinant IPs in the HEK293 cells were analyzed by western blot as well as by agonist-binding assays (n = 3), using [^{3}H]iloprost as described in Fig. 2. (B) Western blot analysis (a) and binding assay (b) for the mutants of IP eLP2. HEK293 cells were cultured on a 24-well plate, and then transfected with cDNA of IP-wt (1), Q162E (2), Q162L (3), L172I (4), R173T (5), M174L (6), A177R (7), or vector (8). The overexpressed recombinant IPs in the HEK293 cells were analyzed by western blot as well as by agonist-binding assays (n = 3), using [^{3}H]iloprost as described in Fig. 2.

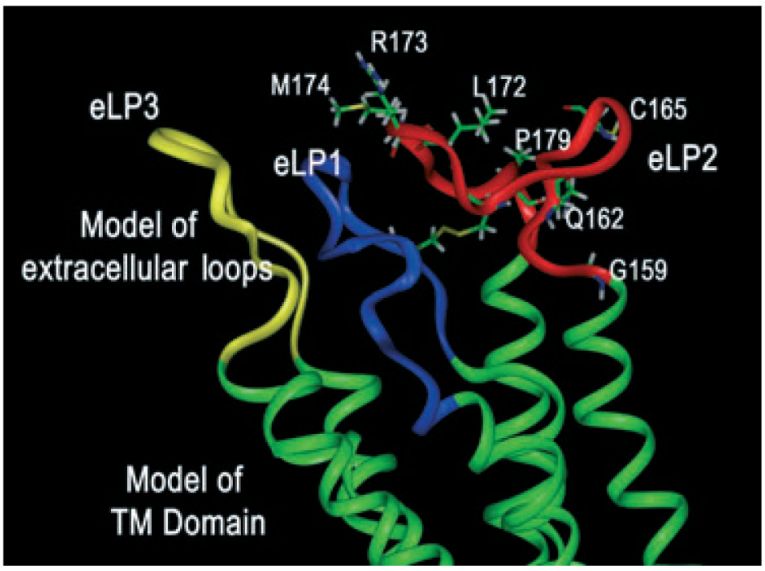

Fig. 7.

The residues important for the ligand interaction in the eLP2 region of TP. The residues that lost binding activity in the mutagenesis studies are labeled in the 3D model of TP.