Molecular cloning and expression of human EP₃ receptors: evidence of three variants with differing carboxyl termini

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1 The polymerase chain reaction (PCR) was used in combination with plaque hybridization analysis to clone four variants of the EP₃ prostaglandin receptor from a human small intestine cDNA library.

2 Three of these variants, i.e. the EP_{3A}, EP_{3E} and EP_{3D}, share the same primary amino acid sequence except for their carboxyl termini, which diverge from one another at the same point, approximately 10 amino acids away from the end of the seventh membrane spanning domain of the receptor. The fourth variant (EP_{3AI}) has a nucleotide coding sequence identical to EP_{3A} but has a completely different 3' untranslated sequence.

3 The carboxyl termini of the three isoforms differ most obviously in length with the EP_{3A} being the longest (41 amino acids) and the EP_{3E} being the shortest (16 amino acids). They also differ in content with the EP_{3A} containing 9 serine and threonines in its carboxyl terminus and the EP_{3E} none.

4 Transient expression in eukaryotic cells showed that the human EP₃ receptor variants had similar but not identical radioligand binding properties and differed in their functional coupling to second messenger pathways. Up to 3 pmol mg⁻¹ protein of [³H]-prostaglandin E₂ binding could be obtained with more than 95% specific binding. Using a reporter gene assay, as a measure of intracellular cyclic AMP levels, the EP_{3A} coupled more efficiently to the inhibition of adenylyl cyclase than did the EP_{3E}.

5 PCR was used to confirm the presence of mRNAs encoding the four human EP₃ receptor variants in tissues of the human small intestine, heart and pancreas. These findings indicate that the EP₃ receptor variants identified here are likely to be expressed in tissues. The differences in the carboxyl termini at the protein level, and in the 3' untranslated regions at the mRNA level, could be profound in terms of the regulation and functional coupling of these receptor isoforms.

Keywords: Prostaglandin; small intestine; cDNA; G-protein coupled; adenylyl cyclase; cyclic AMP inhibition; CAT assays

Introduction

The prostaglandins are locally acting hormones derived from the metabolism of arachidonic acid by prostaglandin endoperoxide synthase (cyclo-oxygenase). They are very widely distributed in animal tissues where they act on cell surface receptors to produce a diverse range of physiological effects. The prostaglandin receptors elicit cellular responses by activating guanine nucleotide regulatory proteins (G-proteins). Like other members of the superfamily of G-protein coupled receptors their molecular structure is dominated by seven hydrophobic regions that appear to represent membrane-spanning α -helices.

The prostanoid receptor family is comprised of several members according to the current classification (Coleman *et al.*, 1982). Separate receptors for prostaglandin D₂ (PGD₂), PGE₂, PGF_{2 α} , PGI₂ and thromboxane A₂ were originally proposed based largely on agonist studies: these receptors were respectively defined as DP, EP, FP, IP and TP. This classification received further support by the development of TP (Coleman *et al.*, 1982) and DP receptor (Giles *et al.*, 1989) antagonists. A further subdivision of EP receptors into 3 subtypes (EP₁, EP₂ and EP₃) was proposed based on functional studies and by the development of an EP receptor antagonist (Coleman *et al.*, 1982; 1987). Similar heterogeneity of the other prostanoid receptors appears likely. Thus, subdivisions within the TP (Masuda *et al.*, 1991; Furci *et al.*, 1992) and DP receptors (Woodward *et al.*, 1993) have been suggested.

The advent of a more detailed classification of the prostaglandin receptors came with the cloning of a human TP receptor (Hirata *et al.*, 1991). Its deduced amino acid sequence confirmed that the TP receptors are in the superfamily of G-protein coupled receptors and provided the basis for the subsequent cloning of the mouse EP₂ (Honda *et al.*, 1993) and EP₃ receptors (Sugimoto *et al.*, 1992). The subsequent cloning of two isoforms of the mouse EP₃ receptor revealed heterogeneity that was not anticipated by previous functional studies (Sugimoto *et al.*, 1993). With respect to their deduced amino acid sequences, the two isoforms, EP_{3 α} and EP_{3 β} , differ only in the sequence of their carboxyl-terminal domains, this difference affecting approximately 30 amino acids changes both the interaction of these receptor isoforms with G-proteins and their patterns of agonist-promoted desensitization (Negishi *et al.*, 1993).

An important benefit of having a cloned receptor is the ability to study its pharmacology in the absence of potentially interfering receptors by the use of heterologous expression. Such types of studies have revealed significant species differences in the pharmacology of cloned rodent G-protein coupled receptors as compared with their human homologues. It has been found that a change in just one amino acid is sufficient to cause differences. For example, the rat 5-HT_{1B} and human 5-HT_{1D} receptors are actually biochemical homologues but a ³⁵⁵T→N mutation in transmembrane (TM) 7 of the human 5-HT_{1D} changes its pharmacology to the extent that these receptors came to be defined as different subtypes (Oksenberg *et al.*, 1992). Similarly, a ²⁰¹S→C mutation in TM5 of the mouse α_{2A} adrenoceptor dramatically

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increases its affinity for the antagonist yohimbine relative to the human α_{2A} (Link *et al.*, 1992). This is significant because [3 H]-yohimbine is one of the main radioligands for the characterization of α_2 -adrenoceptors. To improve our understanding of the pharmacology of the human EP₃ receptors we sought to clone and express these receptors. Using the polymerase chain reaction (PCR) and homology-based screening we found four human variants of this receptor. Three of the variants represent changes at the protein level and one at the mRNA level. One of the variants corresponds to the mouse EP_{3a} but the other three do not correspond to any of the previously described mouse EP₃ receptors.

Methods

Reverse transcription/PCR

A sense primer was designed from nucleotides (nt's) 301–326 of the published mouse EP₃ receptor sequence (Sugimoto *et al.*, 1992) and corresponds to the proposed first extracellular loop of the receptor. Its sequence is:

5'GA(T/C)CC GTCG(T/G) GICGI CTITG (C/T)(C/A)(C/G)ITT^{3'}

An antisense primer was designed from nucleotides 775–797 of the published mouse EP₃ receptor sequence (Sugimoto *et al.*, 1992) and corresponds to the proposed sixth transmembrane domain of the receptor. Its sequence is:

5'AC(A/G)CA CATIA T(A/T/G/C)CCC AT(A/T/G/C)A(A/G)
(T/C)TG^{3'}

The sense and antisense primers were used in a combined reverse transcription/PCR reaction with total RNA from human small intestine (Clontech). The reverse transcription reaction (final volume, 140 μ l) contained 14 μ l 10 $\times$ PCR buffer (Perkin Elmer), 3.5 μ l RNasin (Boehringer Mannheim, 40 u μ l⁻¹), 35 μ l dNTP's (5 mM), 7 μ l random primers (1 mg ml⁻¹), 14 μ l RNA (1 μ g μ l⁻¹) and 7 μ l AMV reverse transcriptase (Boehringer Mannheim, 25 u μ l⁻¹). The reaction was incubated at room temperature ($\sim$ 24°C) for 10 min, 42°C for 1 h, 95°C for 5 min and was placed on ice. The PCR reaction (final volume, 50 μ l) contained 20 μ l from the reverse transcription reaction, 3 μ l 10 $\times$ PCR buffer, 5 μ l dimethylsulphoxide (DMSO), 5 μ l sense primer (20 μ M), 5 μ l antisense primer (20 μ M) and 1 μ l *Taq* polymerase (Perkin Elmer, 2.5 u μ l⁻¹). The following programme was used for the PCR: 94°C, 3 min; 50°C, 2 min; 72°C, 3 min; followed by 59 cycles of 94°C, 1 min; 50°C, 2 min; 72°C, 3 min; and a final cycle of 72°C, 15 min; 4°C, 12 h.

Agarose gel electrophoresis of a sample (15 μ l) of the PCR reaction showed a single product of $\sim$ 500 base pairs (bp). The product was purified (Geneclean, Bio101 Inc.) and was cloned into pBluescript-II KS(+) (Stratagene) using a G/C tailing reaction according to the manufacturer's instructions (Boehringer Mannheim). Briefly, terminal transferase was used to add a poly-dC extension on the purified PCR product and a poly-dG extension on PstI digested pBluescript. The samples were annealed and were used to transform *E. coli* DH10B cells by electroporation. A positive clone (B₁) was obtained and the nucleotide sequence of the PCR product/insert was determined using Δ Taq-PCR sequencing according to the manufacturer's instructions (United States Biochemicals). The sequence of clone B₁ shared $\sim$ 80% identity with the mouse EP₃ receptor and was consistent with its being a human homologue.

Isolation of a genomic clone

A human genomic library in λ FixII (Stratagene) was screened by plaque hybridization analysis (Sambrook *et al.*, 1989) using a 32 P-labelled probe derived from clone B₁. Nitrocellulose filters were used to take impressions of 440,000 recombinants ($\sim$ 20,000 recombinants/filter) which were

denatured, neutralized and baked for 2 h at 80°C. The filters were prehybridized for 2 h at 37°C in 50% formamide/1 M NaCl/1% SDS/100 μ g ml⁻¹ herring sperm DNA and then hybridized with the probe overnight at 37°C in the same solution. The probe was labelled by nick-translation (Gibco/BRL) and was used at a concentration of $\sim$ 5 $\times$ 10⁶ d.p.m. ml⁻¹. The filters were washed for 1 h at 37°C in 2 $\times$ SSC/0.1% SDS and then for 1 h at 53°C in 0.1 $\times$ SSC/0.1% SDS. Autoradiographs were obtained and a positive clone was identified (EP₃ λ 12) and was isolated following 2 additional rounds of plaque hybridization analysis.

DNA was prepared from plate lysates of EP₃ λ 12 using LambdaSorb (Promega). The DNA was digested with EcoRI and an insert of $\sim$ 13 kilobasepairs (kb) was obtained. Further restriction enzyme analysis and Southern blotting (Sambrook *et al.*, 1989) revealed a 2.4 kb BamHI fragment that hybridized with the 32 P-labelled PCR product. This 2.4 kb BamHI fragment was subcloned into pBluescript and its nucleotide sequence was determined using Sequenase (United States Biochemicals). An open reading frame was present that had $\sim$ 80% identity with the mouse EP₃ receptor sequence. The amino terminus of this open reading frame started in approximately the same region as that of the mouse EP₃ amino terminus but the carboxyl terminus of the human sequence ended at a point equivalent to TM6 of the mouse EP₃ receptor. Examination of the nucleotide sequence in this region of the human genomic clone was consistent with an intron/exon boundary.

Isolation of cDNA clones

A human small intestine cDNA library in λ gt10 (Clontech) was screened with the nick-translated PCR product using the same conditions as described above for the genomic library. From $\sim$ 860,000 recombinants, 2 clones were obtained (EP₃27 and EP₃32). Another $\sim$ 460,000 recombinants were screened under the same conditions except that the filters were washed for 1 h at 50°C in 1 $\times$ SSC/0.1% SDS. Four clones were obtained (EP₃1, EP₃4, EP₃19 and EP₃21). All were subcloned into the EcoRI site of pBluescript and were found to contain nucleotide sequence identical to the genomic clone. Although none contained complete open reading frames, between the six clones three distinct alternative splicing variants were present and complete open reading frames could be constructed for all of them (see below). A third screen of $\sim$ 500,000 recombinants yielded a clone (EP₃11.2) that represented a fourth alternative splicing variant and which contained a complete open reading frame. The latter was obtained using a 32 P-labelled RNA probe corresponding to nt's 506–952 (Figure 1) of the genomic clone. Filters were prehybridized at 60°C in 6 $\times$ SSC/20 mM NaH₂PO₄ (pH 7.6)/1 $\times$ Denhardt's/1 mM DTT/100 μ g ml⁻¹ poly-A (United States Biochemical) and were hybridized overnight in the same solution containing 2 $\times$ 10⁶ d.p.m. ml⁻¹ of the probe. Filters were washed under increasingly stringent conditions with the final wash at 60°C for 20 min in 0.1 $\times$ SSC/0.1% SDS. EP₃11.2 was plaque purified and subcloned into the EcoRI site of pBluescript.

Full length plasmids encoding human EP₃ receptors

A sense PCR primer containing adjacent BstXI and SspI restriction sites was made to nucleotide sequence upstream of the apparent translation start site (nt 57 in Figure 1). The sequence of this primer, shown below, contained sequence identical to that in the cDNA and genomic clones (nt's 1–22 in Figure 1) as well as additional 5' sequence (the additional sequence is shown below in the smaller font, the BstXI site is bold and the SspI site is underlined).

5'GATCCACCGCGGTGGAATATGCCCCCTCCCGCTGCGGCTCT^{3'}

This primer was used with an antisense primer (corresponding to nt's 657–679 in Figure 1) in a PCR reaction with the 2.4 kb BamHI genomic clone as a template. A product of ~700 bp was obtained that was purified with GeneClean (Bio101) and digested with BstXI, cleaving it at the 5' end and at a position corresponding to nt's 499–510 in Figure 1. The larger, ~500 bp, fragment was isolated from a low-melting-point agarose gel and was used in a solid phase ligation (Sambrook *et al.*, 1989) with the 4.2 kb fragment isolated from the digestion of the pBluescript EP₃27 cDNA clone with BstXI. [EP₃27 starts at a position corresponding to nt 497 in Figure 1 and thus contains the downstream BstXI site as well as BstXI site immediately upstream in the multiple cloning site of pBluescript.] *E. coli* XL1-Blue cells were transformed and a clone was obtained (KS/EP_{3A}, Figure 1) containing 1170 bases of the complete open reading frame, 56 bases of 5'-untranslated sequence and 504 bases of 3'-untranslated sequence. KS/EP_{3A} was digested with SspI and the coding fragment was ligated to EcoRV digested pBluescript to yield KS/EP_{3D} in which the orientation of the coding sequence was such that the translation start site (nt's 57–59) was downstream of the T3 promoter.

A full length construct of EP_{3E} (KS/EP_{3E}) was obtained by digesting the pBluescript clone of EP₃19 with BglII and HindIII and ligating the resulting small fragment with the large fragment obtained from the digestion of KS/EP_{3A} with the same enzymes. The pBluescript clone of EP₃11.2 was digested with BamHI and the resulting small fragment was ligated to the large fragment obtained from the digestion of KS/EP_{3D} with BamHI to yield KS/EP_{3D}.

Functional expression

The KS/EP_{3A} clone was digested with SspI which cleaved it 2 bases upstream of nt 1 and again at nt 1421. The digest was electrophoresed on a 1% agarose gel and the 1.4 kb fragment was cut out and purified with GeneClean (Bio101). The eukaryotic expression vector, pBC12BI (Cullen, 1987), was digested with HindIII and BamHI, was blunt-ended with Klenow, and was electrophoresed on a 1% low-melting-point agarose gel. The 3.9 kb fragment was isolated and was used in a solid phase ligation with the 1.4 kb SspI fragment from KS/EP_{3A}. Cells were transformed and a clone was obtained (pBC/EP_{3A}).

The KS/EP_{3E} clone was used to prepare pBC/EP_{3E} by digesting pBC12BI with HindIII and BamHI, blunting, and ligating the large fragment to the blunt-ended 1.2 kb fragment obtained from digestion of KS/EP_{3E} with SspI and HinfI. pBC/EP_{3D} was made by digesting KS/EP_{3D} with HindIII and SspI and ligating the coding fragment to the large fragment obtained from pBC12BI which had been cleaved with BamHI, blunt-ended, and then cleaved with HindIII.

Functional expression was determined as previously described (Pepperl & Regan, 1993) in a transient assay using JEG-3 cells and an adenosine 3':5'-cyclic monophosphate (cyclic AMP)-responsive chloramphenicol acetyl transferase reporter gene (CRE-CAT). Briefly, JEG-3 cells were cotransfected with DNA encoding pBC/EP₃ receptor variants and the reporter gene using calcium phosphate precipitation. The cells were maintained in DMEM/5% FBS for 36–40 h and were then rinsed twice with DMEM and drugs were added. They were incubated for 4 h at 37°C and were harvested. CAT assays were done using 50 µl cytosol, 200 nCi [³H]-chloramphenicol and 300 µM butyryl CoA. The butyrylated chloramphenicol was extracted into xylenes and the radio-labelled product was measured by liquid scintillation counting.

Radioligand binding

COS-7 cells were transfected as previously described (Regan *et al.*, 1988) with the pBC/EP₃ receptor constructs. Three days later the cells were harvested and membranes were

prepared by homogenization and differential centrifugation. Membranes were resuspended in 50 mM Tris-HCl/10 mM MgCl₂/1 mM EDTA/pH 7.4 at a concentration of ~1 mg ml⁻¹ and were frozen at -80°C. The membranes were thawed, diluted with ice-cold 50 mM Tris HCl/pH 7.4 (TB), and were dispersed with sonication. Binding was determined in triplicate in a final volume of 200 µl for 30 min at 25°C. Non-specific binding was defined with 10 µM PGE₂. Reactions were started by the addition of membranes and were terminated by the addition of 4 ml of ice-cold TB followed by rapid filtration through Whatman GF/B filters and 3 additional 4 ml washes with a cell harvester (Brandel). The filters were dried, placed in 5 ml of scintillation fluid (Ready Protein, Beckman), and counted (Beckman LS-380). Competition studies were done with a final concentration of 5 nM [³H]-PGE₂. Data were analyzed by computer using the EBDA/Ligand programme (McPherson, 1985).

Northern blot analysis

Preparation of human polyA⁺-RNA, electrophoresis and transfer to a nylon membrane was done by the manufacturer (Clontech, Multiple Tissue Northern, catalogue No. 7760-1). Accordingly, polyA⁺-RNA was prepared by oligo(dT) cellulose chromatography and electrophoresis was on 1.2% agarose/formaldehyde gels. Probes were radiolabelled with ³²P-dCTP by nick translation (Gibco/BRL) and were purified by size exclusion chromatography (Chromaspin-10, Clontech). Prehybridization was in 10 ml of 44% formamide/8.8 × Denhardt's/88 µg ml⁻¹ herring sperm DNA/4.4 × SSPE/1.8% SDS for 3 h at 42°C. Hybridization was for ~16 h at 42°C in 8 ml of the prehybridization buffer containing ~1.5 × 10⁶ c.p.m. ml⁻¹ of probe. Blots were rinsed 6 × 5 min at 24°C in 2 × SSC/0.5% SDS and then 2 × 20 min at 50°C in 0.1 × SSC/0.1% SDS. Exposures were made at -80°C using Kodak AR film.

PCR analysis of variant expression

Sense and antisense primers corresponding to TM5 and the 3'-untranslated regions, respectively, of the human EP₃ receptor variants were used in RT/PCR reactions to confirm the expression of the variants in total RNA from human tissues. The sense primer represents nt's 774–791 (Figure 1) which is common to all of the variants. The antisense primers are as follows: EP_{3A}, nt's 1257–1274; EP_{3A1}, nt's 1356–1373; EP_{3E}, nt's 1164–1181; EP_{3D}, nt's 1166–1183 (Figure 2). The reverse transcription reaction (final volume, 60 µl) contained 6 µl 10 × PCR buffer (Boehringer Mannheim), 2 µl RNasin, 6 µl dNTP's (10 mM), 6 µl random primers (6-mers, 0.04 A₂₆₀, µl⁻¹) or 6 µl p(dT)₁₅ (0.02 A₂₆₀ µl⁻¹), 2 µl denatured (65°C for 10 min) RNA (1 µg µl⁻¹), 12 µl MgCl₂ (25 mM), 0.6 µl acetylated BSA (10 mg ml⁻¹) and 7 µl AMV reverse transcriptase. The reaction was incubated at room temperature (~24°C) for 10 min, 42°C for 45 min, 95°C for 5 min and was placed on ice. The PCR reaction (final volume, 50 µl) contained 4 µl from the reverse transcription reaction, 5 µl 10 × PCR buffer, 0.6 µl sense primer (25 µM), 0.6 µl antisense primer (25 µM), 3 µl MgCl₂, 2 µl dNTP's (1.25 mM), 1 µl tetra-methyl-ammonium chloride (2.5 mM) and 0.3 µl Taq polymerase (Perkin Elmer, 5 u µl⁻¹). An initial denaturation step at 94°C for 2 min was followed by 35 cycles of 94°C, 15 s; 60°C, 15 s; 72°C, 36 s and a final step at 72°C for 6 min. Products were analyzed by agarose gel (1.5%) electrophoresis followed by staining with ethidium bromide.

Results

Using the polymerase chain reaction (PCR) and primers designed from the first extracellular loop and sixth transmembrane (TM) domain of the mouse EP₃ prostaglandin receptor (Sugimoto *et al.*, 1992), a product of ~500 base

pairs was obtained from human small intestine RNA. Analysis of the product's nucleotide and deduced amino acid sequence indicated that it was the human homologue of the mouse EP₃ receptor. The PCR product was labelled with ³²P and was used to screen a human genomic library by plaque hybridization analysis. A clone was isolated and was found to contain nucleotide sequence identical to the PCR product, however, downstream of nt 953 (Figure 1) the sequence of the genomic clone diverged radically from the mouse EP₃ receptor sequence. This divergence, which in the deduced amino acid sequence corresponds to TM6, marks the beginning of an intron. Subsequent Southern blot analysis of the genomic clone with a probe encoding TM7 from the human EP_{3A} receptor cDNA was negative, indicating that the downstream exon(s) were not present in this clone.

The ³²P-labelled PCR product was then used to screen a human small intestine cDNA library. A total of 7 clones were isolated and sequenced. One of these clones appeared to

correspond to the mouse EP₃ (Sugimoto *et al.*, 1993) but it lacked some of the 5' coding region. Between it and the genomic clone, a full length clone encoding the human EP_{3A} was constructed. Later, two of the other cDNA clones were found to have complete 5' coding sequences which confirmed the sequence of the recombinant receptor. The complete nucleotide and deduced amino acid sequence of the human EP_{3A} clone is shown in Figure 1. The overall amino acid identity of this clone with the mouse EP₃ sequence is 79% and identity in the transmembrane domains is 91%. Like the mouse sequence, the human EP_{3A} has a short third intracellular loop (~26 amino acids), two consensus sites for N-linked glycosylation in the amino terminus and an aspartate residue in TM7 that is conserved in other prostanoid receptors but is an asparagine in other G-protein coupled receptors.

Among the other cDNA clones was one (EP_{3A1}) that had a coding sequence identical to the EP_{3A} but which had a completely different 3' untranslated sequence beginning in the

5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95															
TGCC	CCTCC	CGCTG	CGGCT	CTCTG	GACGC	CATCC	CCTCC	TCAAC	TCGAA	GCCAA	C	ATG	AAG	GAG	ACC	CGG	GGC	TAC	GGA	GGG	GAT	GCC	CCC	TTC	TGC								
											M	K	E	T	R	G	Y	G				A	P	F	C	14							
100	105	110	115	120	125	130	135	140	145	150	155	160	165	170	175	180	185	190															
ACC	CGC	CTC	AAC	CAC	TCC	TAC	ACA	GGC	ATG	TGG	GCG	CCC	GAG	CGT	TCC	GCC	GAG	GCG	CGG	GGC	AAC	CTC	ACG	CGC	CCT	CCA	GGG	TCT	GGC	GAG	GAT	46	
T	R	L	N	H	S	Y	T	G	M	W	A	P	E	R	S	A	E	A	R	G	N	L	T	R	P	P	G	S	G	E	D		
195	200	205	210	215	220	225	230	235	240	245	250	255	260	265	270	275	280	285	290														
TGC	GGA	TCG	GTG	TCC	GTG	GCC	TTC	CGG	ATC	AGC	ATG	CTG	CTG	ACT	GGT	TTC	GTG	GGC	AAC	GCA	CTG	GCC	ATG	CTG	CTC	GTG	TCG	CGC	AGC	TAC	CGG	78	
C	G	S	V	S	V	A	F	P	I	T	M	L	L	T	G	F	V	G	N	A	L	A	M	L	L	V	S	R	S	Y	R		
transmembrane 1																																	
295	300	305	310	315	320	325	330	335	340	345	350	355	360	365	370	375	380	385															
CGC	CGG	GAG	AGC	AAG	CGC	AAG	AAG	TCC	TTC	CTG	CTG	TGC	ATC	GGC	TGG	CTG	GCG	CTC	ACC	GAC	CTG	GTC	GGG	CAG	CTT	CTC	ACC	ACC	CCG	GTC	GTC	110	
R	R	E	S	K	R	K	K	S	F	L	L	C	I	G	W	L	A	L	T	D	L	V	G	Q	L	L	T	T	P	V	V		
transmembrane 2																																	
390	395	400	405	410	415	420	425	430	435	440	445	450	455	460	465	470	475	480															
ATC	GTC	GTG	TAC	CTC	TCC	AAG	CAG	CGT	TGG	GAG	CAC	ATC	GAC	CCG	TGG	GGG	CGG	CTC	TGC	ACC	TTT	TTC	GGG	CTG	ACC	ATG	ACT	GTT	TTC	GGG	CTC	142	
I	V	V	Y	L	S	K	Q	R	W	E	H	I	D	P	S	G	R	L	C	T	F	F	G	L	T	M	T	V	F	G	L		
transmembrane 3																																	
485	490	495	↓	500	505	510	515	520	525	530	535	540	545	550	555	560	565	570	575														
TCC	TCG	TTG	TTC	ATC	GCC	AGC	GCC	ATG	GCC	GTC	GAG	CGG	GCG	CTG	GCC	ATC	AGG	GCG	CCG	CAC	TGG	TAT	GCG	AGC	CAC	ATG	AAG	ACG	CGT	GCC	ACC	174	
S	S	L	F	I	A	S	A	M	A	V	E	R	A	L	A	I	R	A	P	H	W	Y	A	S	H	M	K	T	R	A	T		
transmembrane 4																																	
580	585	590	595	600	605	610	615	620	625	630	635	640	645	650	655	660	665	670															
CGC	GCT	GTG	CTG	CTC	GGC	GTG	TGG	CTG	GCC	GTG	CTC	GCC	TTC	GCC	CTG	CTG	CCG	GTG	GCG	CAG	TAC	ACC	GTC	CAG	TGG	CCC	GGG	ACG					
R	A	V	L	L	G	V	W	L	A	V	L	A	F	A	L	L	P	V	L	G	V	G	Q	Y	T	V	Q	W	P	G	T	206	
675	680	685	690	695	700	705	710	715	720	725	730	735	740	745	750	755	760	765	770														
TGG	TGC	TTC	ATC	AGC	ACC	GGG	CGA	GGG	GCG	AAC	GGG	ACT	AGC	TCT	TCG	CAT	AAC	TGG	GCG	AAC	CTT	TTC	TTC	GCC	TCT	GCC	TTT	GCC	TTC	CTG	GGG	238	
W	C	F	I	S	T	G	R	G	N	G	T	S	S	S	H	N	W	G	N														
transmembrane 5																																	
775	780	785	790	795	800	805	810	815	820	825	830	835	840	845	850	855	860	865															
CTC	TTG	GCG	CTG	ACA	GTC	ACC	TTT	TCC	TGC	AAC	CTG	GCC	ACC	ATT	AAG	GCC	CTG	GTG	TCC	CGC	TGC	CGG	GCC	AGC	GCC	ACG	GCA	TCT	CAG	TCC	AGT	270	
L	L	A	L	T	V	T	F	S	C	N	L	A	T	I	K	A	L	V	S	R	C	R	A	K	A	T	A	S	Q	S	S		
transmembrane 6																																	
870	875	880	885	890	895	900	905	910	915	920	925	930	935	940	945	950	↓	960															
GCC	CAG	TGG	GCG	CGC	ATC	ACG	ACC	GAG	ACG	GCC	ATT	CAG	CTT	ATG	GGG	ATC	ATG	TGC	GTC	CTG	TCG	GTC	TGC	TGT	TCT	CCG	CTC	CTG	ATA	ATG	ATG	302	
A	Q	W	G	R	I	T	E	T	A	I	Q	L	M	G	I	M	C	V	L	S	V	C	W	S	P	L	T	A	I	M	M		
transmembrane 7																																	
965	970	975	980	985	990	995	1000	1005	1010	1015	1020	1025	1030	1035	1040	1045	1050	1055															
TTG	AAA	ATG	ATC	TTC	AAT	CAG	ACA	TCA	GTT	GAG	CAC	TGC	AAG	ACA	CAC	ACG	GAG	AAG	CAG	AAA	GAA	TGC	AAC	TTC	TTC	TTA	ATA	GCT	GTT	CGC	CTG	334	
L	K	M	I	F	N	Q	T	S	V	E	H	C	K	T	H	T	E	K	Q	K	E	C	N	F	F	L	I	A	V	R	L		
1060	1065	1070	1075	1080	1085	1090	1095	1100	1105	1110	1115	1120	1125	1130	1135	1140	1145	1150															
GCT	TCA	CTG	AAC	CAG	ATC	TGG	GTT	TAC	CTG	CTG	TTA	AGA	AAG	ATC	CTT	CTT	CGA	AAG	TTT	TGC	CAG	ATC	AGG	TAC	CAC	ACA	AAC	AAC					
A	S	L	N	Q	I	L	D	P	W	V	Y	L	L	L	R	K	I	L	L	R	K	F	C	Q	I	R	Y	H	T	N	N	366	
transmembrane 8																																	
1155	1160	1165	1170	1175	1180	1185	1190	1195	1200	1205	1210	1215	1220	1225	1230	1235	1240	1245	1250														
TAT	GCA	TCC	AGC	TCC	ACC	TCC	TTA	CCC	TGC	CAG	TGT	TCC	TCA	ACC	TTG	ATG	TGG	AGC	GAC	CAT	TTG	GAA	AGA	TAA	T	GAAAG	AACGG	AGTTG	GACAT			390	
Y	A	S	S	S	T	S	L	P	C	Q	C	S	S	T	L	M	W	S	D	H	L	E	R	*									
transmembrane 9																																	
1260	1270	1280	1290	1300	1310	1320	1330	1340	1350																								
TTTAT	TGCAA	TTCTC	GCTTC	CCTGA	ATTTC	CATAT	TTCTT	COCAC	CTGAG	AAGGA	TAATT	ATATA	TTTAT	ATTTC	GATTA	TTTCT	TCATT	TTTAT	CTTTT														
1360	1370	1380	1390	1400	1410	1420	1430	1440	1450																								
TATTT	TAAATG	ATTGT	TTTGT	CAGTA	ATAAC	CATGG	AGATC	AACCT	TATTA	TTATA	ATCCA	TGCCT	CTGAA	TATTA	GATTG	GTTTC	TTGGA	TGGGA	TTTTG														
1460	1470	1480	1490	1500	1510	1520	1530	1540	1550																								
ATATG	CATTT	AAGAA	GTGAG	GAAGA	ATTTC	ACAGA	TGATG	ATTGG	AGGAA	AAGTG	ATGAA	AAGAA	GACCT	GTGTT	CCAGG	AGTTT	TCTCC	AACCT	CAAC														
1560	1570	1580	1590	1600	1610	1620	1630	1640	1650																								
CTTTA	CGTGA	ATCTT	AACCA	AAGTG	ACATC	TTTAC	ATTTC	ATGAT	AGCTT	GCTTT	TGCAA	TATGA	GTTTG	AAAAA	TCANG	ATAAG	CTTAT	GATGG	TGAAA														
1660	1670	1680	1690	1700	1710	1720																											
AGTCA	ACATA	TTGAG	AGTGA	TAATT	CAATT	AATAG	GATAT	GAACT	TAACG	ACATA	TAAAA	GCAAA	TGAGG	GCAGG	AGGG																		

Figure 1 Nucleotide (nt) and deduced amino acid sequence of the human EP_{3A} receptor. The coding sequence is from nt's 57 to 1226 and the 390 amino acid sequence is indicated using the single letter code. Putative transmembrane regions are indicated by the double underline. The beginning of cDNA clone EP_{3A}27 is indicated by the arrow (↓) at nt 497. The beginning of an intron in the genomic sequence is indicated by the arrow (↓) after nt 953. The single underlines are restriction enzymes sites that were used in making recombinants: they are as follows, BstXI, nt's 499–510; BglII, nt's 1072–1077; BamHI, nt's 1079–1084; and SspI nt's 1419–1424.

stop codon at nt 1227. The nucleotide and deduced amino acid sequence of this and 2 other variants are shown in Figure 2. The other 2 variants, EP_{3E} and EP_{3D}, share the same nucleotide sequence as the EP_{3A} up to nt 1133 but then they diverge from each other and from the EP_{3A}. With respect to their amino acid sequences, this gives rise to 3

possible carboxyl terminal variants of the human EP₃ receptor. Assuming that ³⁵⁰R marks the beginning, the carboxyl terminus of the EP_{3A} would consist of 41 amino acids, while the EP_{3E} and EP_{3D} would consist of 16 and 39 amino acids, respectively. There is no amino acid identity between the variants in these regions nor is there any nucleotide sequence

EP_{3A1}

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      1130      1140      1150      1160      1170
AAG TTT TGC CAG ATC AGG TAC CAC ACA AAC AAC TAT GCA TCC AGC TCC ACC TCC TTA
  K   F   C   Q   I   R   Y   H   T   N   N   Y   A   S   S   S   T   S   L

      1190      1200      1210      1220      1230
CCC TGC CAG TGT TCC TCA ACC TTG ATG TGG AGC GAC CAT TTG GAA AGA TGA G AAAAA
  P   C   Q   C   S   S   T   L   M   W   S   D   H   L   E   R   *

      1240      1250      1260      1270      1280      1290      1300
GAAGA CTCAG AGAGC AATTG TGGAG GCCGG CAAGT TCAGG ATCAG GGTGC CAGCA GATTC GGTGT
      1310      1320      1330      1340      1350      1360
CTGAC TGGAG TGCAG TGGAG TGATT TCCGC TCACT GCAAC CTTCA CCTCC TCCAC TCACT GCAAT
      1370      1380      1390      1400      1410      1420      1430
CTTCG CCTCC TGGGT TCAAG TGATT CTCCT GCCTC AGCCT CCCAA GTAGC TGGAA TTGCA CGATG
      1440      1450      1460      1470      1480      1490
CGCCA CAAAG CTGGC TAATT TTTGC ATTTT TAGTA GAGAT GAGTT TCACC ATGTT TGCCA GGCTG
      1500      1510      1520      1530      1540      1550      1560
GTCTT GAACA CCTGA CCTCA AGTAA CCCAC CCACC TTGGC CTCCC AAAGA GCTGG GATTA CAGGC
      1570      1580      1590      1600      1610      1620
ATGAG CCAAC GTGCC TGGCC ATGTT CTGAT CGTTT AATGA TAGCA ACATT TAGTA TTATA GAGCA
      1630      1640      1650
TGAAA ATGTC AAAGC CGCCC GGAAT TC

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EP_{3E}

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      1130      1140      1150      1160      1170
AAG TTT TGC CAG GAG GAA TTT TGG GGA AAT TAA A ACCTG CCTTT CTGCC AGGAT
  K   F   C   Q   E   E   F   W   G   N   *

      1180      1190      1200      1210      1220      1230      1240
CACAT CACTG GAAGC TCCAT GACTC TCTTT TTGTA AAAGA AAAAA AAATC ACAGA AACAC CCACC
      1250      1260      1270      1280      1290      1300
TCCCA AACTA TTCTC TTTTA CTTCT TCCCC CAAGC CCACC CCCAA ATATA ACTGT TATCC AGAAG
      1310      1320      1330      1340      1350      1360      1370
CTGTT ATGTC CTGTT TCCAT ACATG TTTT TTTAC TATAT CTACA TACAT CAATT AAAT
      1380      1390      1400      1410      1420
TATGT CCTAT TGTTT TGTGA ATTTA TATTT GCGTA TACAT TATCG TATGT CCGGA ATTC

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EP_{3D}

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      1130      1140      1150      1160      1170
AAG TTT TGC CAG GTA GCA AAT GCT TCC AGC TGC TCT AAT GAT GGA CAG AAA GGG
  K   F   C   Q   V   A   N   A   V   S   S   C   S   N   D   G   Q   K   G

      1190      1200      1210      1220      1230
CAG CCT ATC TCA TTA TCT AAT GAA ATA ATA CAG ACA GAA GCA TGA AA GAAAA CACTT
  Q   P   I   S   L   S   N   E   I   I   Q   T   E   A   *

      1240      1250      1260      1270      1280      1290      1300
AACTT GCATG TGCAC AGCTT TTGGT AACAA ATATC GCTAA ACCTT ACTGT GAATT AGGCA TCTCT
      1310      1320      1330      1340      1350      1360
GGCAT GCCAC TGTTA TGCAT TGAAT GTGGA ATTTT GGTAT AAAGC TAAAT GGTCT TAGAA GCATA
      1370      1380      1390      1400      1410      1420      1430
GAAAA TCCCC TATGT GCCAA AAGTA GTGAA CACAA CAAAG GAAAA TATAT TAATA ACAGT CTAGT
      1440      1450      1460      1470      1480      1490
GTTTT GTTGA GTCTG CCATT GTAGC TGAAT ATGTG ATTAA TTATG TGATG AAAAC ATTTT TTATA
      1500      1510      1520      1530      1540
AATGA TCTTG GTCTA TTGGG GAGCG GGGAT AGTTA ATATT CCACC GG

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Figure 2 Nucleotide and deduced amino acid sequences of the human EP₃ receptor variants in their carboxyl termini and 3'-untranslated regions. From top to bottom: the EP_{3A1}, the EP_{3E} and the EP_{3D}. Sequences start with nt 1122 (aa. No 356) with upstream sequences the same as in Figure 1 for the EP_{3A}. Amino acid sequences that overlap with the EP_{3A} are in bold type face. The single underlines from nt's 1196–1200 in the EP_{3E} and nt's 1530–1535 in the EP_{3D} are *Hinf*I and *Ssp*I sites, respectively, that were used in making recombinants.

Figure 3 Inhibition of specific [3 H]-PGE $_2$ binding to membranes prepared from COS-7 cells that have been transfected with plasmid DNA encoding the three human EP $_3$ receptor variants. Competitors include natural prostaglandins and compounds that are selective for the various prostaglandin receptor subtypes and are as follows: PGE $_2$ (●), PGE $_1$ (○), MB28767 (△), AH13205 (◇), AH6809 (◆), U46619 (□), PGF $_{2\alpha}$ (▲), PGD $_2$ (■). (a) EP $_{3A}$; (b) EP $_{3D}$; (c) EP $_{3E}$. Binding was done as described in Methods with a final concentration of 5 nM [3 H]-PGE $_2$. Data represent the means $\pm$ s.e. mean from three separate experiments, each performed in triplicate.

Table 1 Competition for the binding of [3 H]-prostaglandin E $_2$ ([3 H]-PGE $_2$) to membranes prepared from COS-7 cells transfected with plasmid DNA encoding alternative splicing variants of the human EP $_3$ prostaglandin receptor¹

Competitor	Receptor variants (IC $_{50}$ in nM)		
	EP $_{3A}$	EP $_{3D}$	EP $_{3E}$
MB28767	3	14	12
PGE $_2$	15	32	20
PGE $_1$	39	60	25
PGF $_{2\alpha}$	2300	9300	1200

¹IC $_{50}$'s were calculated from the data shown in Figure 3 and represent the pooled data from 3 separate experiments. K_{ds} for the equilibrium saturation binding of [3 H]-PGE $_2$ were: 1, 8 and 6 nM for the EP $_{3A}$, EP $_{3D}$, and EP $_{3E}$, respectively.

identity. This is in contrast to the mouse EP $_3$ receptor variants which differ from one another because of a deletion and frame-shift (Sugimoto *et al.*, 1993).

The EP $_3$ receptor variants were cloned into a mammalian expression vector and were expressed transiently in COS-7 cells. Membranes were prepared and radioligand binding was determined using [3 H]-PGE $_2$. Figure 3 shows the results of competition studies using compounds predicted to have varying degrees of affinity for the EP $_3$ receptor. PGE $_2$, PGE $_1$, and MB28767 (EP $_3$ selective) all potently inhibited [3 H]-PGE $_2$ binding; the rank order of potency for all the variants being MB28767 > PGE $_2$ > PGE $_1$. There were, however, differences between the variants in the IC $_{50}$ s for some of the competitors (Table 1). For example, a 13 fold difference separated the IC $_{50}$ s of MB28767 and PGE $_1$ at the EP $_{3A}$, whereas, only a 2 fold difference separated them at the EP $_{3E}$. Most of this difference could be accounted for by a higher affinity of MB28767 for the EP $_{3A}$. PGF $_{2\alpha}$ also seemed to discriminate between the variants to some extent, showing higher affinity for the EP $_{3A}$ and EP $_{3E}$ as compared with the EP $_{3D}$. The other compounds competed very poorly or not at all. These included the selective thromboxane receptor agonist, U46619, the EP $_1$ selective antagonist, AH6809, the EP $_2$ selective agonist AH13205, and PGD $_2$.

The EP $_3$ receptor variants were also co-transfected into JEG-3 cells with a cyclic AMP responsive-chloramphenicol acetyltransferase reporter plasmid (CRE-CAT). This plasmid responds to changes in intracellular cyclic AMP and has been developed into a transient functional assay for receptors that interact with adenylyl cyclase (Pepperl & Regan, 1993). Figure 4 shows the inhibition of forskolin-stimulated CAT activity by sulprostone for the EP $_{3A}$, EP $_{3D}$ and EP $_{3E}$ receptor variants. The curves are biphasic with an inhibition of CAT activity occurring at low concentrations of sulprostone and a reversal at higher concentrations. A similar type of dose-response pattern has also been observed for α_2 -adrenoceptor subtypes (Pepperl & Regan, 1993) and for muscarinic receptors (Jones *et al.*, 1991) using both this assay and/or direct measurements of cyclic AMP. Figure 4 also shows that the EP $_{3A}$ inhibited forskolin-stimulated CAT activity to a greater extent than either the EP $_{3D}$ or EP $_{3E}$. With respect to the potency of sulprostone, large differences were not observed between the variants, although, there was a tendency for sulprostone to have a lower EC $_{50}$ at the EP $_{3A}$.

The full coding sequence of the human EP $_{3A}$ was radio-labelled with 32 P by nick-translation and was used as a probe in Northern blot analyses to examine the distribution of EP $_3$ receptor message in several human tissues. Figure 5 shows that mRNA from human kidney hybridized strongly with the probe. The most abundant message size was $\sim$ 2.4 kb but other bands at 5.1 and 7 kb were also observed. Longer exposures revealed a small amount of the 2.4 kb message in mRNA from the pancreas and heart.

The polymerase chain reaction (PCR) was used with primer combinations that were specific for each of the cDNA variants of the EP $_3$ receptor to determine if human tissues contained mRNA that corresponded to these variants. Figure 6 shows that using human small intestine RNA and reverse transcription followed by PCR, products were obtained that represented each of the cloned EP $_3$ receptor variants. Similar results were obtained with RNA from the human heart and pancreas except that only a single band was observed with primers that were specific for the EP $_{3E}$.

Discussion

A human small intestine cDNA library was screened with a PCR product encoding a portion of a human EP $_3$ receptor and 4 unique cDNA variants of this receptor were identified. One of these variants, the human EP $_{3A}$ appears to be the homologue of the mouse EP $_{3\alpha}$ (Sugimoto *et al.*, 1993). A second variant (EP $_{3A1}$) contains the same coding sequence as

Figure 4 Effects of sulprostone on forskolin-stimulated CRE-CAT reporter gene activity after transient transfection of JEG-3 cells with DNA encoding either: (a) the EP_{3A} (■) and EP_{3D} (□); or (b) the EP_{3A} (■) and EP_{3E} (○). Each curve is expressed as a percentage of the forskolin-stimulated activity in the absence of sulprostone. Transfections and CAT assays were done as described in Methods. Forskolin was used at a final concentration of 1 μ M. For (a) data are representative of 2 experiments and for (b) data are representative of 4 experiments.

the EP_{3A} but has a completely divergent 3' untranslated region beginning with the stop codon. The last two variants, the human EP_{3E} and EP_{3D}, encode receptors with the same amino acid sequence as the EP_{3A} except for their predicted carboxyl terminal domains. These carboxyl terminal sequence differences appear to affect some of the ligand binding properties of these variants and their functional coupling to a cyclic AMP responsive reporter gene. In addition, since the 3'-untranslated region can affect mRNA stability, the existence of EP₃ receptor variants with alternative 3'-untranslated sequences may also be important for regulating the expression of these receptors.

It is interesting that nucleotide sequences of the human EP_{3E} and EP_{3D} diverge from each other and from the EP_{3A} at exactly the same point (nt 1133, Figure 1); which is also where the mouse EP_{3B} diverges from the EP_{3A} (Sugimoto *et al.*, 1993). In the case of cDNAs encoding the mouse EP₃ receptors, the origin of this divergence involves a deletion of 89 nucleotides which results in a frame shift that changes the sequence of the next 26 amino acids before a new termination codon is reached. For our human variants such a mechanism is not in evidence because the nucleotide sequences in the divergent regions do not share identity. In either case mRNA splicing involving exons encoding alternative car-

Figure 5 Northern Blot of human mRNA's hybridized with the full coding sequence (nt's -2 to 1421) of the human EP_{3A} radiolabelled with ³²P by nick-translation. Hybridization, wash and exposure conditions are described in Methods. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. The position of the size standards are indicated. The blot was stripped and probed with a β -actin probe that indicated that similar amounts of RNA were loaded in each lane.

Figure 6 Photograph of ethidium-stained agarose gel of the products of a reverse transcription/PCR reaction with primers specific for each of the human EP₃ receptor cDNA variants. Lane 1 is a positive control for the PCR reaction; lanes 2-5 are negative controls (no templates); lanes 6-9 are positive controls using plasmid DNA encoding each of the human EP₃ receptor variants; lanes 10-13 are the results of the RT/PCR reaction using RNA from human small intestine. In each set of reactions (ie. negative control, positive control, small intestine), primers specific for the following variants were used EP_{3D}, EP_{3E}, EP_{3A} and EP_{3A1}. The expected product sizes (bp) were as follows: EP_{3D}, 409; EP_{3E}, 407; EP_{3A}, 500; and EP_{3A1}, 599. Standards (stds) are HaeIII cleaved Φ X174.

boxyl termini appears to be operational. The use of alternative splicing, as a mechanism of creating receptor heterogeneity, does not appear to be common among the G-protein coupled receptors but it has been described for rhodopsin and for the dopamine receptors. Thus, two variants of the D₂-dopamine receptor are known in which the use of alternative splicing creates 'long' and 'short' forms of the receptor which differ in the lengths of their third intracellular loops (Giros *et al.*, 1989; Monsma *et al.*, 1989; Dal Toso *et al.*, 1989). In addition, splice variants of the D₃-dopamine receptor have been described (Giros *et al.*, 1991).

Besides the cDNAs encoding carboxyl terminal variants of the EP₃ receptor we found evidence of an intron in the human EP₃ receptor gene in a region corresponding to TM6. We did not, however, find any cDNA clones that were variants at this position. Considering that separate exons

may encode TM1–6 and 7, respectively, it appears that the complete human EP₃ receptor gene might be fairly complex with multiple exons. This may also be true for other prostanoïd receptor genes. Thus, PCR products encoding the thromboxane receptor were not obtained from human genomic DNA when primers were used that spanned TM6 but they were obtained with other primer combinations downstream of TM6 (data not shown).

The question arises as to whether or not these cDNA variants are represented in mRNA from human tissues. The answer to this appears to be yes, as judged by reverse transcription coupled with PCR. Thus, antisense primers that were unique to the 3' regions of each of the variants were used in combination with a sense primer common to all of the variants. Four PCR products, corresponding to each of the variants, were obtained using RNA from human small intestine, as well as with RNA from human heart and pancreas. With RNA from human small intestine, however, the primers specific for the EP_{3E} consistently yielded two products: one of expected size and another that was 50–100 nt longer. By analogy to the mouse EP_{3B}, which contains an 89 bp deletion relative to the mouse EP_{3A}, it is possible that the human EP_{3E} receptor represents a deletion of yet another human EP₃ receptor variant. The finding that the larger of the two products was obtained only with RNA from small intestine and not with RNA from heart or pancreas, suggests that EP₃ receptor variants may be uniquely expressed in tissues. Additional studies, however, will be needed to define the tissue specific expression of EP₃ receptor variants.

Further evidence that the EP₃ receptor cDNA clones encoded functional receptors was obtained by radioligand binding studies of the cloned receptors expressed in mammalian cells lacking endogenous EP₃ receptors. Thus, expression of the human EP₃ receptor variants in COS-7 cells followed by radioligand binding with [³H]-PGE₂ have established an EP₃ pharmacology. PGE₂ was found to bind with high affinity as did the EP₃-selective agonist, MB28767. The EP₂-selective agonist, AH13205, and the EP₁ selective antagonist, AH6809, did not inhibit [³H]-PGE₂ binding which further confirms these receptors as EP₃. The inhibitory potency of PGF_{2α} in the human variants was similar to that reported for the cloned murine EP₃ receptors (Sugimoto *et al.*, 1993) and to native EP₃ receptors in the renal, gastrointestinal and reproductive tissues from various nonprimate species (Sonnenberg *et al.*, 1990; Watanabe *et al.*, 1991; Woodward *et al.*, 1994). In contrast, PGD₂ exhibited minimal activity at the human EP₃ variants, whereas, it is more active at some nonprimate EP₃ receptors. Other differences in potency also exist between the cloned murine and human EP₃ receptors. For example, MB28767 is ~10 fold more potent than PGE₁ at the human EP_{3A} as compared with the murine EP_{3A}. In this regard, the murine EP_{3A} is more similar pharmacologically to the human EP_{3E}.

The radioligand binding of [³H]-PGE₂ to membranes prepared from cells transfected with these EP₃ receptors offers some distinct advantages over the use of naturally occurring animal tissues. For example, at a concentration of 5 nM [³H]-PGE₂, 35 µg of membrane protein from COS-7 cells transiently transfected with the EP_{3A} yielded ~37,100 d.p.m. of specific [³H]-PGE₂ binding activity (3.2 pmol mg⁻¹ protein) with only 2% nonspecific binding. Using membranes prepared from the rat uterus, 20 µg of membrane protein yielded ~2,100 d.p.m. of specific binding (309 fmol mg⁻¹ protein) with 33% nonspecific binding (Woodward *et al.*, 1994). The recognition of EP₃ receptor heterogeneity combined with the ability to study these variants in isolation increases the likelihood that the biochemical differences between these variants will be exploited pharmacologically.

The apparent differences in agonist binding between EP₃ receptor variants could reflect differences in the efficiency of coupling, or pattern of coupling, to G-proteins and second messenger pathways. Native EP₃ receptors, previously characterized in cells and tissues, inhibit adenylyl cyclase as part

of their second messenger pathway (Sonnenberg *et al.*, 1990; Matthews & Jones, 1993). The mouse EP₃ receptors that have been stably expressed in cultured CHO cells (Sugimoto *et al.*, 1993) differ markedly in their inhibition of forskolin-stimulated cyclic AMP formation. Thus, PGE₂ is ~3 orders of magnitude more potent at the EP_{3A} than it is at the EP_{3B}. The isoforms also differ with respect to desensitization with the EP_{3A} being relatively sensitive and the EP_{3B} resistant (Negishi *et al.*, 1993). The human EP₃ receptor variants also seem to show differences with respect to their patterns of coupling with adenylyl cyclase. In transiently transfected human JEG-3 cells there was a biphasic effect on forskolin-stimulated cyclic AMP-responsive CAT reporter gene activity: first inhibition, and then reversal. It also appeared that sulprostone could produce a greater degree of inhibition at the EP_{3A} as compared with either the EP_{3D} or EP_{3E}.

Phosphorylation of G-protein coupled receptors on serines and/or threonines present in the carboxyl terminal or third intracellular loop is known to be critical in the process of receptor desensitization (Lefkowitz, 1993). In addition, tyrosines present in the carboxyl terminus of the β-adrenoceptor may have a role in the more specific process of down-regulation, although, it is unknown if this also involves phosphorylation (Valiquette *et al.*, 1990). It is notable, therefore, that the composition of the carboxyl termini of the human EP₃ receptor variants differ markedly in these regards. Thus, the carboxyl terminus of the EP_{3A} contains 7 serines, 3 threonines and 2 tyrosines; the EP_{3E} contains none of these amino acids and the EP_{3D} contains 5 serines, 1 threonine and 1 tyrosine. If desensitization of EP₃ receptors specifically involves phosphorylation of the carboxyl terminus, then the human EP_{3A} and EP_{3D} would be expected to be more affected than the EP_{3E}.

Very recently the cloning of four EP₃ receptor variants (EP_{3A}, EP_{3B}, EP_{3C}, EP_{3D}) from the bovine adrenal medulla was reported (Namba *et al.*, 1993). Interestingly, all of these bovine isoforms were variants starting with the same amino acid in the carboxyl terminus as in the human variants; however, only one is an actual homologue of the human clones. Thus, the bovine EP_{3D}, is a homologue of the human EP_{3D}. The human EP_{3A}, which is a homologue of the mouse EP_{3A}, is not a homologue of the bovine EP_{3A}. In addition, none of the bovine variants are homologues of the mouse EP_{3B}. In total, therefore, up to seven EP₃ receptor variants may exist in any given species.

The identification and characterization of these EP₃ receptor variants demonstrates a greater degree of complexity with respect to alternative RNA splicing than previously thought for the G-protein coupled receptors. It also raises intriguing questions concerning the regulation and expression of these isoforms and their physiological roles in mediating the actions of endogenous prostaglandins. The potential for tissue specific expression of each of the human EP₃ receptor variants exists, as well as the possibility that the regulation of receptor number rests not only at the level of the protein but at the level of the mRNA. The differential expression of these EP₃ receptor variants could affect a target tissue's sensitivity to drugs both in terms of responsiveness and the ability to desensitize. Such differences could potentially be exploited for therapeutic benefit.

Note added in proof

We have recently cloned a cDNA from a human skin fibroblast library (Clontech) that represents an additional carboxyl terminal variant of the EP₃ prostaglandin receptor. This variant, the EP_{3F}, is related to the human EP_{3E} and probably explains the doublet that was observed in the PCR reaction (Figure 6, lane 11; also see paragraph 4 of Discussion). As compared with the EP_{3E} (Figure 2), the EP_{3F} contains an additional 27 bases between nt's 1133 and 1134. The nucleotide and deduced amino acid sequence of this insert is shown as follows.

^{5'}ATG AGA AAA AGA AGA CTC AGA GAG CAA^{3'}
M R K R R L R E Q

The radioligand binding and functional coupling of this variant have not been presently characterized.

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(Received November 15, 1993)

Revised January 27, 1994

Accepted February 3, 1994