Cloning and Expression of a cDNA for Mouse Prostaglandin E Receptor EP₃ Subtype*

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A functional cDNA clone for mouse EP₃ subtype of prostaglandin (PG) E receptor was isolated from a mouse cDNA library using polymerase chain reaction based on the sequence of the human thromboxane A₂ receptor and cross-hybridization screening. The mouse EP₃ receptor consists of 365 amino acid residues with putative seven-transmembrane domains. The sequence revealed significant homology to the human thromboxane A₂ receptor. Ligand binding studies using membranes of COS cells transfected with the cDNA revealed specific [³²P]PGE₂ binding. The binding was displaced with unlabeled PGs in the order of PGE₂ > PGF₂α > PGI₂. The EP₃-selective agonist, M&B 28,767 or GR 63799X, potently competed for the [³²P]PGE₂ binding, but no competition was found with EP₁- or EP₂-selective ligands. PGE₂ and M&B 28,767 decreased forskolin-induced cAMP formation in a concentration-dependent manner in Chinese hamster ovary cells permanently expressing the cDNA. Northern blot analysis demonstrated that the EP₃ mRNA is expressed abundantly in kidney, uterus, and mastocytoma P-815 cells and in a lesser amount in brain, thymus, lung, heart, stomach, and spleen.

Eicosanoids comprising various oxygenated metabolites of arachidonic acid such as prostaglandins (PGs)¹ and leukotrienes exert a variety of biological activities for maintenance of local homeostasis in the body (1, 2). These metabolites act on a cell surface receptor specific for each member to exert their actions. Among them, PGE₂ in particular, produces a broad range of biological actions in diverse tissues. PGE₂ receptors are pharmacologically subdivided into three subtypes, EP₁, EP₂, and EP₃ (3, 4), and these subtypes are suggested to be different in their signal transduction; they are presumed coupled to stimulation of phospholipase C, and stimulation and inhibition of phosphodiesterase, respectively (4-8). Pharmacological actions of these subtypes have been well characterized, and the EP₃ receptor has been suggested to be involved in inhibition of gastric acid secretion (7), modulation of neurotransmitter release in central and peripheral neurons (9), and inhibition of sodium and water reabsorption in kidney tubulus (8, 10, 11). In spite of this information, none of the receptors has been isolated, and their molecular characterization has been carried out only poorly. Recently we cloned a cDNA for the human TXA₂ receptor (12). Based on its sequence we carried out PCR to amplify a homologous sequence from mouse cDNA and, using this fragment as a hybridization probe, performed homology screening. Analysis of nucleotide sequence and expression of the isolated clone revealed that it encodes the mouse EP₃ receptor. We report here the complete nucleotide and deduced amino acid sequences of this receptor, and its ligand binding and biochemical properties analyzed in several mammalian expression systems. This study will be of help in understanding similarity and divergence of eicosanoid receptors.

EXPERIMENTAL PROCEDURES

MATERIALS—M&B 28,767, GR 63799X, butaprost, and SC-19220 were generous gifts from Dr. M. P. L. Caton of Rhone-Poulenc Ltd., Dr. B. M. Bain of Glaxo Group Research Ltd., Dr. P. J. Gardiner of Bayer UK Ltd., and Dr. P. W. Collins of Searle, respectively. [³²P]dCTP (3,000 Ci/rnmol) and [⁵,⁶,¹¹,¹²,¹⁴,¹⁵-³²P]H]PGE₂ (185 Ci/mmol) were obtained from Du Pont-New England Nuclear. PGEs, PGE₂, PGI₂, and PGF₂α were purchased from Funakoshi Pharmaceuticals (Tokyo, Japan). Iloprost and the [¹⁴C]labeled cyclic AMP assay system were obtained from Amer sham Corp. Forskolin and 3- isobutyl-1-methylxanthine were from Sigma. Sources of other materials are shown in the text.

Amplification of a Mouse cDNA Fragment Homologous to the Human TXA₂ Receptor by PCR—First strand cDNA was synthesized from mouse lung total RNA by using random hexanucleotides as primers. PCR primers were designed based on the human TXA₂ receptor cDNA sequences corresponding to the putative third and sixth transmembrane domains of the receptor (12). Mouse lung cDNA served as template in 30 cycles of PCR with 1 min of denaturation at 95 °C, 0.5 min of annealing at 60 °C, and 1.5 min of extension at 72 °C on a Zymatore (Atto Corp., Tokyo, Japan). A single 418-bp fragment was amplified and subcloned into the pluescript SK(+) (Stratagene). A clone isolated (LT3) showed a sequence 78% homologous to the corresponding region of the human cDNA.

Molecular Cloning by Cross-hybridization—Mouse lung cDNA prepared by an oligo(dT) priming method was size-selected (1.8 kb bases) and inserted into the EcoRI site of pZAPII DNA (Stratagene) with EcoRI adaptors (New England Biolabs, Inc.). The 1.9 kb clones derived from the cDNA library were screened by hybridization with LT3. Hybridization was carried out at 58 °C in 6 X SSC (900 mm NaCl and 90 mm sodium citrate) containing 5 X Denhardt's solution (0.1% Yicoll, 0.1% polyvinylpyrrolidone, and 8.1% bovine serum albumin) and 0.5% sodium dodecyl sulfate, and filters were washed at 60 °C in 2 X SSC containing 1% sodium dodecyl sulfate. Among several clones hybridizing positively to LT3, we picked up one showing a signal apparently weaker than others and subjected it to further screening. Nucleotide sequencing of the isolated clone (ML64) revealed that it was a partial clone. Using this clone as a hybridization probe, we then screened the cDNA library of mouse mastocytoma P-815 cells for a full-length clone. From 7.2 X 10⁶ clones

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¹The abbreviations used are: PG, prostaglandin; G protein, heterotrimeric GTP-binding protein; IC₅₀, drug concentration to inhibit response by 50%; PCR, polymerase chain reaction; TX, thromboxane; CHO, Chinese hamster ovary.
of the P-815 xZAPII library, nine clones were isolated and subjected to sequence analysis. Nucleotide sequencing was carried out on double-stranded templates using the dideoxy chain termination method. One clone (MP660) was a full-length clone with a 1,095-base pair open reading frame.

cDNA Expression in COS-1 Cells and Binding Assay—The EcoRI insert of MP660 was subcloned into pCDNA (Invitrogen), a modified eukaryotic vector, and the resultant plasmid DNA was transfected into COS-1 cells by the DEAE-dextran method (13). After culture for 72 h, the cells were harvested, and crude membranes were prepared as described (12). Using these membranes, [3H]PGE2 binding was determined, and protein was measured as described previously (14).

Stable Expression of the Cloned Receptor and cAMP Assay—DNA transfection and cell line establishment were performed essentially as described by Nakajima et al. (15). The EcoRI fragment of MP660 was inserted into pcKCR-dhr, a eukaryotic expression vector containing a mouse gene as a selection marker (16). The plasmids were transfected into CHO cells deficient in dihydrofolate reductase activity as described by Nakajima et al. (12). Using these membranes, [3H]PGE2 binding was determined, and protein was measured as described previously (14). The polypeptide consists of 365 amino acid residues with an estimated molecular weight of 40,077. The hydropathicity profile determined by the Kyte and Doolittle method (22) and the sequence homology analysis indicated that it possesses seven hydrophobic and short segments and shares a significant sequence similarity with other members of G-protein-coupled receptors (23), especially with the human TXA2 receptor (12) (38% in total). Similar to other G protein-coupled receptors (24, 25), potential N-glycosylation sites are extracellular regions; stars, potential phosphorylation sites by cAMP-dependent protein kinase.

![Fig. 1. Nucleotide and deduced amino acid sequences of MP660](attachment:image.png)

The deduced amino acid sequence shown above the nucleotide sequence using single-letter code. Positions of the putative transmembrane segments I-VII are indicated by overlines above the nucleotide sequence. The termini of the open reading frame are shown above the nucleotide sequence.

**RESULTS AND DISCUSSION**

Fig. 1 shows nucleotide and deduced amino acid sequences of MP660. The amino acid sequence was assigned from the longest open reading frame of the cDNA. The nucleotide sequence surrounding the initiation codon agrees reasonably well with the consensus sequence (21). The polypeptide consists of 365 amino acid residues with an estimated molecular weight of 40,077. The hydropathicity profile determined by the Kyte and Doolittle method (22) and the sequence homology analysis indicated that it possesses seven hydrophobic segments and shares a significant sequence similarity with other members of G-protein-coupled receptors (23), especially with the human TXA2 receptor (12) (38% in total). Similar to other G protein-coupled receptors (24, 25), potential N-glycosylation sites are extracellular regions; stars, potential phosphorylation sites by cAMP-dependent protein kinase.
PGIz analogue previously characterized in various tissues (26, 27). Because transfected COS cell membranes. Specificity of this binding is shown in Fig. 2a. The binding of \[^{3H}\]PGEz was inhibited by unlabeled PGs in the order of PGE,; 0, PGE1,; and M&B 28,767, specifically competed for the \[^{3H}\]PGEz binding with equal potency, and they were more potent than PGEz itself. On the other hand, no competition was found at all with either an EPz-specific antagonist, SC-19220, or an EP2-specific agonist, butaprost. [\(^{3H}\]PGEz did not bind to membranes of untransfected cells. These results established that MP660 encodes the EPz subtype of PGE receptor.

Possible association of the EPz receptor with inhibition of adenylate cyclase has been indicated (4). We tested this possibility by permanently expressing the cDNA in CHO cells and examining response of the cells to PGE analogues. As shown in Fig. 3, the transfected CHO cells showed a dose-dependent decrease to PGEz in forskolin-induced cellular cAMP accumulation. M&B 28,767, an EPz-specific agonist, also inhibited forskolin-induced cAMP synthesis and was more potent than PGEz (IC50 of M&B 28,767 = 1 \times 10^{-12} M; IC50 of PGEz = 1 \times 10^{-10} M). This potency of PGEz correlates well with that found in canine kidney and rat uterine membranes (11, 28). Either agonist alone did not increase cAMP accumulation. We also examined phosphatidylinositol turnover in the transfected CHO cells. Addition of up to 1 \mu M M&B 28,767 revealed no significant increase in inositol phosphate content over the basal levels (data not shown). These results demonstrated that the EPz receptor is coupled exclusively to inhibition of adenylate cyclase.

The mouse EPz and human TXAz receptors (12) are significantly similar in size and show highly homologous amino acid sequences, especially in the putative seven-transmembrane segments except segments I and V (Fig. 4). The most highly conserved regions are segment VII and that spanning the latter half of segment IV to the first 12 amino acids in the second extracellular loop (from Leu-165 to Phe-184), 63.6 and 80.0% homology, respectively. As in the human TXAz receptor, there is no Asp in the third transmembrane segment of the EPz, a residue which is presumed to bind the amino group of ligands in the adrenergic receptors (29). Furthermore, Arg-309 in the EPz is equivalent to Arg-295 in the TXAz receptor, which are located at the position analogous to Lys-296 of bovine rhodopsin in the seventh transmembrane segment. The latter amino acid residue was assigned for retinal attachment in the rhodopsin molecule (30). These structural features may reflect the acidic nature of the ligand for the prostaglandin receptors. EPz receptor has the two potential phosphorylation sites by cAMP-dependent protein kinase (31) in the latter half of segment IV to the first 12 amino acids in the second extracellular loop (from Leu-165 to Phe-184), 63.6 and 80.0% homology, respectively. As in the human TXAz receptor, there is no Asp in the third transmembrane segment of the EPz, a residue which is presumed to bind the amino group of ligands in the adrenergic receptors (29). Furthermore, Arg-309 in the EPz is equivalent to Arg-295 in the TXAz receptor, which are located at the position analogous to Lys-296 of bovine rhodopsin in the seventh transmembrane segment. The latter amino acid residue was assigned for retinal attachment in the rhodopsin molecule (30). These structural features may reflect the acidic nature of the ligand for the prostaglandin receptors. EPz receptor has the two potential phosphorylation sites by cAMP-dependent protein kinase (31) in the first cytoplasmic loop, which may be relevant to the finding that \[^{3H}\]PGEz binding is affected by cAMP-dependent phosphorylation in brain membranes (32).

Poly (A) RNAS were prepared from various mouse tissues and hybridized with the EcoRI/BamHI fragment of MP660 (Fig. 5). A positive band was seen at 2.3 kilobases in a number of tissues in which PGEz has pharmacological effects and/or specific binding sites (4). Another hybridizing band was detected at an estimated mRNA size of 7.0 kilonucleotides in kidney, uterus, brain, and mastocytoma P-815 cells. Identity of this latter band is not known at present. The tissue most highly expressing EP1 mRNA was kidney in which PGEz exerts an inhibitory effect on sodium and water reabsorption by inhibiting adenylate cyclase via G, (8, 33). A significant band was also observed in stomach, suggesting that the receptor we cloned is indeed involved in inhibition of histamine-induced gastric acid secretion in this tissue (7).

\begin{figure}
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Binding of \[^{3H}\]PGEz to MP660-transfected COS-1 cell membranes. a, displacement of \[^{3H}\]PGEz binding by various prostaglandins. Unlabeled prostaglandins were added to the binding assay mixture at indicated concentrations, and \[^{3H}\]PGEz binding was determined as described under "Experimental Procedures." O, PGEz; •, PGE1; △, iloprost; A, PGF2α, Δ, PGD2, b, displacement of \[^{3H}\]PGEz binding by subtype-selective PGE analogues. Ligands used are M&B 28,767 (△), GR 63795X (○), butaprost (△), and SC-19220 (○).}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Inhibition by PGEz and EP2-selective agonist of forskolin-induced cAMP accumulation in CHO cells permanently expressing the EPz receptor. CHO cells permanently expressing the EPz receptor were incubated with 1 \mu M forskolin in the presence of the indicated concentrations of PGEz (○) or M&B 28,767 (△), and cAMP accumulation was determined as described (10). The incubation buffer contained 1 mM 3-isobutyl-1-methylxanthine. Each point represents the mean ± S.E. of triplicate determinations.}
\end{figure}
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Fig. 4. Comparison of the amino acid sequences of the mouse EP₃ receptor and the human TXA₂ receptor. The amino acid sequences of the mouse EP₃ (upper) and the human TXA₂ (lower) are aligned to achieve the maximal homology. Asterisks indicate exact matches between the two sequences. Hyphens show deletions of the amino acid residues when compared between the two sequences.

Fig. 5. Northern blot analysis of RNAs isolated from various mouse tissues and P-815 cells. Poly(A)⁺ RNAs were isolated from the tissues listed below and a cell line, and 30 µg of RNA was applied in each lane except that 5 µg was used for P-815 cells. Hybridization analysis was carried out using the 1,072-base pair 1234567891011 12

![Image of a northern blot analysis](image-url)

The exact function of this receptor in this tissue is again not

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