The Second Extracellular Loop of the Prostaglandin EP<sub>3</sub> Receptor Is an Essential Determinant of Ligand Selectivity*

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The prostaglandin EP<sub>3</sub> receptor binds Prostaglandin E<sub>2</sub> in a ligand binding pocket formed in part by seven transmembrane α-helices. The present studies demonstrate that the second extracellular loop of the receptor is involved in prostanoid ligand recognition as well. Site-directed mutagenesis of seven conserved residues clustered in the amino portion of the second extracellular loop was performed. Receptors with single amino acid substitutions at each of these positions were transiently transfected into HEK293tsA201 cells, their ligand binding profiles assessed, and each receptor was tested for its ability to decrease intracellular cAMP levels. Substitution of Trp<sub>199</sub> or Thr<sub>202</sub> with alanine resulted in receptors with increases in affinity up to 12-fold for naturally occurring prostanoid compounds with a C1 methyl ester but wild type affinities for natural prostanoid ligands that have a carboxylate moiety at the C1 position. In contrast, substitution of Pro<sub>200</sub> with serine caused a loss of selectivity up to 20-fold for naturally occurring prostanoid agonists as compared with the wild type EP<sub>3</sub> receptor: the PS200 receptor displayed a decrease in affinity for E-ring compounds and an increase in affinity for F- and D-ring compounds. The EC<sub>50</sub> for inhibition of cAMP remained unchanged for each receptor tested.

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a mediator of a variety of physiological functions. Evidence suggests that many of these effects are the results of PGE<sub>2</sub> interacting with specific seven transmembrane G-protein-coupled receptors (GPCRs). Based upon their ligand binding selectivity and signaling pathway activation, these receptor subtypes are classified as EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub>, and EP<sub>4</sub> (1, 2).

Extensive structural information is available for GPCRs that bind small ligands (biogenic amine neurotransmitters, nucleotides, and opsins), and such studies have provided significant insights regarding the receptor amino acid residues required for ligand recognition (for review, see Ref. 3). Evidence supports the role of residues embedded in the transmembrane as important in receptor-ligand interactions (4). In contrast, the extracellular regions appear to be relatively unimportant for binding of these small ligands, with the exception of a role of an extracellular cysteine disulfide bridge demonstrated in several receptors including the β-adrenergic receptor (5) and the thromboxane A<sub>2</sub> receptor (6) as well as a portion of the second extracellular loop of the adenosine A<sub>1</sub> and A<sub>2a</sub> receptors (7, 8).

Letis is known regarding the structural determinants of EP<sub>3</sub>-receptor-ligand interactions. Several groups have previously identified the importance of an arginine residue found in transmembrane region VII of the EP<sub>3</sub> receptor and conserved throughout prostanoid receptors (9–11). Substitution of Arg<sup>229</sup> in transmembrane VII to either Ala or Glu led to a loss of detectable [H]PGE<sub>2</sub> binding and receptor-mediated inhibition of [cAMP] (9).

Comparisons of the amino acid sequence between the rabbit EP<sub>3</sub> receptor and the other cloned prostanoid receptors have identified several regions of conservation (9). Fourteen conserved amino acid residues were identified outside the putative transmembrane regions, including six amino acid residues clustered in the amino-terminal portion of the second extracellular loop. We hypothesized that conserved extracellular regions of the EP<sub>3</sub> receptor affect receptor/ligand interactions either directly or indirectly, analogous to the proposed interactions between the extracellular regions and ligands of peptide-binding GPCRs such as neurokinin-1 (13), thyrotropin (14), or [Arg<sup>8</sup>]-vasopressin receptors (15). To test whether this conserved primary structure plays a role in receptor-ligand interaction, a series of point mutants were generated and assayed for their ability to bind a panel of natural and synthetic prostanoid analogs. Findings presented herein provide evidence that the second extracellular loop of the prostanoid EP<sub>3</sub> receptor plays a role in ligand selectivity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Misoprostol and misoprostol-free acid were gifts of Dr. Paul Collins (Searle). All other prostanoid analogs were purchased from Cayman Chemical (Ann Arbor, MI). Isoproterenol, ascorbic acid, and indomethacin were purchased from Sigma. [H]PGE<sub>2</sub> was purchased from DuPont NEN. LipofectAMINE, and Opti-MEM were purchased from Life Technologies, Inc.

Site-directed Mutagenesis of the Receptor—Missense mutations were introduced using the polymerase chain reaction (PCR) as described previously (9). The sequence of the flanking oligonucleotides was as follows: upstream oligonucleotide (EP<sub>3</sub> nucleotide 501 coding), 5′ TG TGT TAC ATA CTA ACC GAG 3′; downstream oligonucleotide (EP<sub>3</sub> nucleotide 963 coding), 5′ CCA GGG ATC CAA TAT CTG G 3′.

The internal oligonucleotides used to introduce missense mutations are listed as follows (with underlining indicating nucleotide substitutions): QA198, 5′ A CAG TAC ACQ ATC GCC TGG CCC GG 3′; WA199, 5′ TAC ACC ATC CAA CAC CCT GCC GGG GAC 3′; PS200, 5′ ATG CAG TGA TCA GTG ACC TGG TCG TTC 3′; TA202, 5′ TC CAG TCA TGG CTT GTT GCA CCC CGG GAG 3′; WA203, 5′ C ACQ TCG TGG ACC TGG CTC TCC 3′; FA205, 5′ GT TGG CCC GTG ACC TGG TCG TTC ATC AGC 3′; CA204, 5′ CAG TGG CCC GTG ACC TGG TCT ATC AGC 3′; TA205, 5′ GT TGG CCC GTG ACC TGG TCG TTC ATC AGC 3′.

PCR fragments encoding the target amino acid substitutions were then subcloned into the hemagglutin-tagged 77A isoform of the EP<sub>3</sub> receptor in plasmid 77A hemagglutinin wt pRC/CMV, generating the full-length EP<sub>3</sub> 77A receptor (9). Two independent clones bearing each amino acid substitution were then isolated and characterized in subsequent experiments. The identity of the mutations was confirmed by

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The abbreviations used are: PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PCR, polymerase chain reaction; GPCR, G-protein-coupled receptor; wt, wild type; DP, prostaglandin D<sub>2</sub> receptor; FP, prostaglandin F<sub>2α</sub> receptor; IP, prostaglandin I<sub>2</sub> (prostacyclin) receptor.
performed using Instat (GraphPad).

Statistical analyses were performed using the method of Cheng and Prusoff (17). Statistical analyses were

incubated with 1 nM [3H]PGE2 and varying concentrations of unlabeled

EP3 receptor was performed against all cloned prostanoid rece-

tors. Conserved residues are indicated by hatched circles and invariant residues by filled circles. Residues with an inset symbol □ are conserved across the entire superfamily of GPCRs, those without the inset are unique to prostanoid receptors. In the bottom panel, the region of interest is indicated by the one-letter amino acid code of target amino acids. The resulting substitution followed by the position of the residue is also indicated.

sequencing both strands of the PCR amplified region using a Therm-Sequenase kit (Amersham Life Science, Inc.).

Expression of EP3 cDNAs in Cell Culture—HEK293tsA201 cells were transiently transfected with plasmids bearing wt or mutant EP3 cDNA as described (9). Cells were cultured for 72 h, and the medium was replaced every 24 h. At 72 h cells were lysed and membranes prepared as described (18). Protein concentrations were determined by the BCA assay (Pierce).

Ligand Binding Assays—For saturation binding isotherm experiments, 10–40 μg of membrane protein, representing 20 fmol of receptor, was incubated with various concentrations of [3H]PGE2, and reactions were stopped by filtration onto glass fiber filters as described (9). For competition binding assays, 10–40 μg of membrane proteins were incubated with 1 nM [3H]PGE2 and varying concentrations of unlabeled competitor and assayed as described above.

cAMP Measurements—HEK293tsA201 cells were transiently cotransfected with plasmids containing the human β2AR and either the wt or mutant EP3 receptor. cAMP measurements were performed by radioimmunoassay as described (9).

Data Analysis—Saturation binding isotherms, competition binding isotherms, and dose-dependent responses of cAMP, were analyzed using Prism (GraphPad, San Diego, CA). Kd values were calculated using the method of Cheng and Prusoff (17). Statistical analyses were performed using Instat (GraphPad).

RESULTS

Sequence Alignments of the Second Extracellular Loop Region of Prostanoids—Sequence alignments of the cloned rabbit EP3 receptor were performed against all cloned prostanoid receptors (9). A cluster of six conserved amino acid residues was identified in the putative second extracellular loop (Fig. 1). The sequence was identified as Q156WPCTWCF, where bold characters represent conserved amino acids. Pro200 is conserved throughout all cloned prostanoid receptors with the exception of the FP receptor. We tested whether this conserved portion of the receptor plays a role in the prostaglandin EP3 receptor function and/or structure.

Mutations of Trp799 and Thr202 Cause an Increase in the Affinity of Methyl Ester Compounds—TA202 displayed markedly increased affinities for methyl ester compounds of the E series as compared with the wild type receptor despite displaying similar dissociation constants for [3H]PGE2 (Kd(wt) = 1.4 ± 0.3 nM, Kd(TA202 = 1.3 ± 0.1 nM). As shown in Fig. 2A, TA202 resulted in a 128-fold increase in affinity for misoprostol as compared with the wild type receptor (Kd(wt) = 1600 ± 350 nM, Kd(TA202 = 13 ± 3 nM). In contrast, the affinity of TA202 for the carboxylate derivative misoprostol-free acid increased a modest 2-fold (Kd(wt) = 6.5 ± 1.9 nM, Kd(TA202 = 3.3 ± 0.6 nM). The Kd values for other natural and synthetic prostanoid agonists with a carboxylate at the C1 position were not statistically different from wild type (Table I). Sulprostone, which has a sulfonamide moiety at C1, had a modest 3-fold increase in affinity. To test if the increased affinity for misoprostol could be extended to other E series methyl ester compounds, TA202 was assayed with a panel of paired methyl ester/carboxylate prostanoid analogs that differed in their substituents at positions which differ between PGE2 and misoprostol: substitution at the C15 and C16 position and presence or absence of a double bond between C5 and C6. For each compound tested TA202 displayed in-

Fig. 1. Identification of conserved residues in the EP3 receptor. Sequence alignments were carried as described previously (9). Briefly, the predicted amino acid sequence of the rabbit EP3 receptor was used as the template and aligned against all cloned prostanoid receptors. Conserved residues are indicated by hatched circles and invariant residues by filled circles. Residues with an inset symbol □ are conserved across the entire superfamily of GPCRs, those without the inset are unique to prostanoid receptors. In the bottom panel, the region of interest is indicated by the one-letter amino acid code of target amino acids. The resulting substitution followed by the position of the residue is also indicated.

Fig. 2. Characterization of TA202 using PGE carboxylate and their methyl ester derivatives. Membranes were incubated with 1 nM [3H]PGE2 and varying concentrations of competitor. The data shown are from a single experiment performed in triplicate and are representative of three to five independent experiments. A, competition binding profiles of wt and TA202 receptors. Filled symbols are for wt, and open symbols are for TA202. ▲, TA202 (misoprostol-free acid); ▼, wt (misoprostol-free acid); □, TA202 (misoprostol); ■, wt (misoprostol). B, selectivity ratio of TA202 versus wt for various methyl ester derivatives. Kf values for the methyl ester of each compound was divided by the Kd value (nM) for its respective carboxylate compound. The number of independent experiments is indicated in Table II in parentheses. ■, wt; □, TA202.
TABLE I

Summary of competition binding isotherms of wild type and mutant EP<sub>3</sub> receptors

| Agonists | wt | TA202 |
|----------|----|-------|
| PGE<sub>2</sub> | 1.4 ± 0.1 (3) | 350 ± 20 (3) |
| PGE<sub>2</sub>, methyl ester | 0.9 ± 0.1 (3) | 280 ± 10 (3) |
| 16,16-Dimethyl-PGE<sub>2</sub> | 150 ± 30 (3) | 350 ± 20 (3) |
| 15(S),15-Methyl-PGE<sub>2</sub> | 85.5 ± 3 (3) | 250 ± 10 (3) |
| Misoprostol-free acid<sup>a</sup> | 1.6 ± 0.1 (3) | 1600 ± 160 (3) |
| PGE<sub>2</sub> | 1.4 ± 0.1 (3) | 350 ± 20 (3) |
| PGE<sub>2</sub>, methyl ester | 1.4 ± 0.1 (3) | 350 ± 20 (3) |

<sup>a</sup>p < 0.001.

<sup>b</sup>Data are taken from Table I.

TABLE II

Summary of competition binding isotherms of wild type and TA202 receptors to methyl ester compounds

| Agonists | wt | TA202 |
|----------|----|-------|
| PGE<sub>2</sub> | 3.6 ± 1.1 (3) | 350 ± 20 (3) |
| PGE<sub>2</sub>, methyl ester | 160 ± 20 (3) | 350 ± 20 (3) |
| 16,16-Dimethyl-PGE<sub>2</sub> | 350 ± 20 (3) | 350 ± 20 (3) |
| 15(S),15-Methyl-PGE<sub>2</sub> | 350 ± 20 (3) | 350 ± 20 (3) |
| Misoprostol | 1.1 ± 0.2 (3) | 350 ± 20 (3) |
| Misoprostol methylest | 1.0 ± 0.1 (3) | 350 ± 20 (3) |

<sup>n</sup>Values were averaged from three to five independent experiments ± S.E. The number of independent experiments is indicated in parentheses next to the Ki value. The two-tailed p values were determined by comparing the average values of Ki<sub>wt</sub> versus Ki<sub>mutant</sub>. *, p < 0.05; **, p < 0.01.

Missoprostol demonstrated a 5–7-fold decrease in affinity for PS200 as determined using PGE<sub>2</sub>, PGD<sub>2</sub>, and PGF<sub>2</sub> respectively (Table I, Fig. 3). Similarly, results obtained with the synthetic PGE analogs sulprostone and misoprostol-free acid demonstrated a 5–7-fold decrease in affinity, while misoprostol displayed a 5-fold increase in affinity. Thus, the overall pattern for the PS200 substitution was a loss in affinity for compounds that bind with high affinity to wt and an increase for compounds that bind with lower affinity to wt receptor. These results suggest Pro<sup>200</sup> plays a crucial role maintaining ligand binding selectivity.

The identity of Trp<sup>199</sup> is not highly conserved among prostanoid receptors as tyrosine, phenylalanine, or alanine may be found at this position. Nonetheless, substitution of Trp<sup>199</sup> with alanine resulted in a similar phenotype to the TA202 receptor. WA199 displayed a 9-fold increase in affinity for misoprostol and a 3-fold increase in affinity for sulprostone while the K<sub>i</sub> values for other compounds tested remained unaffected (Table I).

**Mutation of Pro<sup>200</sup> Affects Ligand Selectivity—**Proline 200 is conserved among the EP<sub>1</sub>, EP<sub>2</sub>, EP<sub>3</sub>, DP, IP, and TP receptors across species. In the FP receptor, this position is occupied by a serine residue. Pro<sup>200</sup> of the EP<sub>3</sub> receptor was mutated to Ser, and its binding characteristics were determined. The K<sub>d</sub> of PGE<sub>2</sub>, PS200 receptors. Membranes prepared from cells transfected with each receptor were tested in saturation binding experiments with a panel of natural and synthetic prostanoid analogs, and no statistically significant changes in affinity or order of agonist potency were observed (Table I). These results argue against the role of each individual residues in receptor-ligand interactions.
transduction properties of each receptor variant were determined. As compared with the wild-type receptor (EC_{50} = 480 pm), the various receptor EC_{50} values were: QA198 = 550 pm, WA199 = 360 pm, PS200 = 500 pm, TA202 = 370 pm, WA203 = 400 pm, CA204 = 370 pm, FA205 = 200 pm and did not display any statistically significant differences in receptor evoked signaling in three independent experiments.

**DISCUSSION**

Using site-directed mutagenesis studies of the prostaglandin EP_{3} receptor, the present results demonstrate that the second extracellular loop plays an integral role in receptor-ligand interaction and particularly in ligand selectivity. The gain of agonist affinity observed for several mutant receptor phenotypes argues strongly against gross perturbation of receptor structure by these amino acid substitutions as the cause of the altered ligand binding phenotypes. The precise role of the second extracellular loop in receptor-ligand interaction is unknown. One possible explanation for the observed phenotype is that the second extracellular loop forms part of the binding pocket and is in direct contact with the bound ligand. Alternatively, the effects of these mutations may be the result of an indirect role of the second extracellular loop. This is analogous to the Ca^{2+}, Na^{+}, or K^{+} channels where it has been proposed that extracellular loops fold into the ion channel pore and interact with the transmembrane helices (18). A third possibility is that the loop is important for the overall conformation of the receptor without direct interaction with the transmembrane helices. This interpretation may be consistent with the idea that modification of Trp^{203}, Pro^{200}, or Thr^{202} induces a general relaxation of the receptor conformation, preventing it from discriminating structurally related prostanoid analogs.

Several models supporting a direct role of extracellular domains of peptide/amino acid binding GPCRs have been described in the literature. In the case of the receptor for parathyroid hormone, it was suggested that amino acid residues near the extracellular surface of the transmembrane helices play a “filter” role allowing discrimination between various ligands (19). This concept could be extended to the EP_{3} receptor, where it is conceivable to consider Thr^{202} or Trp^{203} as filters which reduce affinity for compounds with a C1 methyl ester. Elimination of Thr^{202} or Trp^{203} side chains results in the elimination of a “gate” preventing methyl ester prostanoids from accessing the binding cleft. This model argues in favor of a two-step process in terms of receptor-ligand interaction. It has been suggested for the metabotropic glutamate receptor, mGLUR1, that the extracellular region of the receptor may act as a primary point of receptor-ligand interaction or “bait” and subsequently facilitate presentation to the transmembrane ligand pocket (20). Similarly, this model may be applied to the second extracellular loop of the EP_{3} receptor, whereby Trp^{203}, Pro^{200}, and/or Thr^{202} attract ligands and present them to the binding pocket. The filter and bait models are not mutually exclusive and may be complementary to one another.

It is interesting to note that mutation of Cys^{204}, which is conserved among all cloned prostanoid receptors, caused no detectable change in ligand binding affinity or receptor activation. These results are in sharp contrast to the results presented for the thromboxane A_{2} receptor, where replacement of Cys^{183} to Ser (analogous position as Cys^{204}) led a complete loss of binding and signaling (6). These authors suggested that a critical disulfide bond existed between Cys^{183} and Cys^{105} (transmembrane domain III) of the thromboxane A_{2} receptor; however, results presented here argue against the importance of a putative disulfide bridge involving Cys^{204} in the EP_{3} receptor.

It is also of interest that within this highly conserved amino-terminal portion of the second extracellular loop, substitutions at the other absolutely conserved positions Glh^{198}, Trp^{203}, Cys^{204}, and Phe^{205} did not detectably affect ligand binding or signal transduction. It may be that substitutions at several positions are required to disrupt receptor function. Alternatively, an untested function (e.g. internalization/recycling) may have been altered.

We propose a revised version of the model currently described for the EP_{3} receptor (21). We had previously shown that the EP_{3} and EP_{4} receptors displayed increased affinities for carboxylate compounds versus their methyl ester derivatives (9, 12). Furthermore, a large body of literature has suggested that the carboxylate moiety of C1 interacts with the positive side chain of arginine in transmembrane VII. The above results with TA202 suggest that a negative charge on C1 of prostanoids is not required for high affinity interactions with the EP_{3} receptor as shown for the TA202 receptor. Based on the high degree of homology of the second extracellular loop among cloned prostanoid receptors, it is conceivable these findings may be generalized to some or all of the prostanoid receptors. This may suggest that a revised three-dimensional model incorporating the extracellular loop regions is required to interpret receptor-ligand interactions.

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