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FEBS Letters 338 (1994) 170–174

FEBS 13596

FEBS LETTERS

Cloning and expression of three isoforms of the human EP_3 prostanoid receptor

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Received 10 December 1993; revised version received 20 December 1993

Abstract

Functional cDNA clones coding for three isoforms of the human prostaglandin E receptor EP_3 subtype have been isolated from kidney and uterus cDNA libraries. The three isoforms, designated $hEP_{3\text{-}I}$, $hEP_{3\text{-}II}$ and $hEP_{3\text{-}III}$, have open reading frames corresponding to 390, 388 and 365 amino acids, respectively. They differ only in the length and amino acid composition of their carboxy-terminal regions, beginning at position 360. The human EP_3 receptor has seven predicted transmembrane spanning domains and therefore belongs to the G-protein-coupled receptor family. The rank order of potency for prostaglandins and related analogs in competition for [^{3}H]PGE_2 specific binding to membranes prepared from transfected COS cells was comparable for all three isoforms, and as predicted for the EP_3 receptor, with $PGE_2 = PGE_1 >> PGF_{2\alpha} =$ iloprost $> PGD_2 >>$ U46619. In addition, the EP_3-selective agonist MB28767 was a potent competing ligand with an IC_{50} value of 0.3 nM, whereas the EP_1-selective antagonist AH6909 gave IC_{50} values of 2–7 μM and the EP_2-selective agonist butaprost was inactive. In summary, we have cloned three isoforms of the human EP_3 receptor having comparable ligand binding properties.

Key words: Prostaglandin E_2; Prostanoid receptor; EP_3 cDNA; Receptor binding

1. Introduction

The physiological actions of prostaglandin E_2 (PGE_2) are mediated through its interaction with specific G-protein-coupled EP** receptors [1,2]. There are three subtypes of the EP receptor, designated EP_1, EP_2 and EP_3, which have been identified on the basis of their different pharmacological profiles and signal transduction pathways. Thus, activation of the EP_1, EP_2 and EP_3 subtypes results in elevation of intracellular calcium and stimulation and inhibition of adenylate cyclase, respectively. The cloning of the three receptor subtypes has now been reported. The sequences of the mouse and human EP_1 receptors [3,4], the mouse and human EP_2 receptors [5,6] and the mouse, rat and bovine EP_3 receptors [7–11] are all known. In addition, several different isoforms of the EP_3 subtype have also been identified, $EP_{3\alpha}$, $EP_{3\beta}$ and $EP_{3\gamma}$ in the mouse [8,9] and $EP_{3A\text{–}D}$ in the cow [11]. These isoforms are produced by alternative splicing and differ only in the length and amino acid composition of their carboxyl-terminal regions. Most importantly, while the EP_3 receptor isoforms have comparable ligand binding affinities, they have been shown to couple to several signal transduction pathways within the same cell type, including those coupled to both elevation and decrease in cAMP [11]. In this report we describe the cloning, expression and ligand binding properties of three different isoforms of the human (h) EP_3 receptor.

2. Materials and methods

2.1. Screening a human kidney cDNA library

Mouse (m) $EP_{3\alpha}$ receptor [7] cDNA fragments were isolated by polymerase chain reaction (PCR) from reverse-transcribed kidney poly(A)$^+$RNA (Clontech, Palo Alto, CA) using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). Briefly, 25 pmol of upstream primer A 5′-CCACCATGGCTAGCATGTGGGCGCC-3′ and downstream primer B 5′-CTCCAC-GGCCATGGCCGCTGGCCAC-C-3′ or upstream primer A′ 5′-CCAGCGCCATGGCCGTGGAGC-GCGCCC-3′ and downstream primer B′ 5′-GCATCATCTTTCCAG-CTGGTCACTCC-3′, synthesized on a Model 380A DNA synthesizer (Applied Biosystems, Foster City, CA), were added together with 1 μg cDNA, dNTP (200 μM) and *Taq* polymerase (2.5 unit) in a 100 μl reaction volume (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$) for amplification at 95°C/60 s, 60°C/60 s; for 35 cycles in a Perkin Elmer Cetus thermal cycler. The 398 bp A/B product (5′ probe) (nucle-

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** *Footnote.* Prostanoid receptors designated following the recommendation of the IUPHAR Commission on Receptor Nomenclature and Classification [18].

† This manuscript is dedicated to the memory of Gretchen Müller who passed away in 1993.

SSDI 0014-5793(93)E1513-L

otides −5 to 393 [7]) and the 728 bp A′/B′ product (3′ probe) (nucleotides 374 to 1,101 [7]) were isolated using agarose gel electrophoresis followed by Magic PCR purification (Promega, Madison, WI).

A human kidney λgt11 cDNA library (Clontech, Palo Alto, CA) was screened with the ^{32}P-labeled $mEP_{3\alpha}$ receptor 5′ and 3′ probes added together under standard conditions (50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.1% SDS, 100 μg/ml sonicated salmon sperm DNA) at 42°C overnight. Filters were first washed briefly at room temperature with 2 × SSC/0.1% SDS followed by more stringent washing (2 × 30 min) at 50°C with 0.1 × SSC/0.1% SDS. Thirty positive phage clones were obtained of which five hybridized strongly with both the 5′ and 3′ probes. Phage DNA from the positive clones was prepared by the plate lysate method [12].

Clone λhk$EP_3$6–1 was digested with *Eco*RI and was found to contain one insert of 1.8 kb. The 1.8 kb *Eco*RI fragment (hk$EP_3$6–1, designated $hEP_{3\text{-}I}$) was subcloned into the Bluescript vector KS (Stratagene, La Jolla, CA)) and sequenced on both strands using the KS and SK primers, or primers generated from the determined sequences.

2.2. Screening a human uterus cDNA library

An alternative strategy was also used to screen for the hEP_3 and related prostanoid receptors. An antisense 16-fold degenerate 27mer oligonucleotide [13], designated oligo VII(−) [5′-ATA(A,C)ACCCAG-GG(A,G)TCCA(A,G)GATCTG(G,A)TT-3′], based on the 9 conserved amino acids (NQILDPWVY) in transmembrane domain (TMD) VII of the TP [14], EP_1 [3,4] and EP_3 [7–11] receptors, was synthesized as described above. The ^{32}P-labeled oligo VII(−) probe was used to screen a human uterus λgt10 cDNA library (Clontech, Palo Alto, CA) using standard techniques [12]. Briefly, nitrocellulose filters (Schleicher and Schuell, Keene, NH) were prehybridized in 6 × SSC, 2 × Denhardt's solution, 0.2% SDS, 100 μg/ml denatured salmon sperm DNA, at 45°C, and then hybridized in the same solution containing the ^{32}P-labeled probe, at 42°C, overnight. Filters were washed briefly at room temperature with 6 × SSC followed by washing (2 × 30 min) at 45°C. Twenty positive phage clones were obtained and of these 16 hybridized with the mouse EP_3 cDNA probe. Positive phage clones were plaque purified and DNA was prepared by the plate lysate method [12]. Clone λhuEP_3- 21 and λhuEP_3-9, which hybridized with both probes, were digested with *Eco*RI and were found to contain inserts of approximately 1.7 kb and 1.4 kb, respectively. The *Eco*RI fragments (huEP_3-21 and huEP_3-9 and designated $EP_{3\text{-}II}$ and $EP_{3\text{-}III}$) were subcloned into the Bluescript vector SK (Stratagene, La Jolla, CA)) and sequenced as described above.

2.3. Construction of the pcDNAIamp-hEP$_3$ expression vectors

The plasmid pKS-hk$EP_{3\text{-}I}$ was digested with *Hind*III and a 1.7 kb hk$EP_{3\text{-}I}$ cDNA fragment was agarose gel purified. The 1.7 kb *Hind*III cDNA (hk$EP_{3\text{-}I}$) was subsequently subcloned into the *Hind*III site of pcDNAIamp (Invitrogen, San Diego, CA) and the correct orientation was verified by *Eco*RI digestion. The plasmids pSK-hu$EP_{3\text{-}II}$ and pSK-hu$EP_{3\text{-}III}$ were digested with *Eco*RI and the I.7 and 1.4 cDNA fragments were gel purified. They were both subsequently subcloned into the *Eco*RI site of pcDNAIamp and the correct orientations were verified by *Pst*I digestion.

2.4. Expression of the human EP$_3$ receptor isoforms in COS-M6 cells and [^{3}H]PGE$_2$ binding assays

The three isoforms of the hEP_3 receptor ($hEP_{3\text{-}I}$, $hEP_{3\text{-}II}$ and $hEP_{3\text{-}III}$) cDNAs in the pcDNAIamp plasmid (5 μg) were individually transfected into COS-M6 cells using the DEAE-dextran method with chloroquin [15]. The cells were maintained in culture for 72 h, harvested, subjected to lysis by nitrogen cavitation and then membranes were prepared by differential centrifugation (1,000 × *g* for 10 min, then 100,000 × *g* for 30 min). [^{3}H]PGE_2 binding assays were performed in 10 mM potassium phosphate (pH 6.0), containing 10 mM $MgCl_2$, 1 mM EDTA, 0.5 nM [^{3}H]PGE_2 (154 Ci/mmol; Du Pont-New England Nuclear) and 0.5–3 μg of protein from the 100,000 × *g* membrane fraction. Incubations were conducted for 1 h at room temperature prior to separation of the bound and free radioligand by rapid filtration as previously described [16]. Residual [^{3}H]PGE_2 bound to the filter was quantified by liquid scintillation counting. Specific binding was defined as the difference between total binding and non-specific binding, determined in the presence of 1 μM PGE_2.

3. Results and discussion

We have used PCR generated mEP_3 [7] cDNA probes to clone a hEP_3 prostanoid receptor from a kidney cDNA library. In addition, a degenerate oligonucleotide probe, based on the nine conserved amino acids in TMD VII of prostanoid receptors [3,4,7–11,13,14], was used to identify from a uterus cDNA library two additional hEP_3 receptors, which differed only at their carboxy-terminal tails (Fig. 1). Hydropathicity analysis (by the Kyte and Dolittle method [17]) predicted the seven putative TMDs characteristic of G-protein-coupled receptors.

The three isoforms of the hEP_3 receptor, designated $hP_{3\text{-}I}$, $hEP_{3\text{-}II}$ and $hEP_{3\text{-}III}$, code for proteins containing 390, 388 and 365 amino acids with calculated molecular masses of 43,315, 42,688 and 40,507, respectively. All clones contain the same 5′-untranslated and coding sequences until and including the glutamine residue (amino acid 359) located 10 amino acids from the end of TMD VII, after which the sequences diverge. This has also been found for the the mouse [8,9] and bovine [10] EP_3 prostanoid receptors. For the mouse, three isoforms have been described, $EP_{3\alpha}$, $EP_{3\beta}$ and $EP_{3\gamma}$ while for the bovine four isoforms are known, $EP_{3A\text{–}D}$. $hEP_{3\text{-}I}$ is the homologue of the $mEP_{3\alpha}$, $hEP_{3\text{-}II}$ is the homologue of the mouse $EP_{3\gamma}$ and bovine EP_{3D} while $hEP_{3\text{-}III}$ is a new isoform. The sequences of $hEP_{3\text{-}I}$, $mEP_{3\alpha}$, rat EP_3 and bovine EP_{3D} receptors have been compared in Fig. 2. The hEP_3 cDNAs share approximately 85% amino acid identity with the mouse [7–9], rat [10] and bovine [11] EP_3 receptors. Differences between the sequences occur mainly outside of the seven TMDs.

The three isoforms of the hEP_3 receptor subtype were analysed by [^{3}H]PGE_2 binding assays performed with membranes prepared from COS cells transfected with either $hEP_{3\text{-}I}$, $hEP_{3\text{-}II}$ or $hEP_{3\text{-}III}$ cDNA. The specific binding of [^{3}H]PGE_2 was of high affinity and saturable in each case. The equilibrium dissociation constant (K_d) values were comparable at 0.7 nM , 0.8 nM and 0.9 nM for $hEP_{3\text{-}I}$, $hEP_{3\text{-}II}$ and $hEP_{3\text{-}III}$, respectively. High expression levels were achieved in all cases with an estimated maximum number of specific [^{3}H]PGE_2 binding sites (B_{max}) of 14, 5 and 20 pmol/mg of membrane protein for $hEP_{3\text{-}I}$, $hEP_{3\text{-}II}$ and $hEP_{3\text{-}III}$, respectively. There was no [^{3}H]PGE_2 specific binding detectable in binding assays using membranes prepared from non-transfected COS-M6 cells or COS-M6 cells transfected with the pcDNAIamp vector alone.

In competition binding assays PGE_2 and PGE_1 were equipotent in inhibiting [^{3}H]PGE_2 specific binding to all three isoforms with IC_{50} values of approximately 1 nM (Fig. 3 and Table 1). The rank order of affinities for prostaglandins and related analogs was comparable for all three isoforms, with PGE_2 = PGE_1 >> $PGF_{2\alpha}$ = iloprost > PGD_2 >> U46619. In general, all the competing ligands tested displayed the highest affinity for the

Fig. 1. Nucleotide and deduced amino acid sequences of the prostanoid EP_{3-I} to $_{III}$ receptor cDNAs. The start of each cDNA is indicated and the common nucleotide sequence (−195 to 1,077) is shown, followed by specific sequences for each clone. The deduced amino acid sequence (in three letter code) is shown below the nucleotide sequence. The positions of the putative transmembrane domains I–VII (based on the hydropathicity profile) are indicated by overlines above the nucleotide sequence. Asterisks denote potential *N*-glycosylation sites and crosses denote potential protein kinase C phosphorylation sites. An in-frame stop codon, TAG, in the 5′-untranslated region is underlined.

hEP_{3-III} isoform and were 2–5-fold less active at the hEP_{3-I} and hEP_{3-II} isoforms (Table 1). Several EP-subtype selective ligands were also evaluated. The EP_3-selective agonist MB28767 displayed high affinity for all three EP_3 isoforms with IC_{50} values of 0.3 nM (Fig. 3 and Table 1). In contrast, the EP_1-selective antagonist AH6809 was 5–10-fold less active at the hEP_3 subtype, as compared with the hEP_1 subtype, with IC_{50} values ranging from 2 to7 μM against hEP_3 as compared with 0.5 μM against hEP_1 [4]. Finally, the EP_2-selective ag-

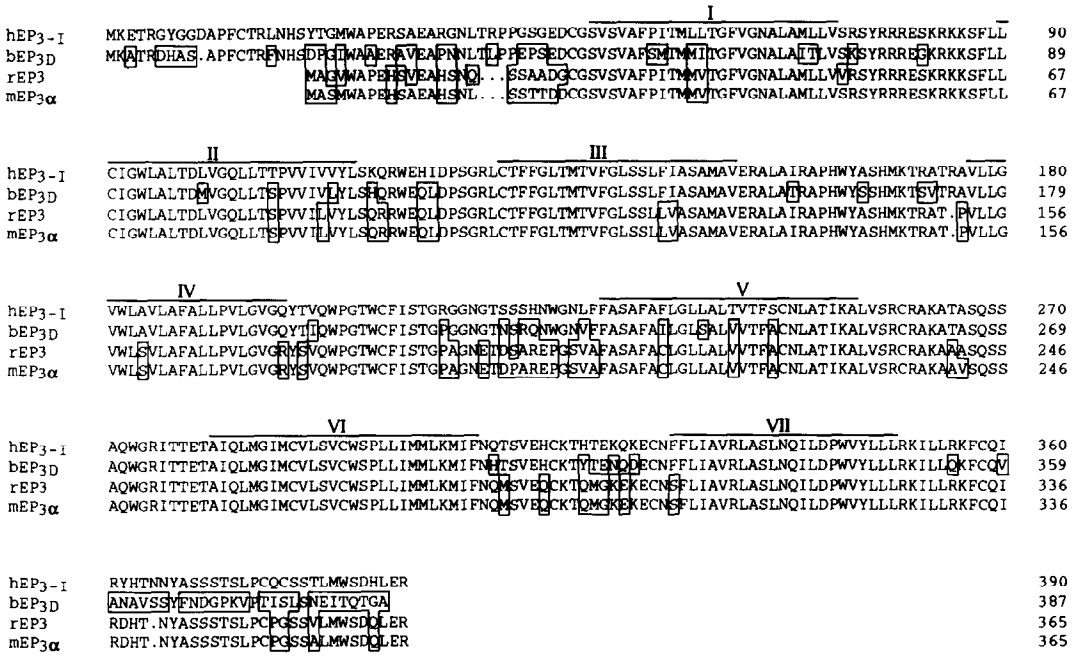
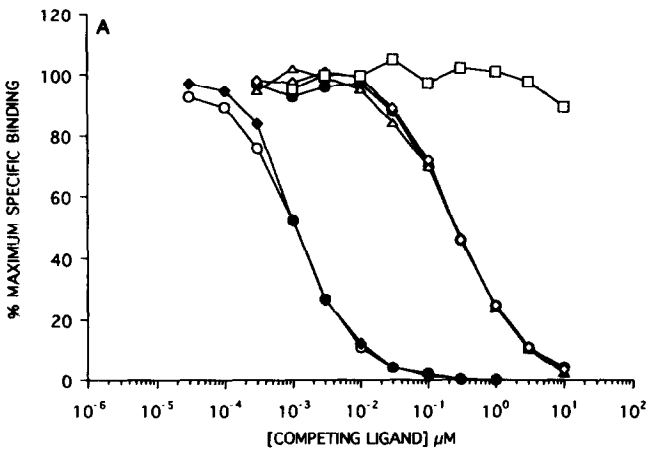
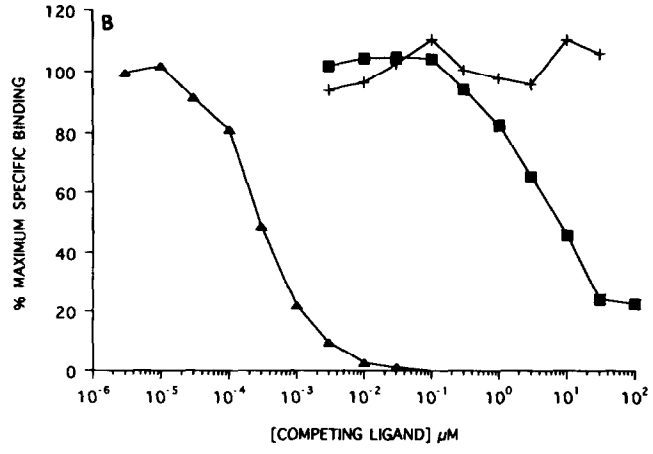


Fig. 2. A comparison of EP_3 receptor amino acid sequences from different species. The deduced amino acid sequences, shown in single letter code, of the $hEP_{3\text{-}I}$, bovine (b) EP_{3D}, rat (r) EP_3 and $mEP_{3\alpha}$ receptors are shown, aligned to optimize homology using a computer program. The boxed residues indicate positions where $hEP_{3\text{-}I}$ differs from the sequences from the other species. Dots indicate gaps introduced in the sequences for alignment purposes.





onist butaprost was inactive up to a concentration of 30 μM. These radioligand binding data demonstrate that the $hEP_{3\text{-}I}$, $hEP_{3\text{-}II}$ and $hEP_{3\text{-}III}$ isoforms all have the ligand binding characteristics predicted for the EP_3 receptor subtype.

In conclusion, we have cloned and expressed three isoforms of the human EP_3 prostanoid receptor. Furthermore, we have shown that these isoforms have comparable affinities for prostaglandins, related analogs and subtype-selective ligands. The cloning of the human EP_3 receptor isoforms will now allow for the elucidation of their signal transduction pathways, distribution and their role in biological processes, both in physiological and disease states.

Acknowledgements: We would like to thank Michel Belley in the Medicinal Chemistry Department of the Merck Frosst Centre for Therapeutic Research for the synthesis of MB 28767.

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Fig. 3. Competition for $[^3H]PGE_2$ specific binding to membranes from pcDNAIamp-$hEP_{3\text{-}I}$ transfected COS-M6 cells. $[^3H]PGE_2$ binding assays were performed as described in Section 2 in the presence of Panel A: 0.03 nM–10 μM PGE_2 (♦), PGE_1 (○), $PGF_{2\alpha}$ (●), iloprost (◇), PGD_2 (△) and U46619 (□) and Panel B: 0.3 nM-100 μM MB28767 (▲), AH6809 (■) and butaprost (+). Butaprost and AH 6809 were generous gifts from Miles Inc and Glaxo Group Research Ltd, respectively.

Table 1
Competition for [3H]PGE_2 specific binding to membranes from pcDNAlamp-$hEP_{3\text{-}I}$, pcDNAlamp-$hEP_{3\text{-}II}$ and pcDNAlamp-$hEP_{3\text{-}III}$ transfected COS-M6 cells

Competing ligand	IC_{50} (nM)		
	$EP_{3\text{-}I}$	$EP_{3\text{-}II}$	$EP_{3\text{-}III}$
Prostaglandins			
PGE_1	1.1	1.0	0.9
PGE_2	1.1	1.1	1.1
$PGF_{2\alpha}$	271	170	72
Iloprost	265	171	87
PGD_2	2,621	1,903	555
U46619	>10,000	>10,000	>10,000
'Selective' Ligands			
MB28767	0.3	0.3	0.3
AH6809	6,278	4,553	2,025
Butaprost	>30,000	>30,000	>30,000

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