Cloning and Expression of a cDNA for the Human Prostanoid IP Receptor

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Yves Boie, Thomas H. Rushmore, Alison Darmon-Goodwin, Ryszard Grygorczyk, Deborah M. Slipetz, Kathleen M. Metters, and Mark Abramovitz†

From the Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, P. O. Box 1005, Pointe Claire-Dorval, Quebec H9R 4P8, Canada

A cDNA clone coding for a functional human prostanoid IP receptor has been isolated from a lung cDNA library. The human IP receptor consists of 386 amino acid residues with a predicted molecular mass of 42,961, and has the seven putative transmembrane domains characteristic of G-protein-coupled receptors. Challenge of Xenopus oocytes expressing the IP receptor and the cystic fibrosis transmembrane conductance regulator (cAMP-activated Cl− channel) with the stable prostanoid analog iloprost resulted in specific inward Cl− currents, demonstrating that the cDNA encoded a functional IP prostanoid receptor coupled to elevation in cAMP. Radioreceptor binding studies using membranes prepared from mammalian COS cells transfected with the IP receptor cDNA showed that the rank order of potency for prostaglandins and prostaglandin analogs in competition for [3H]iloprost specific binding sites was as predicted for the IP receptor, with iloprost >> carbachol, prostaglandin (PG) E2 > PGF2α = PGE2 = U46619. Northern blot analysis showed that IP mRNA was most abundantly expressed in kidney, with lesser amounts detected in lung and liver. In summary, we have cloned and expressed a cDNA for the human prostanoid IP receptor that is functionally coupled to a signaling pathway involving stimulation of intracellular cAMP production.

Prostacyclin (PGI2) is a labile metabolite of arachidonic acid produced in concert with the cyclo-oxygenase pathway. PGI2 plays a major physiological role as a potent mediator of vasodilation and inhibitor of platelet activation (2, 3). Thus, PGI2 causes relaxation of arterial smooth muscle and inhibition of platelet aggregation, degranulation and shape change and is, therefore, thought to be important in maintaining vascular homeostasis (4). Other potential roles for PGI2 are not well established but include regulation of renal blood flow, renin release and glomerular filtration rate in the kidney cortex (2), modulation of neurotransmitter release in the heart (5), and stimulation of secretion in the stomach and large intestine (2). In common with the other prostaglandins, PGI2 is also involved in the inflammatory response eliciting hyperaemia, edema, hyperalgesia, and pyrexia primarily through its role as a vasodilator (3).

The actions of PGI2 are mediated via specific cell surface IP receptor, members of the G-protein-coupled receptor gene superfamily, which upon activation cause an elevation in intracellular cAMP via direct stimulation of adenylate cyclase (6, 7). The known distribution of IP receptors is reflective of the physiological actions of PGI2. They have been characterized extensively by radioligand binding studies in platelets from many species including human and identified in pharmacological studies as being present in coronary, pulmonary, renal, and several other arterial preparations, as well as the heart (6). IP receptors may also be present in myometrium (8), penile erectile tissue (9), and the iris sphincter muscle (10) and have been detected in NCB-20 (11) and NG108-15 (12) neuronal hybrid cell lines and the mouse mastocytoma P-815 cell line (13).

The study of prostanoid receptors is rapidly advancing with the recent cloning of the mouse (m) TP (14), EP (15), EP (16), EP (17, 18) receptors, the bovine EP (19) receptors and the human (h) TP (20), EP (21), and FP (22) receptors. Here we report the cloning of the hIP receptor, and the nucleotide and deduced amino acid sequences are described. In addition, the cystic fibrosis transmembrane conductance regulator (CFTR), which is a cAMP-activated channel, has been expressed in Xenopus oocytes and used to demonstrate that the hIP cDNA codes for a functional receptor coupled to elevation of intracellular cAMP. Finally, we describe the radioligand binding characteristics of the cloned and expressed hIP receptor.

MATERIALS AND METHODS

Cloning of the Human IP Receptor—An antisense 16-fold degenerate 26-mer oligonucleotide (designated oligo EP, d.0. VII(-)) (5'-TACA, GATCCAGGG(A,G/T/C/T)AGGATGGG(G,A/T/T)-3') based on the 9 amino acids (NPILDPWIY) in transmembrane domain (TMD) VII of the mouse EP, receptor (16) and highly conserved within the TP, EP, EP, and EP (17, 18) receptors, the bovine EP (19) receptors and the human (h) TP (20), EP (21), and FP (22) receptors. Here we report the cloning of the hIP receptor, and the nucleotide and deduced amino acid sequences are described. In addition, the cystic fibrosis transmembrane conductance regulator (CFTR), which is a cAMP-activated channel, has been expressed in Xenopus oocytes and used to demonstrate that the hIP cDNA codes for a functional receptor coupled to elevation of intracellular cAMP. Finally, we describe the radioligand binding characteristics of the cloned and expressed hIP receptor.

The abbreviations used are: PGI2, prostacyclin; PG, prostaglandin; CFTR, cystic fibrosis transmembrane conductance regulator; IBMX, 3-isobutyl-1-methylxanthine; ME55, 2-N-morpholinoethanesulfonic acid; h, human; m, mouse; TMD, transmembrane domain; bp, base pairs; kb, kilobase(s).

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† To whom correspondence should be addressed. Tel.: 514-428-8525; Fax: 514-428-8615.
Cloning of the Human Prostanoid IP Receptor

**FIG. 1. Nucleotide and deduced amino acid sequence of the human prostanoid IP receptor (11/6hLXR3).** The deduced amino acid sequence is shown below the nucleotide sequence. The positions of the putative transmembrane segments I–VII (based on the hydrophytaxis profile) are indicated by **overlines** above the nucleotide sequence. An **asterisk** denotes a dominant N-glycosylation site, and **crosses** denote potential protein kinase C phosphorylation sites. The internal NcoI site is **double-underlined**.

The Bluescript vector pKS (Stratagene, La Jolla, CA). The T7 sequencing kit from Pharmacia (Baie d'Urfe, Canada) was used to either partially or fully sequence the cDNA clones with KS and SK primers or primers generated from the determined sequences. Two clones from the lung library, hLXR6 and hLXR11, when digested with EcoRI were found to contain inserts of sizes 1.3 kb (designated hLXR3-11) and 1.5 kb when compared with hWtR3-6. hLXR3-11 was also found to contain a 24-nucleotide direct repeat that occurred in TMD VII, which would cause it to be increased by 8 amino acids. Three additional cDNA clones from the lung did not contain the 24-nucleotide repeat detected in hLXR3-11, and these clones, as well as two from the small intestine and two from the thymus, did not extend beyond the 5'-end and 56 bp at the 3'-end when compared with hWtR3-6. One of these clones contained inserts of sizes 1.3 kb (designated hLXR3-6) and 1.5 kb. The correct orientation was verified by SphI digestion of a S2P-labeled hLXR3-11 5' fragment. Appropriate polymerase chain reactions were carried out using Vent polymerase (1 unit, New England Biolabs, Beverly, MA) in a 100-pL reaction volume containing 100 mM Tris-HCl, pH 8.8, 2 mM MgSO4, 0.1% Triton X-100, 100 μg/mL bovine serum albumin and the DNA thermal cycler 480 (Perkin Elmer, Montreal, Canada). The polymerase chain reaction protocol for the first amplification was as follows: denaturation at 95°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 45 s, for 35 cycles. The second amplification cycles were modified slightly from the first in that the annealing was done at 50°C for 45 s and the extension at 72°C for 45 s. For the first reaction 2 μl (~106 plaque-forming units/ml) of the cDNA library was amplified by polymerase chain reaction using a PstI primer and XbaI-677. For the second amplification (2 μl) of the first polymerase chain reaction was re-amplified with a PstI primer and either XbaI-360 or XbaI-213. Amplified products were then analyzed by Southern blotting according to standard protocols (23) using a 32P-labeled hLXR3-11 5'-fragment. Appropriate polymerase chain reaction fragments were subsequently cloned into Bluescript vector pKS and sequenced as above.

**Construction of the pcDNAIamp-c.11/6hLXR3 Expression Vector**—pKS-hLXR3-11 was digested with SmaI and NcoI, and a 0.4-kb fragment was purified and subsequently ligated into pKS-hLXR3-6, previously cut with EcoRV and NcoI in order to exchange the 5'-end of hLXR3-6 for that of hLXR3-11, thereby creating pKS-11/6hLXR3. Digestion of pKS-11/6hLXR3 with EcoRI released a 1.4-kb cDNA (11/6hLXR3), which was subcloned into the EcoRI site of pcDNAIamp (In-vitrogen, San Diego, CA). The correct orientation was verified by Sphi digestion.

**Northern Blot Analysis**—A Northern blot, containing 2 μg of poly(A)+ RNA from different tissues, was purchased from Clontech and probed with the random-primed, [32P]dCTP-labeled 11/6hLXR3 cDNA, according to the manufacturer’s protocol. The blot was exposed to x-ray film for 48 h.

**Xenopus Oocyte Acquisition and Injection**—Oocytes were taken from adult females of Xenopus laevis using standard surgical procedures (24). Oocytes were treated for 60–120 min with freshly made collagenase (2 mg/mL, type 2, Worthington Biochemical Corp., Freehold, NJ) in Ca2+-free ND96 solution (ND96 contains (in mM): 96 NaCl, 2 KCl, 1 MgCl2, 5 HEPES, 2.5 sodium pyruvate, 0.5 theophylline, as well as 50 μg/mL gentamicin, pH 7.6) to remove follicle cells. Defolliculated stage 5–6 oocytes were selected and maintained in ND96 solution containing 1.8 mM CaCl2. Oocyte nuclei were then injected with 1.0 ng of pcDNAIamp-11/6hLXR3 and/or 2.6 ng of pcDNAIamp-CTP and incu-
Hatched at 18°C for 48–72 h prior to testing.

Detection of Receptor-mediated cAMP Elevation in Xenopus Oocytes—
For this assay an oocyte co-injected with pCDNAIamp-CFTR and pCDNAIamp-11/6hLXR3 was placed in a 0.5-ml perfusion chamber and voltage-clamped at -60 mV with two microelectrodes of 0.5-2.0-megohm resistance filled with 3 mM KCl using Turbo TEC 01C amplifiers (NIPI Electronic GmbH, Tamm, Germany). In order to confirm that CFTR was successfully expressed each oocyte was individually challenged with 3 nM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). Typically in 80–95% of the nuclear-injected oocytes, large IBMX-induced CFTR-mediated Cl- currents were observed ranging from 400 to 1,200 nA depending on the incubation period and the amount of cDNA injected. In addition, control experiments challenging CFTR-injected oocytes with either dibutyryl-cAMP (200 nM) or forskolin (10 μM) also resulted in induction of the CFTR-mediated Cl- current responses (data not shown). Responding oocytes were subsequently challenged with agonists, and the Cl- current responses due to receptor-mediated cAMP elevation were recorded.

pCDNAIamp-11/6hLXR3 Expression in COS-M6 Cells and 1H)Iloprost Binding Assays—The pCDNAIamp-11/6hLXR3 vector was transfected into COS-M6 cells using the DEAE-dextran method with chloroquine (1 μg/mL). The cells were then harvested, subjected to lysis by nitrogen cavitation (24 h), and membranes prepared by differential centrifugation (1000 x g for 10 min, then 100,000 x g for 30 min). [3H]Iloprost binding assays were performed in 10 mM MES/KOH (pH 6.0), containing 1 mM EDTA, 10 mM MnCl2, 4 mM [3H]Iloprost (12.5 Ci/mmol; Amersham International), and 60 μg of protein from the 100,000 x g membrane fraction. Incubations were conducted for 60 min at 30°C prior to separation of the bound and free radioligand by rapid filtration through GF/B filters presoaked in 10 mM MES/KOH (pH 6.0) containing 0.01% (w/v) bovine serum albumin. Filters were washed with 16 mL of soaking buffer, and the residual [3H]iloprost bound to the filter was determined by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding, which was determined in the presence of 2 μM iloprost.

RESULTS AND DISCUSSION

Isolation of a cDNA Coding for the Human IP Receptor—We had previously designed a degenerate oligonucleotide probe based on the 9 conserved amino acids (NQI1DPWYV) in TMD VII found in the TP (14, 19), EP, (17–19), and SP (15, 21) prostanoid receptors in order to clone related cDNAs. Comparison of those 9 amino acids of the recently cloned mouse EP, prostanoid receptor (16) revealed that there were two amino acid differences; glutamine was changed to proline and DVal to Asp. The hydropathy profiles (by the Kyte and Doolittle method; Ref. 27) for two degenerate probes derived from the seven putative TMDs characteristic of G-protein coupled receptors. hLXR3-11 contained a methionine residue 17 amino acids upstream of the putative TMD I with no other upstream in-frame methionine or stop codon. In order to determine if this was the initiating methionine, anchored polymerase chain reaction (see “Materials and Methods” for details) was used to obtain additional sequence information at the 5'-end. Two polymerase chain reaction fragments obtained from a kidney library were sequenced and found to be extended by 19 and 61 nucleotides, as compared with hLXR3-11 (data not shown). No other methionine codon occurred, but an in-frame stop codon, TGA, was found 4 nucleotides upstream of the 5'-end of hLXR3-11. Therefore, the methionine in hLXR3-11 appears to be the start of translation.

hLXR3-11, however, contained a 24-bp direct repeat, which would extend TMD VII by 8 amino acids. Of five human lung XR3 clones sequenced only hLXR3-11 contained this repeat. To confirm that this repeat was an artifact of cloning, additional full-length hIP cDNAs were obtained by polymerase chain reaction of human lung, small intestine, uterus, and kidney cDNA libraries. These cDNAs were analyzed by restriction digestion and sequencing, and none of them were found to contain the 24-bp repeat found in hLXR3-11.

In order to remove this repeat, a hybrid cDNA (11/6hLXR3) was, therefore, constructed in which the 5'-end originated from hLXR3-11 and the 3'-end from hLXR3-6 (see “Materials and Methods” for details). This cDNA, 11/6hLXR3 (Fig. 1), contained an open reading frame of 1,158 bp, which would encode a 386-amino acid polypeptide with a relative molecular mass of 40,961. The 11/6hLXR3 cDNA was employed in the functional studies.

Functional Expression of the Human IP Receptor in Xenopus Oocytes—Prostanoid receptors that had not as yet been cloned included IP and DP, both coupling to intracellular cAMP elevation (2). An assay was, therefore, developed in Xenopus oocytes to detect receptor-mediated elevation in intracellular cAMP.
The activity of the cystic fibrosis Cl⁻ channel (CFTR) is known to be regulated by cAMP-dependent protein kinase A (for review, see Ref. 28). CFTR expressed in oocytes was, therefore, used as a sensor of receptor-mediated changes in intracellular cAMP concentrations.

Functional activity was determined in oocytes injected with pcDNAIamp-lUGhLXR3 and/or pcDNAIamp-CFTR using electrophysiological techniques. Challenge of control oocytes injected with pcDNAIamp-CFTR alone with 3 mM IBMX, a phosphodiesterase inhibitor, gave rise to an accumulation of CAMP in oocytes due to basal adenylate cyclase activity (30, 31). This, in turn, resulted in large CAMP-dependent CFTR-mediated Cl⁻ current responses. Challenge with IBMX was used to confirm expression of CFTR. These oocytes did not respond, however, to 100 nM of the metabolically stable prostacyclin analog iloprost (32), PGE₂ or PGD₂, demonstrating the absence of endogenous prostanoid receptors coupled to elevation of CAMP in defolliculated oocytes (Fig. 2B).

When oocytes were co-injected with 1 ng of pcDNAIamp-l116hLXR3 and 2.6 ng of pcDNAIamp-CFTR, challenge with 0.5-100 nM iloprost gave prominent dose-dependent CFTR-mediated Cl⁻ current responses. These responses were specific for iloprost, with no detectable Cl⁻ current observed in response to PGD₂ or PGE₂ at 100 nM (Fig. 2A).

Thus, injection of pcDNAIamp-l116hLXR3 into oocytes resulted in the expression of a functional hIP receptor coupled to stimulation of intracellular CAMP.

Expression of the Human IP Receptor in COS-M6 Cells—[³H]Iloprost binding assays were performed using membranes prepared from COS-M6 cells transfected with pcDNAIamp-1116hLXR3 in order to determine the relative affinities of prostaglandins and related synthetic analogs for the hIP receptor (Fig. 3). The most effective competing ligand was iloprost, which displayed an IC₅₀ value of 4.0 ± 0.14 μM. The related prostacyclin analog carbacyclin was 100-fold less potent, with an IC₅₀ value of 431 ± 71 nM (33). PGE₂ was considerably less effective as a competing ligand, with an IC₅₀ value of approximately 10 μM, while PGD₂, PGF₂, and the thromboxane analog U46619 were essentially inactive in competition for [³H]Iloprost specific binding to the hIP receptor at a concentration of 30 μM.

The rank order of affinity for prostaglandins and related syn-

Fig. 3. Competition for [³H]Iloprost specific binding to pcDNAIamp-1116hLXR3-transfected COS-M6 membranes by prostaglandin-receptor agonists. [³H]Iloprost binding assays were performed as described under "Materials and Methods." The percentage maximum [³H]Iloprost specific binding at each competing ligand concentration was determined for iloprost (▲), carbacyclin (●), PGE₂ (▲), PGF₂α (Ο), PGD₂ (▲), and the TP-receptor agonist U46619 (Ο), over a concentration range up to 100 μM.

Fig. 4. A comparison of prostanoid receptor amino acid sequences. The deduced amino acid sequences of the hIP, hEP₂, hEP₃, hFP, and mEP₂ receptors are shown, aligned using GCG Wisconsin DNA software. Identical amino acids in at least four sequences are boxed. Dots indicate gaps introduced in the sequences for alignment purposes.
The hIP receptor was therefore: iloprost > carbacrylin > PGE2 > PGF2a = PGD2 = U46619, as predicted from pharmacological studies (2, 3). In addition, Scatchard analysis showed that [3H]iloprost specific binding to the hIP receptor confirmed to a two-site model (Affiniti Two-Site saturation software, Beckmann Instruments) with high affinity and low affinity equilibrium dissociation constants of 1 and 44 nM, respectively. Both high and low affinity specific binding sites were saturable, with a maximum number of specific binding sites of 2.0 and 2.3 pmol/mg membrane protein, respectively.

Characteristics of the Human IP Receptor—Comparison of the amino acid sequences for the different prostanoid receptors is presented in Fig. 4. The hIP receptor shares the highest identity (37%) with the mEP3 receptor (16) and the lowest identity (28%) with the hFP receptor (21). The sequence identity in the TMDs of the hIP and the mEP3, both of which couple to elevation in cAMP, increases to 48%, but when hIP is compared with any other prostanoid receptor the identity is never more than 38%. Interestingly, TMD IV is the most conserved between hIP and mEP3 receptors (16 of 22 residues are identical), suggesting a possible role for this TMD in receptor coupling. TMD VII, which has the highest identity among all of the prostanoid receptors, includes 8 amino acids invariant in all the prostanoid receptor family (Fig. 4). Of note is the observation that the hIP receptor has a phenylalanine at position 292 in TMD VII, which in virtually all other G-protein-coupled receptors is a tyrosine (34).

The hIP receptor shares 23 residues (18 of which are in the TMDs) in common with the other prostanoid receptors and another 10 residues (7 of which are in the TMDs) in common with all G-protein coupled receptors. It has been postulated that conserved amino acids within a family may be important in defining its particular functional and structural properties, while those residues conserved throughout the superfAMILY are critical in maintaining the overall structure of the receptor (35). In vitro mutagenesis studies focusing on the invariant residues found in the prostanoid receptor family may help define those residues that play an important role in ligand binding and signal transduction mechanisms.

Tissue Distribution of the Human IP Receptor—Northern blot analysis of the hIP receptor is shown in Fig. 5. The hIP mRNA was approximately 2.4 kb, although less prominent higher molecular weight bands could be seen in some of the lanes, as has been reported for other prostanoid receptors (mouse EP1, EP2, and TP). The hIP mRNA was shown to be most abundantly expressed in kidney, with lower but comparable levels detected in lung and liver. This distribution is consistent with several of the known biological effects of the IP receptor. In the kidney, for example, the glomerulus is the major site of prostaglandin synthesis, and in the human glomerulus PGI2 is the principle prostaglandin synthesized. Within this organ, the hIP receptor is thought to play an important role in renal blood flow, renal release and glomerular filtration rate. PGI2 can also affect vascular tone within the pulmonary circulation (for review, see Ref. 2). hIP mRNA was also detected in the liver, and although its role in this tissue is not known, perhaps the hIP receptor is involved in hepatic circulation. Lower levels of hIP expression could also be seen in skeletal muscle and heart.

In conclusion, we have cloned and expressed a cDNA from a human lung library which encodes a functional prostanoid IP receptor. The hIP receptor has been shown to couple to elevation in intracellular cAMP and the rank order of affinity for prostaglandins and related synthetic analogs in competition for the hIP receptor was as predicted. Further detailed studies at the molecular level, as well as more detailed localization studies, will now be possible in order to help understand the physiological role played by PGI2 through its interaction with the IP receptor.

REFERENCES

Cloning of the Human Prostacyclin IP Receptor

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