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Two Isoforms of the EP_3 Receptor with Different Carboxyl-terminal Domains

IDENTICAL LIGAND BINDING PROPERTIES AND DIFFERENT COUPLING PROPERTIES WITH G_i PROTEINS*

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Functional cDNA clones for two isoforms of the mouse prostaglandin E receptor EP_3 subtype derived from alternative RNA splicing were obtained. The two isoforms are only different in the sequence of the putative cytoplasmic carboxyl-terminal tail and their hydrophobicity; one isoform, named $EP_{3\alpha}$, has a hydrophilic tail, and the other, named $EP_{3\beta}$, has a hydrophobic tail. When expressed, the two receptors displayed identical ligand binding properties but different responses to guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS). Without a change in the B_{max} value, GTPγS increased K_d for prostaglandin E_2 of $EP_{3\beta}$ and decreased that of $EP_{3\alpha}$. These effects were abolished by the treatment of membranes with pertussis toxin and restored by the addition of G_{i2}. Although both isoforms exerted inhibition of forskolin-induced cAMP accumulation, three orders lower concentrations of agonists were required for $EP_{3\alpha}$ than $EP_{3\beta}$ for 50% inhibition of cAMP formation. A similar difference in agonist potency was observed also for agonist-induced stimulation of GTPase activity in membranes. Thus, the two receptors with different carboxyl-terminal tails show different coupling to the G_i protein, leading to the opposite responses to GTP in the ligand binding affinity and to different affinities of the agonist-occupied receptors to the G proteins.

Prostaglandin (PG)[1] E_2 has a broad range of biological actions in diverse tissues through specific receptors on plasma membranes for maintenance of local homeostasis in the body (1, 2). The pharmacological actions of PGE_2 are diverse among tissues; PGE_2 causes contraction or relaxation of vascular and nonvascular smooth muscle and stimulates or suppresses the secretion of neurotransmitters and hormones (3). Pharmacological studies suggested that at least three types of PGE receptors, EP_1, EP_2, and EP_3, exist, which were thought to differ in their signal transduction (4). Among PGE receptor subtypes, the EP_3 receptor has been the most well characterized pharmacologically and has been suggested to be involved in such PGE_2 actions as inhibition of gastric acid secretion (5), modulation of neurotransmitter release (6, 7), inhibition of lipolysis in adipose tissue (8), and inhibition of sodium and water reabsorption in kidney tubulus (9). We recently cloned the mouse EP_3 receptor from a mouse mastocytoma P-815 library and demonstrated that this receptor is a G protein-coupled rhodopsin-type receptor and engaged in inhibition of adenylate cyclase (10). Although various EP_3-mediated actions are believed to be mediated by inhibition of adenylate cyclase (11, 12), the concentration of PGE_2 that inhibits cAMP formation varies with the tissue, the IC_{50} values of PGE_2 being 10^{-8}, 10^{-10}, and 10^{-12} M in rat hepatocytes (13), rat kidney cells (14), and rat myometrium (15), respectively. In rhodopsin-type G protein-coupled receptors, guanine nucleotides have been demonstrated to modulate ligand binding affinity to receptors (16, 17). Depending on the tissue, PGE receptors show different responses to guanine nucleotides in their ligand binding affinity. GTP decreases the binding affinity in bovine adrenal medulla (18) but increases it in hamster adipocytes (19) and canine renal medulla (20), suggesting that there are two manners of association of PGE receptors with G proteins. These differences in efficiency as to agonists as well as responses to GTP of the EP_3 receptor imply heterogeneity of this type of receptor.

We report here identification of two isoforms of the EP_3 receptor with different carboxyl-terminal tails produced by alternative splicing and the resultant frame shift. They show different efficiencies in activation of coupling G protein and inhibition of adenylate cyclase by distinct interactions with the inhibitory G protein. These findings will facilitate understanding of the diversity of cellular responses to PGE_2. They further show the importance of the cytoplasmic carboxyl-terminal tail in modulating the coupling of a rhodopsin-type receptor with a G protein.

EXPERIMENTAL PROCEDURES

Materials—M&B28767, butaprost, and SC-19220 were generous gifts from Dr. M. P. L. Caton of Rhone-Poulenc Ltd., Dr. P. J. Gardiner of Bayer UK Ltd., and Dr. P. W. Collins of Searle, respectively. [α-^{32}P]dCTP (3,000 Ci/mmol) and [5,6,8,11,12,14,15-^{3}H]PGE_2 (185 Ci/mmol) were obtained from Du Pont-New England Nuclear. PGE_1, PGD_2, $PGF_{2\alpha}$, and unlabeled PGE_2 were purchased from Fu-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D13321.

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[1] The abbreviations used are: PG, prostaglandin; G protein, heterotrimeric GTP-binding protein; CHO, Chinese hamster ovary; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PT, pertussis toxin; PCR, polymerase chain reaction; MES, 4-morpholineethanesulfonic acid; AppNHp, adenyl-5′-yl β,γ-imidodiphosphate; bp, base pair(s).

nakoshi Pharmaceuticals (Tokyo, Japan). The cyclic AMP ^{125}I assay system was obtained from Amersham Corp. Forskolin and 3-isobutyl-1-methylxanthine were from Sigma. G_{i2} was purified from bovine spleen as described (21). The sources of other materials are given in the text.

cDNA Cloning—Mouse mastocytoma P-815 cDNA was synthesized by an oligo(dT) priming method using a cDNA synthesis kit (Amersham), as described (22), size-selected (>1.8 kilobases), and inserted into the *Eco*RI site of λZAPII DNA (Stratagene) with *Eco*RI adaptors (New England Biolabs). The 7.2×10^5 clones derived from the cDNA library were screened by hybridization (23) with ML64, a partial clone for EP_3 obtained from a mouse lung library by cross-hybridization as described previously (10). The clones isolated were subjected to restriction and sequence analyses. Nucleotide sequencing was carried out on double-stranded templates using the dideoxy chain termination method.

Transient Expression in COS-1 Cells and Binding Assay—The 2.1-kilobase pair *Eco*RI insert of MP660 and the 2.0-kilobase pair insert of MP653 were individually subcloned into pcDNA I (Invitrogen), a eukaryotic vector, and then each resultant plasmid DNA was transfected into COS-1 cells by the DEAE-dextran method (24). After culturing for 72 h, the cells were harvested and homogenized in a solution comprising 25 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, using a Dounce homogenizer (40 strokes). The homogenate was centrifuged at $800 \times g$ for 10 min, and the pellet was suspended in the same buffer, homogenized, and then centrifuged. The two supernatants were combined and centrifuged at $100{,}000 \times g$ for 1 h. The crude membranes thus isolated were suspended in 20 mM MES, pH 6.0, containing 10 mM $MgCl_2$ and 1 mM EDTA (buffer A). 50 μg of membrane protein was used for the [^{3}H]PGE_2 binding assay.

For ligand binding to the two molecular forms of EP_3, each type of cell membrane was incubated with various concentrations (Scatchard plot analyses) or 2.5 nM (displacement experiments) of [^{3}H]PGE_2 in 100 μl of buffer A at 30 °C for 1 h. The reaction was terminated by the addition of 2 ml of ice-cold buffer A, and the mixture was rapidly filtered through a Whatman GF/C filter. The filter was then washed four times with 2 ml of ice-cold buffer A, and the radioactivity associated with the filter was measured in 5 ml of Clearsol (Nacalai Tesque, Kyoto, Japan). Nonspecific binding was determined using a 1,000-fold excess of unlabeled PGE_2 in the incubation mixture. The specific binding was calculated by subtracting the nonspecific binding from the total binding. The specific [^{3}H]PGE_2 binding to the membrane reached equilibrium within 50 min under these conditions.

Stable Expression in CHO Cells—cDNA transfection and cell line establishment were performed essentially as described (25). The 2.1-kilobase pair *Eco*RI fragment of MP660 and the 2.0-kilobase pair *Eco*RI fragment of MP653 were individually inserted into the *Eco*RI site of pdKCR-dhfr, a eukaryotic expression vector containing a mouse dihydrofolate reductase gene as a selection marker (26). The resultant plasmids, pdKCR-dhfr-660 and pdKCR-dhfr-653, were transfected into CHO cells deficient in dihydrofolate reductase activity (CHO-dhfr$^-$) (27) by the calcium phosphate method (28). Cell populations expressing the cDNA together with dihydrofolate reductase were selected in the α-modification of Eagle's medium lacking ribonucleosides and deoxyribonucleosides and containing 10% dialyzed fetal bovine serum (Cell Culture Laboratories). From these cell populations, clonal cell lines were isolated by single-cell cloning. Expression of the cDNA was assessed by RNA blotting. As a control, CHO cells were mock-transfected (transfected only with the vector) and isolated. These cells gave no signal on RNA blotting. Crude membranes of CHO cells were prepared in the same way as those of COS cells.

Reconstitution of the EP_3 receptor with G_{i2} in PT-treated membranes was performed according to the method of Asano *et al.* (29). CHO cells stably expressing the EP_3 receptor isoforms were cultured in the presence or absence of 20 ng/ml PT for 9 h. CHO cell membranes (50 μg) were incubated with or without G_{i2} purified from bovine spleen (10 pmol/mg of the membrane protein) at 0 °C for 1 h. PGE_2 binding assays were carried out with 2.0 nM [^{3}H]PGE_2 in the presence or absence of 100 μM GTPγS.

cAMP Assay—$EP_{3\alpha}$- or $EP_{3\beta}$-CHO cells (5×10^5 cells/well) cultured in 24-well plates were washed with 0.5 ml of HEPES-buffered saline (140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, and 15 mM HEPES, pH 7.4) and then preincubated for 10 min in 450 μl of the solution and 1 mM 3-isobutyl-1-methylxanthine at 37 °C. Then 50 μl of the test agent and forskolin (final, 1 μM) in HEPES-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine was added to each well. After incubation for 10 min at 37 °C, the reaction was terminated by the addition of 500 μl of ice-cold 10% (w/v) trichloroacetic acid. The cyclic AMP formed was measured by a radioimmunoassay using an Amersham cAMP assay kit.

GTPase Activity Assay—Measurement of GTPase activity in $EP_{3\alpha}$- and $EP_{3\beta}$-CHO cell membranes was performed as follows. The final reaction mixture contained 10 μg of each CHO cell membrane, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM 3-isobutyl-1-methylxanthine, 0.1 mM phenylmethylsulfonyl fluoride, 0.25 mM ATP, 0.5 mM AppNHp, the indicated concentrations of PGE_2 or M&B28767, and 0.25 μM [γ-^{32}P]GTP (0.3 μCi) in 100 μl of 20 mM Tris-HCl, pH 7.5. The reactions were initiated by the addition of each CHO cell membrane, conducted for 10 min at 30 °C, and terminated by the addition of 0.5 ml of ice-cold 5% Norit A and 0.1% bovine serum albumin in 20 mM potassium phosphate, pH 7.5. The tubes were centrifuged for 5 min at $2{,}000 \times g$ at 4 °C, and the $^{32}P_i$ in 300 μl of each supernatant was counted in scintillation fluid. Under these conditions, the time course for GTPase activity of membrane adequately showed a linear relationship over a 10-min incubation period.

Amplification of RNA—Measurement of the relative amounts of the two receptor mRNAs expressed in each tissue was performed according to the method of Wang *et al.* (30). Total RNA was isolated from mouse brain, thymus, lung, heart, liver, stomach, spleen, ileum, kidney, testis, and uterus by the acid guanidinium thiocyanate-phenol-chloroform method (31), and the RNAs were transcribed into cDNA by the random hexanucleotide priming method using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). Each cDNA derived from 2.5 μg of RNA was used as a template in a PCR with primers corresponding to nucleotide positions 651–680 (PCR I) and 1264–1293 (PCR II). The 5′-end ^{32}P-labeled PCR II (0.3 pmol; 1.0×10^6 cpm/pmol) was incubated in each PCR reaction (final, 25 μl). Twenty-three cycles of PCR were performed using the following temperature profile: 94 °C, 40 s; 60 °C, 40 s; 72 °C, 1.5 min. The number of amplification cycles was optimized for quantification of RNA in preliminary experiments. The DNA-resolved gel was dried and then subjected to autoradiography, and the radioactivity of the gel corresponding to the bands was counted. An autoradiogram obtained in one of three independent experiments is shown in Fig. 7.

RESULTS

cDNA Cloning of Two Molecular Forms of the Mouse EP_3 Receptor—On the basis of the homology to thromboxane A_2 receptor sequences (32, 33), we recently isolated cDNA for the mouse PGE receptor (10). In this homology screening, we first obtained a partial PGE receptor clone from a mouse lung cDNA library, and using a fragment of this clone as a probe, we isolated several clones from a mouse mastocytoma P-815 library. Restriction analysis of the isolated clones revealed at least two types of cDNAs; five clones belong to one type represented by MP660, and three clones belong to another type represented by MP653. We first characterized MP660, found that it is a functional mouse EP_3 receptor cDNA, and reported it in a previous paper (10). We then characterized the other group of clones represented by MP653. Sequencing analyses revealed that MP653 had a 1,083-base pair (bp) open reading frame and that it was identical to MP660 in the nucleotide sequence except for the deletion of an 89-bp sequence in the coding region of the putative carboxyl-terminal tail of the MP660-encoded receptor (Fig. 1*a*). Fig. 1*b* shows the cDNA and deduced amino acid sequences of MP653 as compared with those of MP660. Deletion of this 89-bp sequence creates another reading frame downstream from this junction, which extends the coding region to a new stop codon located 77 bp downstream from the stop codon of MP660. As a consequence, a 30-amino acid carboxyl-terminal fragment of the MP660-encoded receptor (peptide-α) was replaced with a new 26-amino acid fragment (peptide-β) in the carboxyl-terminal end of the MP653-encoded receptor. Thus, the cytoplasmic carboxyl-terminal domains of the two EP_3 isoforms

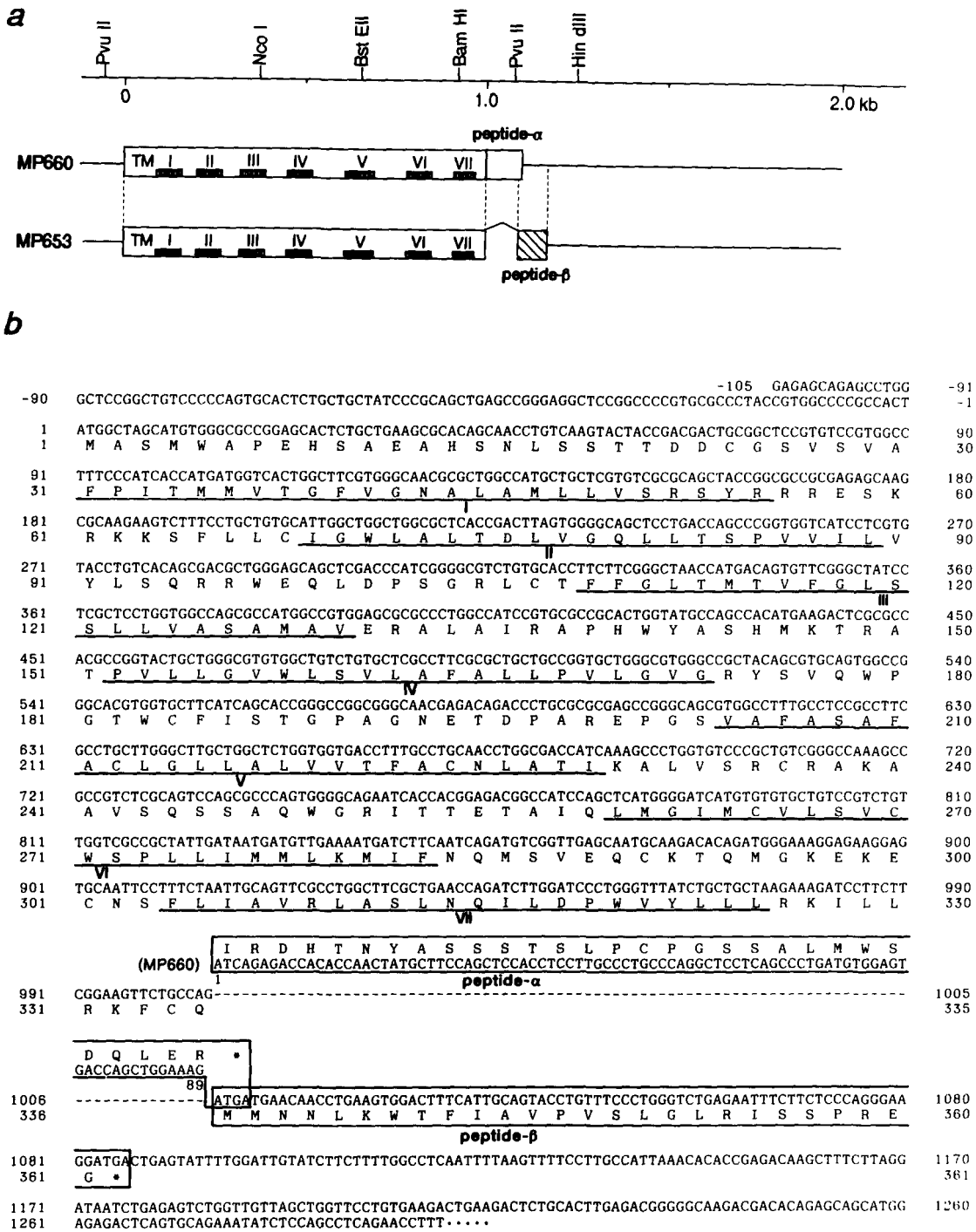

FIG. 1. **Comparison of the cDNA structures of the two EP_3 receptor isoforms.** *Panel a*, schematic representation of mouse EP_3 receptor cDNA clones, MP660 and MP653. *Boxes* represent coding sequences; the *open box* is the corresponding coding sequence in the two cDNAs, the *gray* one is the sequence coding peptide-α, and the *hatched* one is the sequence coding peptide-β. The putative transmembrane domains are indicated by *striped boxes*. *Panel b*, nucleotide and deduced amino acid sequences of MP653. The deduced amino acid sequence is shown under the nucleotide sequence using the single-letter code. The positions of putative transmembrane segments I–VII are indicated by *underlines* below the amino acid sequence. The region deleted in MP653 is represented by *hyphens*. The deleted 89-bp sequence is indicated above them, and deduced translation in MP660 is shown over the nucleotide sequence. Peptide-α and peptide-β are *boxed* with the termination codons indicated by *asterisks*.

consist of the 10 common amino acids in the amino-terminal region followed by these different peptides. Hydrophobicity analyses according to the method of Kyte and Doolittle (34) revealed that peptide-β forms a hydrophobic domain, whereas peptide-α is of a hydrophilic nature (Fig. 2). Peptide-α contains 9 serine and threonine residues, potential phosphorylation sites (35), whereas peptide-β contains only 4.

The two cDNAs were expressed in COS-1 cells, and their ligand binding properties were compared. Specific [^{3}H]PGE_2 binding was observed in the membranes of COS-1 cells transfected with MP660 and of those with MP653. Fig. 3 shows the effects of various unlabeled PGs and PGE receptor subtype-specific ligands on this binding. The binding of [^{3}H]PGE_2 to MP660- or MP653-transfected cell membranes was

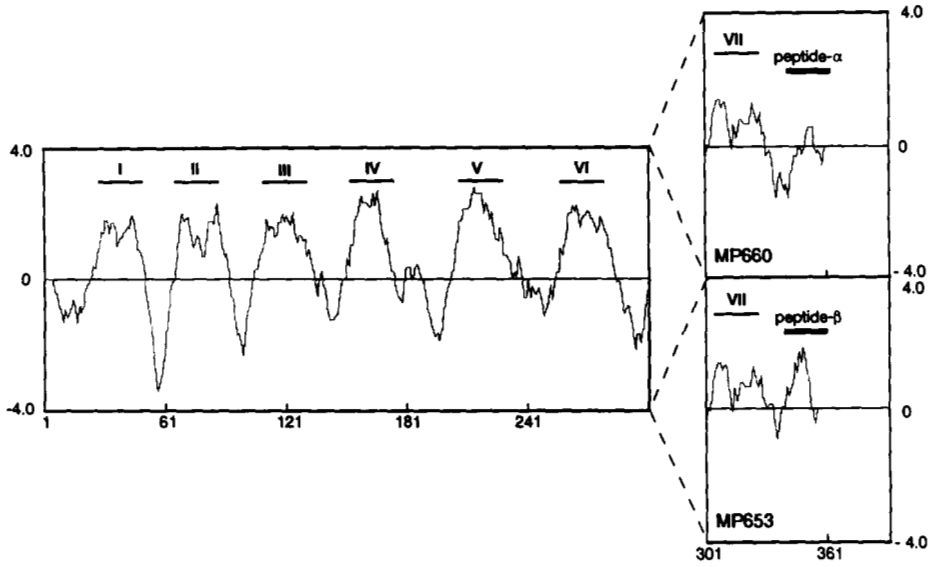

FIG. 2. **Comparison of the hydrophobicities of the amino acid sequences coded by MP660 (*upper*) and MP653 (*lower*), as analyzed by the method of Kyte and Doolittle (34).** The positions of the transmembrane domains and of peptide-α and peptide-β are indicated by *plain* and *bold lines*, respectively. Amino acids are numbered underneath.

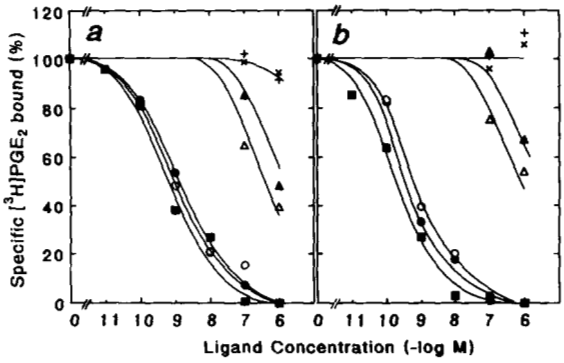

FIG. 3. **Displacement of [^{3}H]PGE_2 binding to the two EP_3 isofoms in transfected COS-1 cells by various prostaglandins and PGE subtype-selective ligands.** Unlabeled ligands were added to the binding assay mixture at the indicated concentrations, and [^{3}H]PGE_2 binding to MP660-transfected COS-1 cell membranes (*panel a*) and MP653-transfected cell membranes (*panel b*) was determined as described under "Experimental Procedures." The prostaglandins used were: PGE_2 (●), PGE_1 (○), $PGF_{2\alpha}$ (△), and PGD_2 (▲). The subtype-selective ligands used were: SC-19220 (×) for EP_1, butaprost (+) for EP_2, and M&B28767 (■) for EP_3.

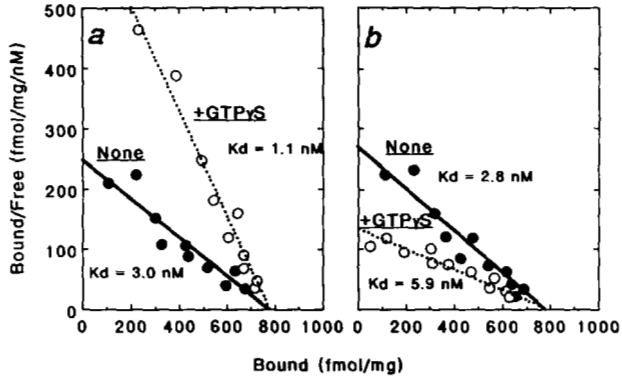

FIG. 4. **Scatchard plot analyses of the PGE_2 binding to each isoform in COS cell membranes in the presence or absence of GTPγS.** The specific binding of [^{3}H]PGE_2 (0.5–30 nM) to MP660 ($EP_{3\alpha}$; *panel a*) or MP653 ($EP_{3\beta}$; *panel b*) in transfected COS-1 cell membranes (50 μg) was determined in the presence (○) or absence (●) of 100 μM GTPγS as described under "Experimental Procedures." The Scatchard plot was transformed from the values of specific [^{3}H]PGE_2 binding.

inhibited in a similar concentration-dependent manner by unlabeled PGs and by an EP_3-selective ligand in the order of M&B28767 > PGE_2 = PGE_1 > $PGF_{2\alpha}$ > PGD_2. No competition was observed with either an EP_1-specific antagonist, SC-19220, or an EP_2-specific agonist, butaprost, in either type of membrane. These binding properties indicate that the two receptors have identical specificities and can be classified as the EP_3 subtype of PGE receptor (36). Hence, we have designated the MP660-encoding receptor as $EP_{3\alpha}$ (containing peptide-α) and the MP653-encoding receptor as $EP_{3\beta}$ (containing peptide-β).

GTPγS Conversely Affects the Binding Affinity for [^{3}H]PGE_2 of the Two Receptors via the Same G Protein—Binding characterization of the two expressed receptors revealed that their carboxyl-terminal sequences have no effect on their binding affinities or specificities. The carboxyl-terminal peptide of receptors has been shown to participate in receptor-G protein coupling (37), suggesting that the two receptors couple to a G protein differently. Receptor-G protein coupling can be examined in several ways. One way is to examine modulation of the binding affinity of a receptor by guanine nucleotides (16). We therefore examined the effect of a guanine nucleotide on [^{3}H]PGE_2 binding to the two EP_3 isoforms. Fig. 4 shows the results of Scatchard plot analyses of the specific [^{3}H]PGE_2 binding to each receptor in the presence or absence of GTPγS, a nonhydrolyzable GTP analogue. In the absence of GTPγS, both receptors exhibited indistinguishable binding affinities with dissociation constants (K_d) of 3.0 nM for $EP_{3\alpha}$ and 2.8 nM for $EP_{3\beta}$. The addition of GTPγS at 100 μM decreased K_d of $EP_{3\alpha}$ (1.1 nM), but it increased K_d of $EP_{3\beta}$ (5.9 nM), without any change in B_{max}.

The opposite responses of $EP_{3\alpha}$ and $EP_{3\beta}$ to GTPγS might be because of a difference in the type of G protein coupling to each receptor. This was examined in Chinese hamster ovary (CHO) cells stably expressing the two receptors. We first examined the effect of pertussis toxin (PT) treatment (Table t a fixed concentration of the ligand (2.0 nM), the addition of GTPγS enhanced [^{3}H]PGE_2 binding in $EP_{3\alpha}$-expressing cell membranes. PT treatment also enhanced this binding and additional stimulation by GTPγS was not observed. On the other hand, the GTPγS addition reduced [^{3}H]PGE_2 binding in $EP_{3\beta}$-expressing cells. PT treatment of this type of membrane reduced this binding to the same level as that with GTPγS and abolished the inhibitory effect of GTPγS. As a

TABLE I

Effects of pertussis toxin treatment and subsequent G_{i2} reconstitution on [^{3}H]PGE_2 binding to CHO cell membranes expressing the two EP_3 isoforms

CHO cells stably expressing the EP_3 receptor isoforms were cultured in the presence or absence of 20 ng/ml pertussis toxin for 9 h. CHO cell membranes (50 μg) were incubated with or without G_{i2} purified from bovine spleen (10 pmol/mg of the membrane protein) at 0 °C for 1 h. PGE_2 binding assays were carried out with 2.0 nM [^{3}H]PGE_2 in the presence or absence of 100 μM GTPγS as described under "Experimental Procedures." Values shown are means ± S.E. for triplicate experiments.

Treatment	Specific PGE_2 binding			
	$EP_{3\alpha}$		$EP_{3\beta}$	
	−GTPγS	+GTPγS	−GTPγS	+GTPγS
	pmol/mg protein			
None	0.723 ± 0.084	1.63 ± 0.043	1.59 ± 0.084	1.05 ± 0.058
Pertussis toxin	1.73 ± 0.095	1.70 ± 0.062	1.06 ± 0.081	0.970 ± 0.060
Pertussis toxin + Gi_2	0.699 ± 0.10	1.65 ± 0.11	1.54 ± 0.057	0.956 ± 0.062

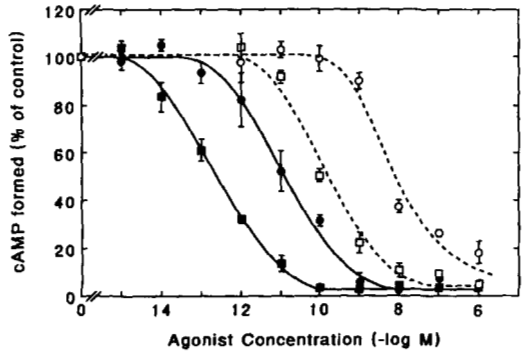

FIG. 5. **Comparison of adenylate cyclase inhibition by the two EP_3 receptor isoforms permanently expressed in CHO cells.** $EP_{3\alpha}$-CHO cells (*closed symbols*) or $EP_{3\beta}$-CHO cells (*open symbols*) were incubated at 37 °C for 10 min with 1 μM forskolin in the presence of the indicated concentrations of PGE_2 (●, ○) or M&B28767 (■, □), and then cAMP formation was determined as described under "Experimental Procedures." Each point represents the mean ± S.E. for triplicate determinations. cAMP formed in $EP_{3\alpha}$- and $EP_{3\beta}$-CHO cells treated with forskolin amounted to 16.6 ± 0.42 and 17.2 ± 0.32 pmol/10^5 cells, respectively.

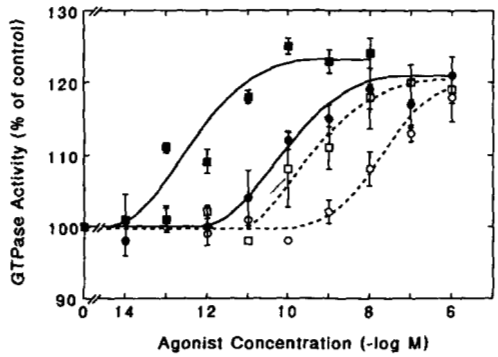

FIG. 6. **Comparison of agonist-induced stimulation of the GTPase activity in CHO cell membranes expressing the two EP_3 receptor isoforms.** GTPase activity of $EP_{3\alpha}$-CHO (*closed symbols*) or $EP_{3\beta}$-CHO (*open symbols*) cell membranes was measured in the presence of the indicated concentrations of PGE_2 (●, ○) or M&B28767 (■, □), as described under "Experimental Procedures." Each point represents the mean ± S.E. for triplicate determinations. The basal GTPase activity of $EP_{3\alpha}$- and $EP_{3\beta}$-CHO cell membranes was 16.6 ± 0.09 and 14.5 ± 0.18 pmol/min/mg of protein, respectively.

result, for both isoforms, PT treatment mimicked the effect of GTPγS and abolished it in these membranes. This suggests that both the $EP_{3\alpha}$ and $EP_{3\beta}$ receptors couple to a PT-sensitive tein, probably G_i. To determine whether or not G_i participates in these receptor systems, we reconstituted G_{i2} purified from bovine spleen into each type of PT-treated cell membrane. The addition of G_{i2} restored the effect of GTPγS on PGE_2 binding in each type of membrane (Table I), indicating that the opposite responses of $EP_{3\alpha}$ and $EP_{3\beta}$ to GTPγS are caused not by a difference in the type of coupling G protein, but by that in the carboxyl-terminal tails of the two receptors.

The Two EP_3 Isoforms Stimulate GTPase Activity and Inhibit Adenylate Cyclase with Different Efficiencies—Because both $EP_{3\alpha}$ and $EP_{3\beta}$ couple to G_i, as described above, and $EP_{3\alpha}$ mediates inhibition of adenylate cyclase (10), we compared the potencies of PGE_2 and its analogue as to inhibition of adenylate cyclase in the two receptor systems. As shown in Fig. 5, PGE_2 and M&B28767 dose-dependently inhibited forskolin-induced cAMP formation in $EP_{3\alpha}$-transfected CHO cells, the half-maximal concentrations for the inhibition being 1×10^{-11} M and 1×10^{-13} M, respectively. PGE_2 and M&B28767 also showed dose-dependent inhibition in $EP_{3\beta}$-transfected CHO cells, but the half-maximal concentrations for the inhibition (1×10^{-8} M for PGE_2 and 1×10^{-10} M for M&B28767) were 3 orders of magnitude higher than those in $EP_{3\alpha}$-transfected CHO cells. $EP_{3\alpha}$- and $EP_{3\beta}$-transfected CHO cells showed comparable levels of receptor expression (approximately 1.7 pmol of receptors/mg of membrane protein for both receptors), indicating that the difference in the half-maximal concentration for the inhibition is not caused by a difference in the expression levels of the receptors. No significant increases in the contents of cAMP and inositol phosphates were observed in either type of CHO cells on the addition of up to 1 μM M&B28767 (data not shown). These results suggest that both EP_3 receptors are engaged in an identical intracellular function, *i.e.* inhibition of adenylate cyclase, and that the efficiency of signal transduction in the $EP_{3\alpha}$ system is much higher than that in the $EP_{3\beta}$ receptor.

Because the two receptors showed identical affinities to the ligands, the above results suggested that the different efficiencies of the inhibitory actions of the two EP_3 systems are caused by a difference in the efficiency of the ligand-receptor complexes to associate with and activate G_i. We therefore analyzed the agonist-mediated activation of G proteins by examining the effects of PGE analogues on GTPase activity in each type of CHO cell membrane. As shown in Fig. 6, PGE_2 and M&B28767 dose-dependently stimulated the GTPase activity in $EP_{3\alpha}$-transfected CHO cell membranes, the half-maximal concentrations for the stimulation being 1×10^{-10} M and 1×10^{-12} M, respectively. The two agonists also stimulated the GTPase activity in $EP_{3\beta}$-transfected CHO cell

membranes, but the half-maximal concentrations (5×10^{-8} M for PGE_2 and 1×10^{-9} M for M&B28767) were about 3 orders of magnitude higher than those in $EP_{3\alpha}$-transfected CHO cell membranes. The maximal increase in GTP hydrolysis caused by PGE_2 and M&B28767 in $EP_{3\alpha}$-transfected CHO cell membranes was about 20% above the control level and was the same as that in $EP_{3\beta}$-transfected CHO cell membranes. This demonstrated that the different efficiencies as to the inhibition of adenylate cyclase are because of the differences in the G protein activation by the two receptor isoforms.

Expression of $EP_{3\alpha}$ and $EP_{3\beta}$ in Various Tissues—The relative abundance of mRNAs for the two isoforms in various tissues was investigated by PCR using primers at both sides of the 89-bp sequence in the presence of a 5′-end radiolabeled primer at one side, as described by Wang *et al.* (30) (Fig. 7). The upper and lower bands of the products corresponded to $EP_{3\alpha}$ and $EP_{3\beta}$, respectively. In any tissue expressing EP_3, $EP_{3\alpha}$ was dominantly expressed over $EP_{3\beta}$. In uterus and ileum, the $EP_{3\alpha}$ isoform was expressed at 3–4-fold higher levels than $EP_{3\beta}$. In brain, very little expression of $EP_{3\beta}$ could be detected under the PCR conditions used in spite of the significant level of expression of $EP_{3\alpha}$. In P-815 cells and stomach, on the other hand, the mRNA ratio for $EP_{3\alpha}/EP_{3\beta}$ was about 1.5, which is in accordance with the results of our P-815 cDNA library screening. Although the same level of $EP_{3\alpha}$ expression was observed in uterus and P-815 (Fig. 7), an apparently much higher level of expression of $EP_{3\beta}$ was detected in P-815 cells.

DISCUSSION

We report here the identification of two isoforms of the EP_3 receptor represented by MP660 and MP653 (Fig. 1). The two isoforms show identical ligand binding specificities, couple to the same G protein, probably G_i, and inhibit cAMP accumulation. The second isoform cDNA, MP653, has the same coding sequence except for the deletion of an 89-bp sequence in the cytoplasmic carboxyl-terminal domain. The nucleotide sequences delineating the boundaries of the 89-bp sequence in MP660 are consistent with consensus exon sequences for RNA splice junctions (38, 39). In fact, at the putative downstream splice junction of the 89-bp sequence, we have identified a 2.1-kilobase intron sequence by means of genomic PCR (data not shown). These results suggest that these two isoforms are generated from a single gene by alternative RNA splicing (40). Among G protein-coupled rhodopsin-type receptors, two dopaminergic D_2 receptors were the first reported to be produced by alternative splicing, which results in the addition or deletion of 29 amino acids in the third cytoplasmic loop (41–43). However, the presence or absence of the 29 amino acids did not significantly affect the ability of a receptor to inhibit cAMP production. On the other hand, the two carboxyl-terminal end peptides of the EP_3 isoforms, which are much the same in length but different in sequence, showed significant effects on the mode of receptor-G protein interaction. Two effects were observed on analyses of the binding and signaling properties of the two isoforms. The first effect is on the binding affinity of a receptor in the G protein-free form. As shown in Fig. 4, the two isoforms showed identical binding affinities in their G protein-bound forms. The addition of GTPγS increased the K_d for PGE_2 of $EP_{3\beta}$ and decreased that of $EP_{3\alpha}$. PT treatment, by causing the loss of the ability of the G_i protein to bind to a receptor (16), mimicked the effect of GTPγS. These results demonstrated that the ligand binding affinities of the two isoforms are either positively or negatively modulated by their cytoplasmic carboxyl-terminal tails in the G_i-free form. They also suggest that the G_i protein, by binding to these receptors, suppresses such actions of the cytoplasmic tails. The second effect is on the efficiency of a receptor to activate G protein. Although the two EP_3 isoforms couple to the same G protein, the EC_{50} values of PGE_2 and M&B28767 for G protein activation were different by 3 orders between the two EP_3 systems, and this difference was reflected in the different IC_{50} values of the agonists as to the inhibition of adenylate cyclase. Although the different EC_{50} values may be partly attributed to the difference in the K_d values for the G_i-free receptors, they are not enough to explain the 3 orders of magnitude difference in G protein activation by the two isoforms. Thus, the two cytoplasmic carboxyl-terminal tails differentially affect the affinity of the ligand-occupied receptors for the G_i protein; peptide-α allows the EP_3 receptor to associate with the G_i protein much more easily than peptide-β.

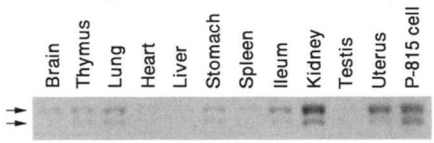

FIG. 7. **Relative amounts of $EP_{3\alpha}$ and $EP_{3\beta}$ in various tissues.** Aliquots (3 μl) of each PCR product were resolved in 1.5% agarose gel. *Arrows* indicate the 732-bp (*upper*; $EP_{3\alpha}$) and 643-bp (*lower*; $EP_{3\beta}$) DNA fragments, as ascertained by sequencing. The expression level was calculated by measurement of ^{32}P in each fragment. The PCR conditions and the number of amplification cycles were optimized in preliminary experiments. This figure shows an autoradiogram obtained in one of three independent experiments.

How do the two carboxyl tails cause such different effects on the receptor-G protein coupling? Hydrophobicity analyses demonstrated that peptide-α and -β show different hydrophobicities (Fig. 2). Peptide-α is hydrophilic, whereas the hydrophobicity of peptide-β was even higher than that of the seventh transmembrane segment. Peptide-β may be incorporated into the membranes and interact with other transmembrane segments, whereas peptide-α may be present in the cytoplasm. If so, these different hydrophobicities may influence the structures and functions of the two otherwise identical isoforms of the EP_3 receptor. Alternatively, the difference in the two receptors may be explained by the occurrence or not of palmitoylation. For the β-adrenergic receptor, it has been reported that the cysteine residue within the carboxyl-terminal tail region is palmitoylated with the fatty acid inserted in the membrane (44), and this conformation of the carboxyl-terminal domain is essential for the receptor-G protein coupling. The two isoforms contain a cysteine residue (Cys^{334}) in common at the ninth position out of the seventh transmembrane segment. An additional cysteine residue (Cys^{351}) is found in peptide-α at the 26th position, whereas no cysteine is found here in peptide-β. However, neither position of cysteine is consistent with the consensus position of the amino acid for palmitoylation (45). The difference in the carboxyl-terminal tails may confer an additional difference in receptor behavior. Carboxyl-terminal peptide-α contains 8 serine and threonine residues, whereas peptide-β contains only 4. In the rhodopsin or β-adrenergic receptor, these residues within this region are proposed to be phosphorylated by specific kinases that regulate the signaling activity of the ligand-occupied receptors (46, 47). Such regulation has been suggested for PGE receptors (48, 49). Therefore, the two carboxyl-terminal peptides might be differentially regulated by phosphorylation and involved in receptor desensitization and down-regulation.

The binding displacement experiment revealed that PGE_2 and M&B28767 showed almost equal binding affinities. For both agonists, K_d in the $EP_{3\alpha}$ system is about 1×10^{-9} M. On

the other hand, the IC_{50} values of agonists for inhibition of adenylate cyclase were lower (1×10^{-10} M for PGE_2 and 1×10^{-12} M for M&B28767) than K_d, indicating that agonist binding to a part of the $EP_{3\alpha}$ receptor is enough to inhibit adenylate cyclase. Furthermore, for inhibition of adenylate cyclase activity and stimulation of GTPase activity, M&B28767 showed 2 orders lower IC_{50} and EC_{50} than PGE_2 in both EP_3 receptor systems. This suggests that the M&B28767-receptor complex couples to the G protein and, hence, to adenylate cyclase more efficiently than the PGE_2-receptor complex.

The results on amplification of RNA (Fig. 7) demonstrated that in every tissue expressing EP_3, $EP_{3\alpha}$ is dominantly expressed, but the two EP_3 mRNAs coexist in different relative amounts. The pattern of the effect of GTPγS observed in $EP_{3\alpha}$ closely resembles that reported previously for the PGE binding sites in hamster adipocytes (19) and canine renal outer medulla (20), which are believed to be EP_3 receptors. Thus, $EP_{3\alpha}$ appears to be responsible for the GTP sensitivity of these tissues. Considering that PGE_2 exhibits different IC_{50} values for adenylate cyclase inhibition in the two EP_3 systems, the ratio of the two isoforms would affect the ability of individual tissues to respond to PGE_2. Different proportions of the two EP_3 isoforms might, therefore, underlie the diversity of EC_{50} values of PGE_2.

Thus, our study on the two EP_3 receptor isoforms will facilitate understanding of not only the heterogeneity of the actions of PGE_2 but also the functions of the cytoplasmic carboxyl-terminal tails of the G protein-coupled receptors. The carboxyl-terminal tail plays a role in signaling from both a G protein to a receptor and from a receptor to a G protein by modulating the ligand binding affinity of the receptor and the affinity of the ligand-receptor complex for the G protein.

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