A Cluster of Aromatic Amino Acids in the i2 Loop Plays a Key Role for Gs Coupling in Prostaglandin EP2 and EP3 Receptors*

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To assess the structural requirements for Gs coupling by prostaglandin E receptors (EPs), the Gs-coupled EP2 and Gs-coupled EP3 receptors were used to generate hybrid receptors. Intercalation of the whole i2 loop and its N-terminal half (i2N) had no effect on the binding of both receptors expressed in HEK293 cells. Agonist-induced cAMP formation was observed in wild type EP2 but not in the i2 loop- or i2N-substituted EP2. Wild type EP3β left cAMP levels unaffected, whereas i2 loop- and i2N-substituted EP3 gained agonist-induced adenylyl cyclase stimulation. In EP2, the ability to stimulate cAMP formation was lost by mutation of Tyr145 into Ala but retained by mutations into Phe, Trp, and Leu. Consistent with this observation, substitution of the equivalent His140 enabled EP3β to stimulate cAMP formation with the rank order of Phe > Tyr > Trp > Leu. The point mutation of His140 into Phe was effective in another EP3 variant in which its C-terminal tail is different or lacking. Simultaneous mutation of the adjacent Trp141 to Ala but not at the following Tyr142 weakened the acquired ability to stimulate cAMP levels in the EP3 mutant. Mutation of EP2 at adjacent Phe144 to Ala but not at Tyr145 reduced the efficiency of agonist-induced cAMP formation. In Chinese hamster ovary cells stably expressing Gs-acquired EP3 mutant, an agonist-dependent cAMP formation was observed, and pertussis toxin markedly augmented cAMP formation. These results suggest that a cluster of hydrophobic aromatic amino acids in the i2 loop plays a key role for Gs coupling.

Individual members of the superfamily of G protein-coupled receptors (GPCRs) efficiently interact only with a subset of the many structurally similar G protein heterotrimers (1–3). The spectrum of cellular responses triggered by activation of a specific GPCR is determined by the type of G proteins recognized by the activated receptor. It is therefore very important to elucidate the molecular basis governing the selectivity of receptor/G protein interaction for understanding cellular signal transduction.

Accumulating evidence indicates that multiple receptor regions of GPCRs are involved in G protein coupling and determining the selectivity of G protein recognition. Numerous studies have shown that the second intracellular loop (i2 loop), the membrane-proximal portions of the third intracellular loop (i3 loop), and the N-terminal segment of the cytoplasmic tail all contain amino acids predicted to play roles in regulating selectivity of receptor/G protein interactions (4, 5). Traditional mutagenesis approaches, including the use of hybrid receptors and alanine-scanning mutagenesis techniques, have led to important insights into the structural basis underlying the selectivity of receptor/G protein interactions (6). For example, intracellular loop 1 (i1 loop) is less important in determination of G protein selectivity but may indirectly contribute to G protein recognition. The i2 loop and i3 loop are of critical importance in determining the selectivity of receptor/G protein coupling and the efficiency of G-protein activation. The C-terminal tail plays a role in constraining basal activity, by preventing access of the G-protein to the receptor surface. Despite such information, it still remains controversial which receptor elements are critical for G protein selectivity and activation, and thus it is still difficult to predict whether a particular receptor can couple to a G protein.

Prostaglandin E2 (PGE2), one of the best known arachidonic metabolites, exhibits a broad range of biological actions in diverse tissues through their binding to specific receptors on the plasma membrane (7). We and other groups have revealed the primary structures of eight types of prostanoid receptors, including four subtypes of PGE receptor (EP1, EP2, EP3, and EP4), and demonstrated that they belong to the subfamily of rhodopsin-type (class I) GPCRs (8, 9). Prostanoid receptors thus have several unique features specific to prostanoid receptors in addition to those in common with other rhodopsin-type receptors; for example, they contain fewer basic or acidic amino acids throughout their putative transmembrane domains (10).

To assess the roles of such unique structural features, we have investigated the properties of receptors with mutations within such unique regions and demonstrated that the arginine residue within the putative seventh transmembrane domain conserved in all prostanoid receptors is important not only for interaction with the carboxylic acid group of agonists but also for particular signal activation (11–14). Furthermore, we found that the aspartate residue within the seventh transmembrane domain of the EP3 receptor plays a key role in governing G protein association and activation (15). On the other hand,
multiple EP3 receptor isoforms exist, which are different only in their C-terminal structures (16, 17). We found that these isoforms are different in their constitutive Gs activities and thus concluded that the C-terminal tail plays a role in constraining the basal activity, by preventing access of the Gi to the receptor surface (18–21). Thus, structurally close members of the GPCR subfamily such as the prostanooid receptors are useful not only for understanding prostanooid receptor-specific events but also for elucidating the general molecular basis of the structure and function relationship of GPCRs, including G protein selectivity.

To gain new insight into the mechanisms governing receptor-G protein coupling selectivity, we here designed a series of experiments using two members of the prostanooid receptors, aiming to identify structural requirements for selective Gi coupling. We first constructed Gi-coupled EP2 and Gi-coupled EP3 hybrid receptors with the i1, i2, or i3 loops interchanged and examined possible functional interchanges in Gi coupling in these mutant receptors. Second, we searched for the functional amino acids critical for Gi coupling.

EXPERIMENTAL PROCEDURES

Materials—Sulprostone and butaprost were generous gifts from Dr. M. P. L. Caton of Rhone-Poulenc Ltd. [5,6,8,11,12,14,15-3H]PGE2 (185 Ci/mmol) and a [3H]-labeled cAMP assay system were obtained from Amersham Biosciences. Forskolin was from Sigma, and pertussis toxin (gent grade). Amersham Biosciences. Forskolin was from Sigma, and pertussis toxin was from Seikagaku (Tokyo, Japan). All other chemicals were of reagent grade.

Construction of cDNAs for the mEP2, mEP3β, EP3α-based, and EP3-based Mutant Receptors—The functional cDNAs for mouse EP2 (mEP2), EP2β (mEP2β), EP3β, and T335 were previously cloned or generated in our laboratory (16, 22). The construction of pcDNA3-based expression plasmids (Invitrogen) encoding for wild-type mEP2 and mEP3β has been described previously (23). Various EP2/EP3 chimeric receptors and various mutant EP2 and EP3 receptors were prepared by standard PCR-based mutagenesis techniques (QuikChangeTM site-directed mutagenesis kit; Stratagene, La Jolla, CA). For EP2-based chimeras, the following mEP2 receptor sequences were replaced with the corresponding mEP3β receptor segments: EP2-i1, mEP2 47–67 → mEP3β 50–64; EP2-i2, mEP2 136–151 → mEP3β 133–148; EP2-i3, mEP2 222–262 → mEP3β 231–256; EP2-i2N, mEP2 136–143 → mEP3β 133–140; EP2-i2C, mEP2 144–151 → mEP3β 141–148. For EP3-based chimeras, the following mEP3β receptor sequences were replaced with the corresponding mEP2 receptor segments: EP3-i1, mEP3β 50–64 → mEP2 47–67; EP3-i2, mEP3β 133–148 → mEP2 136–151; EP3-i3, mEP3β 231–256 → mEP2 222–262; EP3-i2N, mEP3β 133–140 → mEP2 136–143; EP3-i2C, mEP3β 141–148 → mEP2 144–151. Single amino acid substitutions in mEP2, mEP3β, and T335 were introduced in a similar manner. All PCR-derived sequences were verified by dideoxy sequencing of the mutant plasmids.

Cell Culture, Transient Expression, and Surface Expression of EP2-based or EP3-based Mutant Receptors in HEK293 Cells—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum under humidified air containing 5% CO2 at 37 °C. For the transfection using the LipofectAMINE 2000 reagent (Invitrogen), cells in 60-mm tissue culture dishes were incubated at 37 °C for 4 h with a transfection mixture composed of 3 ml of Dulbecco’s modified Eagle’s medium, containing 10% heat-inactivated fetal bovine serum, 1 μg of DNA, and 15 μl of LipofectAMINE 2000 reagent. For the cAMP assay, HEK293 cells were then trypsinated, and aliquots of recovered cells were transferred to 24-well tissue culture plates. Surface expression of receptor proteins on HEK cell membranes was confirmed by an immunofluorescence assay using antibodies against the N-terminal region of the mouse EP2 and EP3 receptors under nonpermeabilized conditions.

PGE2-binding Assay—The harvested HEK293 cells expressing each receptor were homogenized using a Potter-Elevehem homogenizer in 20 mM Tris-HCl (pH 7.5), containing 10 mM MgCl2, 1 mM EDTA, 20 μM indomethacin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 250,000 g for 20 min, the pellet was washed, suspended in 20 mM Mes-NaOH (pH 6.0) containing 10 mM MgCl2 and 1 mM EDTA, and was used for the [3H]PGE2-binding assay. The membranes (50 μg) were incubated with various concentrations of [3H]PGE2 at 30 °C for 1 h, and [3H]PGE2 binding to the membranes was determined by adding a 1000-fold excess of unlabeled PGE2 into the incubation mixture. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

Measurement of cAMP Formation—Cyclic AMP levels in HEK293 cells were determined as reported previously (24). The receptor-expressing HEK293 cells cultured in 24-well plates (2 × 105 cells/well) were washed with HEPES-buffered saline containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 1.2 mM MgCl2, 1.1 mM KH2PO4, 11 mM glucose, 10 μM indomethacin, and 15 mM HEPES, pH 7.4, and preincubated for 10 min. Reactions were started by the addition of test reagents along with 100 μM Ro-20–1724. After incubation for 10 min at 37 °C, reactions were terminated by the addition of 10% trichloroacetic acid. The content of cAMP in the cells was measured by radioimmunoassay with a cAMP assay system (Amersham Biosciences).

Stable Expression of mEP3β, EP3-H1410F, mEP2, and EP2-Y143A in the Chinese Hamster Ovary (CHO) Cells—cDNAs for mEP3β, EP3-H140F, mEP2, and EP2-Y143A were transfected into CHO cells using the LipofectAMINE PLUS system according to the manufacturer’s instructions, and stable transformants were cloned as described previously (25). CHO cells expressing each receptor (5 × 105 cells) were pretreated with or without PT (20 ng/ml) for 7 h before the addition of the agonist. The cells expressing EP3 receptors were incubated at 37 °C for 10 min with or without sulprostone in the absence or presence of 10 μM forskolin. The cells expressing EP2 receptors were incubated at 37 °C for 10 min with or without butaprost. The cAMP contents were determined as described above.

Statistical Analysis—All data shown are expressed as means ± S.E. of three independent experiments. Statistical analysis was carried out by Student’s t test. p values of <0.005 were considered to indicate a significant difference.

RESULTS

Agonist Binding Properties in Hybrid EP2/EP3 Receptors—Wild type and mutant EP receptors analyzed in this study were transiently expressed in HEK293 cells and assayed for their ability to mediate agonist-dependent stimulation of adenyl cyclase (mediated by Gi). Consistent with its reported profile, the wild-type EP2 receptor (mouse, mEP2) caused a pronounced increase in intracellular cAMP levels upon stimulation with butaprost, an EP2 agonist. On the other hand, sulprostone stimulation of the wild type EP3β receptor (mouse, mEP3β) left cAMP levels unaffected. To explore the structural basis underlying Gi coupling, a series of hybrid EP2/EP3 receptors were created in which the intracellular domains were systematically exchanged between the two wild type receptors (Fig. 1A). EP2-i1 and EP3-i1 represent EP2 and EP3β with interchanged i1 loops, respectively. Moreover, we created hybrid receptors in which the N-terminal (i2N) or C-terminal (i2C) halves of the i2 loops (i2C) were individually exchanged between the wild type receptors as described below. For every mutant receptor used in this study, the expression of receptor proteins in HEK293 cells was examined by immunofluorescent analysis using antibodies against the N-terminal region of the mouse EP2 and EP3 receptors under nonpermeabilized conditions, and membrane surface expression and the expression levels of each mutant receptor were found to be comparable with those of wild-type receptors (Fig. 1A and data not shown).

Saturation binding studies showed that among the EP2-based hybrid receptors, EP2-i2, EP2-i2N, and EP2-i2C retained the ability to bind to the agonist [3H]PGE2 with high affinity, but EP2-i1 and EP2-i3 failed to bind to the agonist (Table I). The EP2-i2, EP2-i2N, and EP2-i2C hybrid receptors exhibited Ki values close to that obtained for the wild type EP2 receptor (Table I). [3H]PGE2 binding to these mutants was displaced by the addition of butaprost with Ki values similar to that of the wild type EP2 receptor (Ki for butaprost, 1.3–3.0 μM). These three hybrid receptors were expressed at levels similar to that found for the wild-type EP2 receptor (Bmax = 890–1110 fmol/mg; Table I). On the other hand, all EP3-based hybrid receptors except for EP3-i1 retained the ability to bind to [3H]PGE2. These hybrid receptors exhibited Ki values close to that ob-
Fig. 1. Structures and agonist-dependent G<sub>s</sub> activities of EP2/EP3 hybrid receptors. A, diagrams showing structures of mEP2, mEP3β, and the 10 mutant receptors used in this study and immunocytochemistry showing surface expression of the wild-type receptors and their chimeras. The part of the receptors derived from mEP2 is shown in black, and that from mEP3β is shown in gray. The amino acid sequences of the i1–i3 loops of EP2 and EP3 are shown below the diagrams, and the region interchanged between the two receptors is boxed. Extracellular N-terminal sequences were detected using corresponding antibodies on nonpermeabilized transfected HEK293 cells. The surface expression was visualized using secondary antibodies labeled by fluorescence. Background was compared using cells transfected with empty vector, pcDNA3 (Mock). B and C, agonist-dependent cAMP formation in HEK293 cells expressing mEP2 and EP2-based mutant receptors (B) and in HEK 293 cells expressing mEP3β and EP3-based mutant receptors (C). HEK293 cells expressing each receptor or pcDNA3-transfected HEK293 cells were seeded and cultured for 24 h before the assay (2 × 10<sup>5</sup> cells/well). For the mEP2 and EP2-based mutant receptors, the cells were stimulated for 10 min by adding media with the indicated concentrations of butaprost, an EP2-selective agonist (B). For the mEP3β and EP3-based mutant receptors, the cells were stimulated for 10 min by adding media with the indicated concentrations of sulprostone, an EP3-selective agonist (C). Amino acid
that found for the wild-type EP3 receptor (fmol/mg; Table I). Consistent with the previous reports (16, 25), the EP3-derived sequences are able to stimulate cAMP formation in a fashion about 1.5-fold higher than wild type EP2. In this transient expression system in HEK293 cells, the expression levels of wild type EP3 were also about 1.5-fold higher than wild type EP2.

**Agonist-dependent Stimulation of Adenylyl Cyclase by Hybrid EP2-EP3 Receptors**—The hybrid receptors showing considerable binding affinities for PGE<sub>2</sub> (EP3-2, EP3-3, EP3-i2N, and EP3-i2C) were then subjected to cAMP formation analysis. Wild-type mEP2 mediated a butaprost-dependent increase in cAMP. In contrast, the mutant EP2 receptor (EP2-i2) containing the EP3 receptor sequence in the i2 loop almost completely lost the ability to mediate agonist-dependent stimulation of adenylyl cyclase; butaprost failed to elicit a significant increase in cAMP production over the background level (Fig. 1B). These results suggested that the i2 loop of EP2 might be essential for G<sub>G</sub> coupling. On the other hand, substitution of the i3 loop of the EP3 receptor with the EP2 receptor resulted in a mutant receptor (EP3-i3) that was similar to the wild type EP3 receptor and lacked the ability to mediate stimulation of adenylyl cyclase. However, the mutant EP3 receptor (EP3-i2) in which the i2 half region of the EP3 receptor with the EP2 sequence resulted in a mutant receptor (EP3-i2N) that was similar to the wild type EP3 receptor (Table I). In contrast, EP2-Y143A showed an ~10-fold higher affinity to [3H]PGE<sub>2</sub> than wild type EP2.

In this transient expression system in HEK293 cells, the expression levels of wild type EP3 were also about 1.5-fold higher than wild type EP2.

**Effects of Point Mutations at Tyr<sup>143</sup> on G<sub>G</sub> Coupling of the EP2 Receptor**—Among the 8 amino acids in the i2N region, 3 amino acids were identical between mEP2 and mEP3β, which were candidates for key amino acids (Fig. 2A). In addition, the rat EP2 receptor contains an Ala residue at position 138 instead of Ser, indicating that Ser<sup>138</sup> is less important for G<sub>G</sub> coupling. We therefore constructed four mutant receptors with Ala mutations at each of the four candidate positions (EP2-Y136A, EP2-G140A, EP2-Y141A, and EP2-Y143A). Among these mutants, EP2-Y136A showed cAMP formation in an agonist dose-dependent manner similar to wild type EP2, whereas EP2-G140A and EP2-Y141A showed high efficacies of cAMP production similar to that of the wild type receptor, although they showed rightward shifted butaprost dose-response curves. In contrast, EP2-Y143A failed to increase cAMP formation above background levels (Fig. 2B). The binding properties of EP2-Y143A was similar to those of the wild-type receptor (Table I), suggesting that loss of cAMP producing activity is due to a loss of G<sub>G</sub> coupling and that Tyr<sup>143</sup> in EP2

### Table I

Summary of binding properties in mEP2, mEP3β, and their mutant receptors

| Receptor | K<sub>d</sub> (nM) | B<sub>max</sub> (fmol/mg) | K<sub>i</sub> for selective agonist<sup>a</sup> |
|----------|-----------------|----------------------|-------------------------------|
| mEP2     | 19.2 ± 2.1      | 934 ± 97             | 1.8 ± 0.09                    |
| EP2-i1   | ND              | ND                   | NP                            |
| EP2-i2   | 23.3 ± 1.1      | ND                   | NP                            |
| EP2-i3   | ND              | 1110 ± 83            | 2.2 ± 0.11                    |
| EP2-i2N  | 16.2 ± 1.4      | 1040 ± 96            | 1.7 ± 0.15                    |
| EP2-i2C  | 12.2 ± 1.8      | 803 ± 71             | 3.0 ± 0.12                    |
| EP2-Y143A| 32.0 ± 2.9      | 709 ± 82             | 3.3 ± 0.29                    |
| EP2-Y143F| 19.4 ± 2.2      | 638 ± 65             | 2.3 ± 0.18                    |
| EP2-Y143W| 21.1 ± 1.7      | 749 ± 38             | 2.0 ± 0.51                    |
| EP2-YAA  | 12.8 ± 2.0      | 110 ± 14             | NP                            |
| mEP3β    | 2.24 ± 0.33     | 1680 ± 123           | 4.3 ± 0.18                    |
| EP3-i1   | ND              | ND                   | NP                            |
| EP3-i2   | 1.53 ± 0.21     | 1520 ± 142           | 1.5 ± 0.09                    |
| EP3-i3   | 1.43 ± 0.18     | 1503 ± 64            | 1.1 ± 0.10                    |
| EP3-i2N  | 2.89 ± 0.32     | 1610 ± 105           | 2.3 ± 0.15                    |
| EP3-i2C  | 1.93 ± 0.13     | 1920 ± 99            | 3.1 ± 0.24                    |
| EP3-i140Y| 3.14 ± 0.26     | 1010 ± 118           | 4.0 ± 0.39                    |
| EP3-H140F| 2.68 ± 0.26     | 1092 ± 95            | 3.6 ± 0.78                    |
| EP3-H140A| 3.23 ± 0.35     | 1170 ± 87            | 2.9 ± 0.49                    |
| EP3-YAA  | 1.86 ± 0.22     | 1840 ± 124           | NP                            |

<sup>a</sup> K<sub>i</sub> values for butaprost (μM) and for sulprostone (nM) are indicated, respectively.
plays a critical role for $G_s$ coupling. We further examined the effects of various amino acid substitutions of Tyr$^{143}$ of EP2 on agonist-induced cAMP accumulation. All mutant EP2 receptors with single amino acid substitutions showed binding properties similar to the wild-type EP2 receptor (Table I and data not shown). Substitution of Tyr$^{143}$ with Phe (EP2-Y143F) resulted in a receptor stimulating cAMP production with an efficiency higher than that of the wild-type EP2 receptor (Fig. 2D). Agonist-dependent cAMP accumulation was observed in EP2-Y143W and EP2-Y143L, but their agonist dose dependence was lower than that of the wild type EP2 receptor. Substitution with other residues resulted in a great loss in the ability to stimulate the cAMP response (Fig. 2C). The potency order of mutants in butaprost-induced cAMP producing ability was as follows: EP2-Y143F > wild type > EP2-Y143W, EP2-Y143L = EP2-Y143N, EP2-Y143D, EP2-Y143R, EP2-Y143P, EP2-Y143I, EP2-Y143A = 0. These results suggested that the aromatic ring nature of tyrosine at this position in the EP2 receptor appears to be required for $G_s$ coupling with high efficiency.

Substitution of His$^{140}$ with an Uncharged Aromatic Residue Is Sufficient to Confer $G_s$ Coupling on the EP3 Receptor—In order to explore whether a single or a few amino acid mutations can confer $G_s$ coupling on mEP3, we constructed three mutant EP3 receptors, EP3-H140Y, EP3-R137G/H140Y, and EP3-A133Y/H140Y, all of which include conversion of His$^{140}$ into Tyr (Fig. 3A). Surprisingly, all three mutant EP3 receptors exerted sulprostone-dependent cAMP formation in a fashion similar to that of the mutant EP3-i2N receptor (Fig. 3B). This finding indicated that the single amino acid substitution of His$^{140}$ into Tyr is sufficient to confer $G_s$ coupling on EP3. We further constructed mutant EP3 receptors with His$^{140}$ replaced with various amino acids (Fig. 3A). All mutant EP3 receptors with single amino acid substitutions showed $[^{3}H]$PGE$_2$ binding properties similar to the wild-type EP3 receptor (Table I and data not shown). Substitution of His$^{140}$ with Phe resulted in a mutant EP3 receptor (EP3-H140F) with the most potent ability to stimulate cAMP production; its maximal cAMP production was 2-fold that of the EP3-H140Y receptor (Fig. 3D). Moreover, the mutant receptors with His$^{140}$ replaced with Trp and Leu (EP3-H140W and EP3-H140L) exerted moderate and slight increases in cAMP accumulation upon sulprostone stimulation, respectively. The EC$_{50}$ values for sulprostone of these four mutant receptors were similar (8.5–20 nM). In contrast, the mutant EP3 receptors with substitution of His$^{140}$ into other amino acids elicited no significant increase in cAMP levels (Fig. 3C). The potency order of mutants for sulprostone-induced cAMP-producing activity was as follows; EP3-H140F > EP3-H140Y > EP3-H140W > EP3-H140L = EP3-H140D, EP3-
H140N, EP3-H140R, EP3-H140A, EP3-H140P, EP3-H140I, wild-type mEP3β/H9252/H11005. The binding affinities of EP3 mutants for PGE2 and sulprostone were similar to that of the wild-type receptor (Table I and data not shown), suggesting that the difference in the cAMP response was not caused by an altered binding affinity for the agonist. These results indicate that substitution of His140 into a noncharged aromatic residue is sufficient to confer Gs coupling on the EP3 receptor. Moreover, the preference of aromatic residues in the efficiency of Gs coupling at the equivalent positions in both EP3 and EP2 receptors suggested that this amino acid contributes to Gs coupling in similar mechanisms for both EP2 and EP3 receptors.

A Cluster of Aromatic Residues at the Center of the i2 Loop Is Required for Efficient Gs Coupling—The present study suggested that the bulky aromatic amino acid at the center of the i2 loop may be one of determinants for Gs coupling in prostanoid receptors. However, when we examined the sequences of the i2 loop of the prostanoid receptors, we found that the EP2 receptor has two more aromatic amino acids, Phe144 and Tyr145, just after Tyr143. The existence of three aromatic amino acids at this position is conserved among all members of Gs-coupled prostanoid receptors. Interestingly, the EP3 receptors of various species also contain the latter two aromatic residues, Trp141 and Tyr142, just after the key position, His140 (Fig. 4). As shown above (Fig. 1, B and C), interchanging the i2C regions had little effect on the ability of the EP2 and EP3 receptors to stimulate adenyl cyclase activity, suggesting less importance of the i2C region for Gs coupling. However, this interchange did not alter the existence of the latter two aromatic residues in the cluster. We therefore hypothesized that the latter two residues in the cluster may have potential roles in Gs coupling, and we examined the effects of mutations at both or either aromatic residues in the i2 loop of the EP2 and EP3 receptors (Fig. 5). In the EP2 receptor, simultaneous alanine mutations of Phe144 and Tyr145 (EP2-YAA) led to a great reduction in the efficiency of agonist-induced cAMP production. A single alanine mutation at Phe144 (EP2-YAY) resulted in a significant reduction of the butaprost-dependent cAMP response, whereas mutation of Tyr145 to Ala (EP2-YFA) led to a slight increase in the efficiency of the agonist-induced cAMP response. The rank order of cAMP-producing activity (at 10^{-6} M) of these mutants was as follows: EP2-YFA ≥ EP2-YAY > EP2-Y143A (AFY) = 0. These results suggest that Tyr143 is the most critical for Gs coupling, but Phe144 is also required for highly efficient coupling, and Tyr145 contributes to Gs coupling only when an aromatic residue is not present at position 144. We investigated whether a similar tendency could be observed in the Gs-acquired EP3 mutant. As discussed above, EP3-H140Y (TWY), which has a cluster of three aromatic residues at the center of the i2 loop, exhibited agonist-dependent adenyl
cyclase activity, whereas wild type EP3β (HWY) showed no response upon sulprostone treatment. Simultaneous introduction of Ala residues at positions Trp 141 and Tyr 142 led to a complete loss of the ability to stimulate cAMP formation (EP3-YAA). A single alanine mutation at Trp141 (EP3-YAY) resulted in a receptor almost unable to stimulate cAMP production, whereas mutation of Tyr142 to Ala left agonist-dependent cAMP levels unaffected (EP3-YWA).

The rank order of these mutants in cAMP-producing activity was as follows: EP3-YWA > EP3-H140Y (YWY) > EP3-YAY > EP3-YAA, mEP3 (HWY).

Thus, similar results were obtained for the EP3 point mutants, indicating that the existence of a hydrophobic aromatic residue at position 140 is the most critical, but Trp141 and Tyr 142 also contribute significantly and little to Gs coupling, respectively. These results suggest that a cluster of aromatic residues at the center of the i2 loop plays a key role in high efficiency Gs coupling of the prostanoid receptors.

**A Gain-of-function Mutation Does Not Alter Intrinsic Gi Activity of the EP3 Receptor**—In this study, we used the mEP3β receptor as a prostanoid receptor that does not couple to stimulation of adenylyl cyclase and found that the point mutation at His140 is sufficient to confer Gs coupling on the EP3 receptor.
Critical Amino Acids for Gs Coupling in Prostanoid Receptors

A Gain of Function Is Independent of the C-terminal Structure of the EP3 Receptor—We previously reported that mouse EP3 isoforms with different C-terminal tails (EP3α, EP3β, and EP3γ) and C-terminal truncated form (T335) differ in their agonist-dependent Gs activity (21). Since these isoforms are different only in C-terminal structure, we previously demonstrated that the C-terminal tail could play a role in Gs coupling of EP3 receptor. Based on this notion, the effects of i2 loop mutations can be explained by modification of the C-terminal function in Gs coupling. To explore this possibility, we examined the effects of H140F mutation on cAMP-producing activity in other EP3 isoforms (Fig. 7). We employed EP3γ and C-terminally truncated T335, both of which increased cAMP levels in an agonist-dependent manner when expressed in CHO cells (21). In our previous report, the Gs activity elicited by EP3γ observed in CHO cells requires more than 10⁻⁶ M of agonist, and its maximal response is not as high as EP2 or EP4 receptors, and thus the Gs coupling is considered to be less efficient. Indeed, the increase in cAMP formation by wild-type EP3γ or T335 was hard to detect even in the presence of 10⁻⁵ M of agonist in the current expression system. On the other hand, introduction of H140F mutation into EP3γ or T335 resulted in a receptor showing agonist-dependent cAMP-producing activity with similar EC₅₀ values around 10⁻⁸ M. Moreover, a significant increase in basal cAMP levels in the absence of agonist was observed in both EP3γ-H140F and T335-H140F but not in EP3β-H140F. The increase in basal cAMP levels by the T335-H140F was significantly higher than that by the EP3γ-H140F. Instead, the agonist-dependent increase in cAMP levels in the mutant T335 appeared lower than that in the mutant EP3γ. However, in the current system, we could hardly detect cAMP increases with any significant difference in wild-type EP3γ and T335 even in the presence of 10⁻⁵ M agonist. These results suggested that the effects of i2 loop mutation on Gs coupling of EP3 are independent of C-terminal structure, which is likely to govern the balance of constitutive and agonist-induced G protein activation as observed in the Gs activity of the EP3 isoforms.

**Discussion**

One of the most important findings in this study is the "gain of function" of Gs activity of the EP3 receptor by a point mutation; conversion of the amino acid His¹⁴⁰ to the center of the i2 loop into an uncharged aromatic residue is sufficient to confer Gs coupling with high efficiency on the EP3β receptor without affecting intrinsic Gi coupling. We further established CHO cells stably expressing the wild-type EP2 (CHO-EP2) and EP2-Y143A receptor (CHO-EP2Y143A) and examined the effects of pertussis toxin on cAMP formation. The two cell lines exhibited same order of PGE₂ binding sites, but the CHO-EP2Y143A cells did not show any cAMP responses upon butaprost treatment (Fig. 6B). Moreover, pertussis toxin failed to restore butaprost-induced cAMP response, indicating that loss of agonist-induced cAMP-producing activity in EP2-Y143A is not a result of gain of Gs activity.

Since the bulky hydrophobic amino acid equivalent to His¹⁴⁰ of EP3 was proposed to be important in the general interaction with G proteins, we examined whether this point mutation affects intrinsic Gi activity. We established CHO cells stably expressing the Gi coupling-acquired mutant EP3 receptor (CHO-EP3H140F) and compared its functional properties with those of CHO cells expressing wild-type EP3β (CHO-EP3β). As observed in HEK293 cells, the two EP3 receptors showed similar binding affinities (EP3β, Kᵢ = 2.84 nM; EP3H140F, Kᵢ = 3.17 nM), but the expression level of EP3H140F was lower than that of EP3β cells (CHO-EP3β, B₅₀ = 1240 fmol/mg; CHO-H140F, B₅₀ = 367 fmol/mg). In CHO-EP3β cells, sulprostone did not elicit cAMP formation but inhibited forskolin-induced cAMP formation in a dose-dependent manner with an EC₅₀ of 3.1 nM (Fig. 6A). This inhibition by sulprostone was completely abolished by pretreatment of the cells with pertussis toxin. In contrast, in CHO-EP3H140F cells, sulprostone dose-dependently stimulated cAMP formation with an EC₅₀ of 22 nM, and the compound exhibited no more inhibition against forskolin-induced cAMP production. However, once the cells were pre-treated with pertussis toxin, sulprostone-induced cAMP formation was significantly potentiated even in the presence of forskolin. It should be noted that the potentiating effects of pertussis toxin were significantly observed even at 10⁻⁹ M, suggesting that this mutant receptor is capable of Gi coupling with high efficiency. These results indicate that the EP3-H140F receptor still has an intrinsic Gi activity. Thus, we conclude that the H140F point mutation is sufficient to confer Gs coupling with high efficiency on the EP3β receptor without affecting intrinsic Gi coupling.
acid residues at the position corresponding to Tyr143 of mEP2 in G protein coupling has been pointed out in studies on several kinds of rhodopsin-type receptors (26–29). This site is located at the C-terminal end of a highly conserved i2 loop motif with the following most common sequence: DRYXSIV or IXXPL, where X is any amino acid (2). The last residue in the consensus sequence, Leu, is replaced with Phe or Met in some members of Gα-coupled receptors. According to the report by Moro et al. (27), Leu131 in the human M1 muscarinic receptor, which is equivalent to Tyr143 of the mEP2 receptor, is critical for stimulation of phosphatidylinositol turnover (Gq coupling). Moreover, they showed that introduction of the equivalent point mutation F193A into the β2 adrenoreceptor caused a significant loss in isoproterenol-induced cAMP accumulation (Gα coupling). Based on these findings, they concluded that the bulky hydrophobic amino acid at this position is an important amino acid that governs general coupling with any kind of G protein.

However, the current finding that conversion of His140 into Phe in the EP3 receptor failed to alter Gγ coupling (Fig. 6) may suggest that EP3 does not require the particular amino acid at this position for efficient Gγ coupling. Indeed, the importance of the hydrophobic amino acid at the corresponding position has not been reported for Gγ-coupled receptors. However, the His residue is completely conserved in EP3 receptors derived from various species and is quite unique to EP3 in the GPCR family (Fig. 4). Interestingly, the His residue observed at the key position of EP3 also has a positively charged imidazole structure, which is ineffective in Gγ coupling. This can also be interpreted to signify that the His residue participates in the Gγ selectivity of EP3 receptor by preventing efficient Gγ coupling. In this respect, Gγ-coupled receptors contain a nonhydrophobic amino acid at this position; the EDG2 and EDG3 receptors have a basic amino acid, and chemokine receptors, CXCR4 and CXCR6, have a threonine residue (Fig. 4). The variety of amino acids at this position in Gγ-coupled receptors may reflect a variety in the way to exert their Gγ selectivity and the existence of some other domains such as the C-terminal region of the i3 loop to be required for Gγ activation with high efficiency as suggested for the M2 muscarinic receptor (30, 31).

The current study indicated that both EP2 and EP3 require one of the following amino acid residues: Phe, Tyr, Trp, or Leu at position 143 or 140, respectively, for efficient Gγ coupling. However, the identity of the side chain moiety (Phe, Tyr, Trp, or Leu) affected different parameters of Gγ coupling between EP2 and EP3. In the EP2 receptor, the identity of aromatic moiety seems to affect the EC_{50} values of cAMP production only slightly, suggesting that Gγ coupling of the EP2 is also governed by other domains such as the i3 loop, as suggested by previous studies (32). In contrast, in the EP3 receptor, the identity of the aromatic moiety affected the maximal cAMP response without great changes in EC_{50} values. Thus, it seems that the Gγ activation efficiency by EP3 completely depends on the side chain moiety at this position, indicating an absolutely pivotal role of this amino acid in Gγ coupling of EP3. However, we cannot entirely exclude the possibility that the amino acid identity may alter the Gγ activation efficiency, which is usually included in outcomes in a detection system for Gγ activity.

Previously, we reported that three C-terminal variants, EP3α, EP3β, and EP3γ, and C-terminal truncated T335 differ in their agonist-dependent Gγ activity (EP3γ > T335 > EP3α > EP3β = 0) (21). Since these variants are different only in C-terminal sequence, we speculated that the C-terminal tail may function as a key regulator of Gγ coupling of EP3 receptor; β-tail prevents and γ-tail allows the interaction of Gγ with the common structure of the EP3 receptor. However, the current study demonstrated that “Gγ-excit able” EP3γ further acquired drastic Gγ activity, and such gain of function by the H140F mutation is reproduced in C-terminally truncated T335 (Fig. 7). Thus, the gain of Gγ activity is independent of C-terminal structure. In our previous report, the Gγ activity elicited by EP3γ observed in CHO cells requires agonist concentrations of more than 10^{-6} M, and its maximal response is still not as high as that observed for EP2 or EP4 receptors, and thus the activity is considered to be less efficient. Indeed, the agonist-dependent Gγ activity of wild-type EP3γ was undetectable in the current expression system. In contrast, the acquired Gγ activity in the mutant receptor is comparable to EP2 and EP4 in terms of the degree of maximal activity and agonist dose dependence and is thought to be essentially different from intrinsic Gγ activity appearing in EP3γ. However, the common EP3 structure that allows intrinsic Gγ activity may serve as a premise factor for point mutation resulting in gain of Gγ coupling with high efficiency. Whether an introduction of a cluster of aromatic residues at the center of i2 loop enables other Gγ-coupled receptors to gain Gγ coupling is an interesting issue to be examined. We also previously reported that EP3 variants are different also in their constitutive Gγ activity (T335 > EP3γ > EP3α > EP3β = 0) (18, 21). It is quite interesting that H140F mutants exhibited basal Gγ activity with rank order of T335 > EP3γ > EP3β = 0, which is in good accordance with the potency order of constitutive Gγ activity in EP3 variants. Moreover, it should be noted that the agonist-dependent Gγ activity in the mutant T335 appeared less than that in the mutant EP3γ; the Gγ activity elicited by T335 receptor has been shown to be completely constitutive. These results suggested that the effects of i2 loop mutation on Gγ coupling of EP3 is independent of C-terminal structure, which is likely to govern the balance of constitutive and agonist-induced G protein activation as observed in the Gγ activity of the EP3 isoforms. Importantly, these results suggest a general role for the C-terminal tail in G protein coupling; the
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C-terminal tail plays a critical role in constraining the constitutive activity irrespective of class of coupling G proteins.

One of the remarkable findings in this study is that a cluster of aromatic amino acids beginning with Tyr143 or the corresponding residue is required for Gs coupling with high efficiency in prostanoid receptors (Fig. 5). This feature, the existence of three bulky aromatic amino acids following the conserved proline residue, is unique to Gs-coupled prostanoid receptors (Fig. 4). The 3 amino acids just after the proline in the four Gs-coupled prostanoid receptors are YFY, FYF, or YLY, whereas the other members contain HWY, LIH, IFH, or FSR. The present study demonstrated that the existence of an uncharged aromatic residue at the first position is the most critical for Gs coupling. However, the simultaneous introduction of alanine mutations at the following two residues resulted in a significant loss of efficiency in Gs activity in EP2 and EP3-H140Y receptors. Moreover, the existence of an aromatic residue (Phe144 in EP2 and Trp144 in EP3) at the second position appears to be required for Gs coupling with high efficiency. In contrast, the Tyr residue at the third position is dispensable if the first two residues are aromatic, but this residue is likely to take part in Gs coupling in the absence of an aromatic residue at the second position. Based on these results, we concluded that Gs coupling is controlled by the three aromatic amino acids following the conserved Pro residue with a rank order of contribution of first > second > third residue in the prostanoid receptors.

How does the aromatic residue contribute to Gs coupling with high efficiency? The rank order of amino acids critical for efficient Gs coupling of EP2 and EP3 receptors is as follows: Phe > Tyr > Trp > Leu >> other amino acids = 0 (Figs. 2 and 3). There is no doubt that the C-terminal 5 amino acids of the Go subunit are important for its selective binding to the receptors; both Gs and Go11 families contain a Tyr residue at −4 from the C-terminal end, whereas the Gi family contains a quite different amino acid, cysteine, at this position (33–35).

Recently, Liu et al. demonstrated that the aromatic moiety of the Tyr residue conserved at −4 from the C-terminal end of the Goα and Goq plays a key role in receptor/G protein interactions with high efficiency (36). By point mutation analysis, they demonstrated that agonist-induced Goα11 activation is controlled by the identity of the −4 residue with the rank order of Phe > Tyr > Trp >> other amino acids. Although they did not examine the effect of the Leu mutation, the three most effective amino acids, Phe, Tyr, and Trp are completely identical to the amino acids critical for Gs coupling at the key position in both EP2 and EP3 receptors. From these results, we speculate that the bulky aromatic amino acid in the i2 loop takes part in recognition of the Tyr residue conserved in Gs and Gq through a mechanism such as π electron interactions. In such a case, a cluster of aromatic residues may contribute to strengthen the interaction with or to accelerate the recognition of the tyrosine residue at the −4 position of Giα. Recently, Erlenbach et al. (29) employed an yeast screening system, in which random mutations were introduced into the Giα-coupled vasopressin V2 receptor, to detect amino acid mutations affecting receptor interaction with the C-terminal tail of Gi proteins. They found that a single amino acid substitution at Met145 into Leu or Trp within the i2 loop equivalent to Tyr143 of mEP2 allowed the V2 receptor to couple to both Giα and Giβ (29). They also discussed the possibility that Met145 is a strong candidate site for interaction with G proteins based on the analogy of the high resolution x-ray structure of bovine rhodopsin. According to the original report, the i2 loop exhibits an L-like structure when viewed parallel to the membrane plane but lacks regular secondary structure (37). Because the cytoplasmic extension of TM III and the N-terminal segment of the i2 loop show considerable sequence homology among GPCRs of the rhodopsin family, it is likely that the i2 loop of the EP2 receptor adopts a structure similar to that observed in rhodopsin. If this is correct, the cluster of aromatic residues from Tyr143 to Tyr145 is predicted to be located just N-terminal of the bend of the L-like structure that is a characteristic feature of the i2 loop where it is easily accessible for interactions with G proteins. Taken together, we propose a cluster of aromatic amino acids in the i2 loop as a strong candidate for an interaction site with the Gi protein.

The current study demonstrated that interchanging of the i2 loop or the N-terminal or C-terminal half region of the i2 loop between EP2 and EP3 left the individual binding affinities and the specificity and expression levels of the receptors unaffected. These results may reflect the fact that the i2 loops do not directly contribute to the formation of the ligand binding pocket. On the contrary, the interchanging of the i1 loops resulted in loss of binding ability of both receptors. Although all EP receptors can recognize PGE2 as a natural ligand, it has long been suggested that each EP receptor recognizes different functional groups of agonists (38). Since it was recently proposed that both TM I and II contribute to receptor recognition of different functional groups of prostanoid ligands (39), the i1 loop of the prostanoid receptors may be critical in the formation of subtype-specific ligand binding pockets. Interchanging of the i3 loops differently affected the binding properties of the wild type receptor; EP2 lost but EP3 retained the ability to bind to PGE2. This finding may reflect the fact that EP2 requires an i3 loop of appropriate length to form a binding pocket that can hold prostaglandin derivatives with a bulky structure (25).

In summary, we have demonstrated that a cluster of aromatic amino acids at the center of the i2 loop plays a key role in Gs coupling, at least in the prostanoid receptors. This study will be of help to understand the molecular mechanisms of G protein coupling selectivity by the individual GPCRs.

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A Cluster of Aromatic Amino Acids in the i2 Loop Plays a Key Role for \( G_S \) Coupling in Prostaglandin EP2 and EP3 Receptors

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