Amino Acid Residues Conferring Ligand Binding Properties of Prostaglandin I and Prostaglandin D Receptors

IDENTIFICATION BY SITE-DIRECTED MUTAGENESIS*

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Prostaglandins are a group of cyclooxygenase metabolites of C-20 unsaturated fatty acids and consist of the prostaglandins (PGs)1 and the thromboxanes (1). PGs share a prostanoid structure as a common structure and have two structural features to discriminate each other. First, they have functional groups attached at the 9- and 11- positions of a cyclopentane ring, which classifies them into four types: D, E, F, and I. Second, they are classified into three series, 1, 2, and 3, by the number of double bonds in the side chains (Fig. 1). Thromboxane A2 has an oxane ring instead of the cyclopentane ring. It is believed that PGE1 without a double bond in the α-side chain can mimic this configuration of PGI molecule and bind to IP, but this is not achieved by PGE2 (12). These results suggest that the ligand binding specificity of mIP is determined by two ways; one is the less strict specificity for the cyclopentane ring structures, and the other is the recognition of the configuration of the side chains.

The prostaglandin receptors can be functionally grouped into three categories: the relaxant receptors, the contractile receptors, and the inhibitory receptor (13). The relaxant receptors, consisting of IP, DP, EP2, and EP4, mediate increases in cAMP and induce smooth muscle relaxation. Sequence homology among these functionally related receptors is higher than that between the three separate groups (11, 14). According to the phylogenetic tree constructed upon these homologies, the EP2 and EP4 subtypes evolved from a primitive EP, and that IP, DP, and EP2 evolved together after the evolution of EP4 subtype. The amino acid sequences of mDP, mEP2, and mIP show 58% identity each other in the transmembrane domains (Fig. 2), whereas those of mIP and mEP4 show 44% identity. Taking advantage of the high homology between IP and DP, we previously constructed various chimeric receptors from mIP and mDP, and examined the regions conferring the ligand binding properties of these receptors (15). When the region extending from the sixth transmembrane domain to the carboxyl terminus of mIP was replaced with the corresponding region of mDP, this IPN-V/DPL-C receptor acquired the ability to bind PGD2 and PGE2 without decreasing the affinities of mIP to iloprost and PGE1. These binding characteristics did not change when the fourth and fifth transmembrane domains of mIP was further replaced with the corresponding regions of mDP. However, when the COOH-terminal end of the second transmembrane domain (Phe102) to the second intracellular neighborhood of mIP was replaced with the corresponding regions of mDP, this receptor still bound only iloprost. The amino acid substitutions in this chimeric suggest that Ser50 in the first transmembrane domain of mIP confers the broad ligand recognition of mIP and that Lys75 and Leu83 in the second transmembrane domain of mIP confers the high affinity to PGD2 and the strict specificity of ligand binding of mDP, respectively.

Using chimeras of the mouse prostaglandin (PG) I receptor (mIP) and the mouse PGD receptor (mDP), we previously revealed that the cyclopentane ring recognition by these receptors is specified by a region from the first to third transmembrane domain of each receptor; recognition by this region of mIP is broad, accommodating the D, E, and I types of cyclopentane rings, whereas that of mDP binds the D type of PGs alone (Kobayashi, T., Kiriyama, M., Hata, T., Hirata, M., Ushikubi, F., and Narumiya, S. (1997) J. Biol. Chem. 272, 15154–15160). In the present study, we performed a more detailed chimeric analysis, and narrowed the domain for the ring recognition to a region from the first transmembrane domain to the first extracellular loop. One chimera with the replacement of the second transmembrane domain and the first extracellular loop of mDP with that of mIP bound only iloprost. The amino acid substitutions in this chimera suggest that Ser50 in the first transmembrane domain of mIP confers the broad ligand recognition of mIP and that Lys75 and Leu83 in the second transmembrane domain of mDP confer the high affinity to PGD2 and the strict specificity of ligand binding of mDP, respectively.

Prostanoids are a group of cyclooxygenase metabolites of C-20 unsaturated fatty acids and consist of the prostaglandins (PGs)1 and the thromboxanes (1). PGs share a prostanoid structure as a common structure and have two structural features to discriminate each other. First, they have functional groups attached at the 9- and 11- positions of a cyclopentane ring, which classifies them into four types: D, E, F, and I. Second, they are classified into three series, 1, 2, and 3, by the number of double bonds in the side chains (Fig. 1). Thromboxane A2 has an oxane ring instead of the cyclopentane ring. It is believed that PGE1 without a double bond in the α-side chain can mimic this configuration of PGI molecule and bind to IP, but this is not achieved by PGE2 (12). These results suggest that the ligand binding specificity of mIP is determined by two ways; one is the less strict specificity for the cyclopentane ring structures, and the other is the recognition of the configuration of the side chains.

The prostaglandin receptors can be functionally grouped into three categories: the relaxant receptors, the contractile receptors, and the inhibitory receptor (13). The relaxant receptors, consisting of IP, DP, EP2, and EP4, mediate increases in cAMP and induce smooth muscle relaxation. Sequence homology among these functionally related receptors is higher than that between the three separate groups (11, 14). According to the phylogenetic tree constructed upon these homologies, the EP2 and EP4 subtypes evolved from a primitive EP, and that IP, DP, and EP2 evolved together after the evolution of EP4 subtype. The amino acid sequences of mDP, mEP2, and mIP show 58% identity each other in the transmembrane domains (Fig. 2), whereas those of mIP and mEP4 show 44% identity. Taking advantage of the high homology between IP and DP, we previously constructed various chimeric receptors from mIP and mDP, and examined the regions conferring the ligand binding properties of these receptors (15). When the region extending from the sixth transmembrane domain to the carboxyl terminus of mIP was replaced with the corresponding region of mDP, this IPN-V/DPL-C receptor acquired the ability to bind PGD2 and PGE2 without decreasing the affinities of mIP to iloprost and PGE1. These binding characteristics did not change when the fourth and fifth transmembrane domains of mIP was further replaced with the corresponding regions of mDP. However, when the COOH-terminal end of the second transmembrane domain (Phe102) to the second intracellular

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‡ The abbreviations used are: PG, prostaglandin; mDP, mouse prostaglandin D receptor; mIP, mouse prostaglandin I receptor; mEP2, mouse prostaglandin E receptor; DP, prostaglandin D receptor; EP2, prostaglandin E receptor; FP, prostaglandin F receptor; IP, prostaglandin I receptor; TP, thromboxane A2 receptor; CR, chimeric receptor; PCR, polymerase chain reaction.
loop of mIP was further replaced with those of mDP, this IPN-II(Val101)/DPII-Leu83-C receptor (formerly designated as IPN-II/DP1LC) markedly decreased the affinities to PGE₂, PGE₃, and iloprost, and bound the D type of PGs alone, as did IPN₁/DP1LC and mDP. These results suggest that the domains recognizing the ring structure and the side chain configuration are located in different regions. The sixth to seventh transmembrane domain of mIP confers the specificity of mIP to discriminate a structural difference in the α-side chain between PGE₁ and PGE₂, and hence determines the selectivity between PGE₁ and PGE₂. The binding domain for the cyclopentane ring localizes in a region containing the first to third transmembrane domain. This region of mIP shows the broad binding properties for the cyclopentane ring and accommodates the D, E, and I types, whereas that of mDP is strict between mIP and mDP.

A membrane topology model of the mIP and mDP in the transmembrane domains. A membrane topology model of the mIP receptor based on the hydrophobicity analysis by the method of Kyte and Doolittle (27) is shown. Solid circles indicate the residues that are identical between mIP and mDP. Solid lines indicate sites and restriction endonucleases used for construction of chimeric receptors (see “Experimental Procedures”).

Fig. 1. Structures of prostaglandins. Structures of prostanoic acid and PGs and a PG analogue used in this study, PGD₂, PGE₁, PGE₂, PGE₂a, and a PG₁ analogue, iloprost, are shown.

Fig. 2. Homology between mIP and mDP in the transmembrane domains. A membrane topology model of the mIP receptor was constructed by the method of Kyte and Doolittle (27). Solid circles indicate the residues that are identical between mIP and mDP. Solid lines indicate sites and restriction endonucleases used for construction of chimeric receptors (see “Experimental Procedures”).

Amino Acids Specifying Ligand Binding of DP and IP Receptors

EXPERIMENTAL PROCEDURES

Materials—PGD₂, PGE₁, PGE₂, and PGF₂α were generous gifts from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). (5,6,8,9,12,14,15-T-IPGD₂ (115 Ci/mmol), [H]iloprost (15.3 Ci/mmol), and iloprost were obtained from Amersham Pharmacia Biotech.

Construction of cDNA for Chimeric Receptors and Receptors with Point Mutations—The mIP and mDP cDNA were first subcloned into pCMX expression vector (16). The Ball-EcoRV fragment of CP302, a cDNA of mIP (8), and the Asp718-BamHI fragment of PG9, a cDNA of mDP (9), were subcloned into the EcoRV sites and the Asp718 and BamHI sites of pCMX, respectively. Four chimeric receptors and 10 point mutants (Fig. 3A) were then constructed by a PCR-based strategy using primer pairs and templates described in Table I. Five ng of each template was used for amplification by PCR in a reaction mixture containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM MgCl₂, 1 mM 2-mercaptoethanol, 10% dimethyl sulfoxide, 0.2 mM dNTPs, 2.5 units of Ex Taq polymerase (Takara Co. Ltd., Osaka, Japan), and 20 pmol of each primer in a total volume of 20 μl. After a denaturation step at 94 °C for 3 min, 20 cycles of an amplification step (94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min) were carried out and followed by a final elongation step of 3 min at 72 °C. PCR products were electrophoresed, excised, purified using a DNA fragment purification kit (Toyobo Co. Ltd., Osaka, Japan), inserted into pCMX, and sequenced by the dideoxy chain termination method. These mutant receptors have no insertion or deletion in the amino acid sequences.

The Chimeric IPN₁/DPII-C Receptor—Fragments N-1 and C-1 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-1 was digested with Asp718 and Psp1406I, and fragment C-1 was digested with Psp1406I and BamHI. Both digested fragments were ligated into the Asp718 and BamHI sites of pCMX-mIP.

The Chimeric IPN₁/DPII-C Receptor—Fragments N-2 and C-2 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-2 was digested with Asp718 and SpeI, and fragment C-2 was digested with SpeI and BamHI. Both digested fragments were ligated into the Asp718 and BamHI sites of pCMX-mIP.

The Chimeric IPN₁/DPII-C Receptor—Fragments N-3 and C-3 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-3 was digested with Asp718 and SnaBI, and fragment C-3 was digested with SnaBI and BamHI. Both digested fragments were ligated into the Asp718 and BamHI sites of pCMX-mIP.

The Chimeric IPN₁/DPII-C Receptor—Fragments N-4 and C-4 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-4 was digested with Asp718 and Eco47III, and fragment C-4 was digested with Eco47III and BamHI. Both digested fragments were ligated into the Asp718 and BamHI sites of pCMX-mIP.

Four mutants were constructed by site-directed mutagenesis of this chimeric DP₃₁/DP₃₂/DP₃₃ receptor.
The Chimeric CRT94K Receptor—Fragments N-5 and C-5 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-5 was digested with Asp718 and BsiWI, and fragment C-5 was digested with BsiWI and BspEI. Both digested fragments were ligated into the Asp718 and BspEI sites of pCMX-mDP.

The Chimeric CRT94K Receptor—Fragment N-6 was amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-6 was digested with PstI and fragment C-6 was obtained by digestion with PstI and BspEI of pCMX-PstI/EcoRI-DPIII-C. Both digested fragments were ligated into the PstI and BspEI sites of pCMX-mDP.

The Chimeric CRT94K Receptor—Fragments N-7 and C-7 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-7 was digested with Asp718 and Narl, and fragment C-7 was digested with NarI and BspEI. Both digested fragments were ligated into the Asp718 and BspEI sites of pCMX-mDP.

The Chimeric CRT94K Receptor—Fragments N-8 and C-8 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-8 was digested with Asp718 and Eco47III, and fragment C-8 was digested with Eco47III and BspEI. Both digested fragments were ligated into the Asp718 and BspEI sites of pCMX-mDP.

Five mutants were then constructed by site-directed mutagenesis of CRT94K receptor.

The Chimeric CRT94K/V103A Receptor—Fragments N-9 was amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-9 was digested with PstI and fragment C-9 was obtained by digestion with PstI and BspEI of pCMX-PstI/EcoRI-DPIII-C. Both digested fragments were ligated into the PstI and BspEI sites of pCMX-mDP.

The Chimeric CRT94K/A100M Receptor—Fragments N-10 and C-10 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-10 was digested with Asp718 and BsiWI, and fragment C-10 was digested with BsiWI and BspEI. Both digested fragments were ligated into the Asp718 and BspEI sites of pCMX-mDP.

The Chimeric CRT94K/S109Q Receptor—Fragments N-11 was amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-11 was digested with PstI and BspEI, and fragment N-11 was obtained by digestion with PstI of pCMX-CRT94K. Both digested fragments were ligated into the PstI and BspEI sites of pCMX-mDP.

The Chimeric CRT94K/V103A Receptor—Fragments N-12 was amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment C-12 was digested with PstI and BspEI, and fragment N-12 was obtained by digestion with PstI from pCMX-CRT94K. Both digested fragments were ligated into the PstI and BspEI sites of pCMX-mDP.

The Chimeric CRT94K/100M Receptor—Fragments N-13 and C-13 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-13 was digested with Asp718 and BsiWI, and fragment C-13 was digested with BsiWI and BspEI. Both digested fragments were ligated into the Asp718 and BspEI sites of pCMX-mDP.

The Chimeric CRT94K/100M Receptor—Fragments N-14 and C-14 were amplified by PCR with primer pairs and the template shown in Table I (Fig. 3B). Fragment N-14 was digested with Asp718 and Eco47III, and fragment C-14 was digested with Eco47III and BspEI. Both digested fragments were ligated into the Asp718 and BspEI sites of pCMX-mDP.

Ligand Binding Studies—Each receptor was transiently expressed in COS-7 cells cultured in 15-cm dishes by transfecting with 20 μg of plasmid DNA by the lipofection method (17). After culture for 60 h, the cells were harvested, washed once, and suspended in a buffer containing 20 mM Hapes-NaOH (pH 7.4) containing 5 mM MgCl₂, 140 mM NaCl, and 5 mM KCl. Binding assays were performed at 4°C for 1 h essentially as described previously (9). In competition experiments, the cells were incubated with 20 nM [3H]PGD₂ or 20 nM [3H]Iloprost in the presence of various concentrations of PGD₂, PGE₁, PGE₂, PGE₃, or iloprost. The incubation was terminated by the addition of 2 ml of ice-cold 10 mM Tris-HCl (pH 7.4) (the washing buffer), and the mixture was rapidly filtered through GF/C filters (Whatman International Ltd., Maidstone, United Kingdom). The filter was then washed with 5 ml of the washing buffer three times. The radioactivity in the filter was measured in 5 ml of Clear-Sol scintillation mixture (Nakalai Tesque, Kyoto, Japan). Non-specific binding was determined in the presence of 500-fold excess of unlabelled ligands in the incubation mixture. Kᵢ values were calculated from IC₅₀ values of radioligand binding as described previously (18).

RESULTS

We previously revealed that the binding domain for the cyclopentane ring of mIP extending from the first to third transmembrane domains has the broad recognition, accommodating the D, E, and I types of cyclopentane rings, whereas that of mDP has the strict recognition, accommodating the D type alone. We also found that further replacement of the region containing the COOH-terminal end of the second transmembrane domain (Phe¹⁰²) to the second intracellular loop of mIP with that of mDP resulted in loss of the broad recognition of mIP and gain of the strict specificity of mDP. This IP/V₁₀³/A₁₀⁰/M receptor, formerly designated as IP/V₁₀³/A₁₀⁰/M (15), markedly decreased the affinities to PGE₁, PGE₂, and iloprost, and bound PGD₂ alone. These results suggest that this region of mDP has amino acid residue(s) determining the strict specificity of ligand binding of mDP.

To define the regions conferring the ligand binding properties of mIP and mDP to discriminate the cyclopentane ring structure in more detail, the mIP, mDP, and six chimeric receptors including IP/V₁₀³/A₁₀⁰/M, IP/V₁₀³/A₁₀⁰/M, IP/V₁₀³/A₁₀⁰/M, IP/V₁₀³/A₁₀⁰/M, IP/V₁₀³/A₁₀⁰/M, and IP/V₁₀³/A₁₀⁰/M were expressed in COS-7 cells for binding studies. The cells were incubated with 20 nM amounts of either [³H]Iloprost or [³H]PGD₂, and the binding properties were analyzed by competition with PGD₂, PGE₁, PGE₂, and iloprost. We used in the present study intact cell suspensions for the binding assay, because more reproducible results were obtained in cell suspensions than in cell lysates used in the previous study (15). Representative analyses are shown in Fig. 4, and the results of several analyses are summarized in Table II. As shown in Fig. 4A, mIP showed a selective binding to iloprost and PGE₂ with the Kᵢ values of 62 ± 4 and 417 ± 59 nM, respectively (Table II), consistent with previous reports on the cloned mouse IP receptor (8) and on native IP receptor in various cells (19, 20). Also consistent with previous reports on the cloned mouse and human DP receptor (9, 21) and on native human DP receptor (22), only PGD₂ effectively displaced [³H]PGD₂ binding to mDP with a Kᵢ value of 11 ± 2 nM (Fig. 4B, Table II). We then examined the binding properties of the chimeric receptors. As described previously (15), IP/V₁₀³/A₁₀⁰/M bound PGD₂, PGE₁, PGE₂, and iloprost with the Kᵢ values of 555 ± 48, 100 ± 15, 155 ± 9, and 51 ± 7 nM, respectively (Fig. 4A, upper right, and Table II). Similar ligand binding properties were shown by IP/V₁₀³/A₁₀⁰/M, which has a further substitution of the third transmembrane domain. They bound PGD₂, PGE₁, PGE₂, and iloprost with the Kᵢ values of 275 ± 88, 55 ± 6, 55 ± 6, and 21 ± 3 nM, respectively (Fig. 4A, lower left, and Table II). These results indicate that the domain determining the binding specificity for the cyclopentane ring of mIP localizes in a region containing the first transmembrane domain to the first extracellular loop, and the third transmembrane domain is exchangeable. In contrast, the binding of PGE₁, PGE₂, and iloprost was almost abolished when the COOH-terminal end of the second transmembrane domain (Phe¹⁰²) to the first extracellular loop (Cys¹²²) of mIP was then further replaced (Fig. 4B, upper left, and Table II). This IP/V₁₀³/A₁₀⁰/M receptor bound PGD₂ alone. This binding specificity was consistent with that obtained by our previous study using the lysates of cells expressing this chimeric receptor (15), although the affinity was much lower than that found in the previous analysis; we found a difficulty in reproducing the previous high affinity of [³H]PGD₂ binding to this chimera in cell lysates. The above results suggest that some amino acid residue(s) from Leu⁸³ to Cys¹⁰⁴ of mDP are responsible for the exclusion of prostanoid molecules other than the D type from binding to the receptor. Similar strict
binding specificity was exhibited by IP$_{N}$/DP$_{I}$ and IP$_{N}$/DP$_{II}$, which have further substitution of the second and the first transmembrane domains (Fig. 4B, Table II). Notably, these IP$_{N}$/DP$_{I}$ and IP$_{N}$/DP$_{II}$ receptors showed approximately 4- and 160-fold increases in the binding affinity to PGD$_{2}$ compared with that of IPN/IPII/Ex1/DPIII-C, respectively. IPN/IPII/Ex1/DPIII-C receptor bound only iloprost but not PGE$_{1}$, PGE$_{2}$, or PGD$_{2}$ when Ser$_{50}$(mIP) was substituted for Gly$_{22}$(mDP) of DP N-I/IPII-Ex1/DPIII-C, whereas IPN-I/DPII-C and IPN/DPI-C receptors showed approximately 4- and 65-fold increases in the binding affinity to PGD$_{2}$, PGE$_{1}$, and PGE$_{2}$, respectively. IPN-I/DPII-C receptor bound iloprost with an affinity almost comparable to that of mIP (Table II). These results indicate first that the region of mIP from the second transmembrane domain to the first extracellular loop contains amino acid residue(s) conferring the iloprost binding, and second that amino acid residue(s) in the first transmembrane domain of mIP contribute to its broad binding properties. To identify the amino acid residue(s) determining the broad binding properties of mIP, four amino acid residues in the first transmembrane domain of mIP, Pro$_{47}$, Ser$_{50}$, Met$_{53}$, and Val$_{58}$, which were identical in mIP and mEP$_{2}$ but differed in mDP, were identified (Fig. 5). The corresponding mDP amino acid residues in the first transmembrane domain of DP N-I/IPII-Ex1/DPIII-C (Ala$_{19}$, Gly$_{22}$, Leu$_{25}$, and Leu$_{30}$) were changed to these amino acid residues in mIP by site-directed mutagenesis (Fig. 3A). The resultant CRA19P, in which Pro$_{47}$(mIP) was substituted for Ala$_{19}$(mDP) of DP N-I/IPII-Ex1/DPIII-C, the resultant CRG22S receptor recovered the ability to bind PGE$_{1}$, PGE$_{2}$, and PGD$_{2}$ with the $K_{d}$ values of 389 ± 76 nM, and the binding of PGD$_{2}$, PGE$_{1}$, or PGE$_{2}$ was negligible (Fig. 4A, lower right, and Table II). These results indicate first that the region of mIP from the second transmembrane domain to the first extracellular loop contains amino acid residue(s) conferring the iloprost binding, and second that amino acid residue(s) in the first transmembrane domain of mIP contribute to its broad binding properties.

### Table I

| Fragment | Sequence of 5’ primer$^a$ | Sequence of 3’ primer$^b$ | Template |
|----------|----------------------------|---------------------------|----------|
| N-1      | TAATACGACTCACTATAGGG       | CTAGCGCTGGGCATCCTGGGTGC   | mIP      |
| C-1      | GAAACCTTGCCCTCTCGATTGC     | TGACTTAGCTGCCAAAGGCGGAT  | mIP      |
| N-2      | TAATACGACTCACTATAGGG       | TGTACGTAGTTGATATTCAGCA   | mIP      |
| C-2      | GTACTGTTGATGCTGACGTGGT    | CCATATGGTCACACCA         | mDP      |
| N-3      | TAATACGACTCACTATAGGG       | CGGACATCGACTGCCAGATCAG   | mDP      |
| C-3      | GTAGCCTAGAAAAAGGCTCTCG    | CGGACATCGACTGCCAGATCAG   | mDP      |
| N-4      | TAATACGACTCACTATAGGG       | CCATATGGTCACACCA         | mDP      |

$^a$ All the sequences shown are from 5’ to 3’ direction. Underlined are restriction enzyme sites and codons for mutated amino acids shown in single-letter code.
mIP with the $K_i$ value of 2167 nM (Figs. 4A and 6C, Tables II and III). Neither $[^{3}H]PGD_2$ nor $[^{3}H]iloprost binding was observed in CR L25M/L30V, which contained substitutions of both Met53(mIP) and Val58(mIP) for Leu25(mDP) and Leu30(mDP) (data not shown).

To identify the amino acid residue(s) conferring the high affinity to PGD$_2$, two amino acid residues in the second transmembrane domain of mIP, Thr94 and Arg107, which were identical in mIP and mEP2 but differed in mDP, were identified (Fig. 5). These amino acid residues of DPN-I/IPII-Ex1/DPIII-C was individually changed to the corresponding mDP amino acid residues, Lys75 and Gln88 (Fig. 3A). Ligand binding of the resultant CR T94K and CR R107Q receptors was analyzed using $[^{3}H]iloprost as a radioligand (Fig. 6). CR R107Q, in which Gln88(mDP) was substituted for Arg107(mIP) of DPNI/IPII-Ex1/DPIII-C, showed no difference in the binding properties from DPN-I/IPII-Ex1/DPIII-C (Fig. 6D, Table III). On the other hand, CRT94K acquired the ability to bind PGD$_2$ with the $K_i$ value of 2367 nM, an affinity comparable to that of mDP, 1162 nM (Figs. 4B and 6E, Tables II and III). This mutant receptor retained its iloprost binding, indicating that the exclusion of iloprost is exerted by other residue(s).

To identify the amino acid residue(s) to exclude iloprost from binding to mDP, five mutant receptors were constructed from CRT94K. Because mEP2, like mDP, does not bind iloprost, four amino acids that are identical in mDP and mEP2 but differ in mIP, and one amino acid that differs between mDP, mEP2, and mIP were identified in the second transmembrane domain and...
the first extracellular loop (Fig. 5). Four of five amino acids were in the second transmembrane domain, and one was in the first extracellular loop. These mIP amino acid residues of CRT94K were individually changed to the corresponding mDP residues by site-directed mutagenesis (Fig. 3A). Ligand binding was analyzed using [3H]PGD$_2$ as a radioligand (Fig. 7). CRT94K bound PGD$_2$ and iloprost with the $K_i$ values of $7 \pm 1$ and $93 \pm 11$ nM (Fig. 7A, Table IV). Individual substitution of Leu$^{77}$(mDP), Met$^{81}$(mDP), Ala$^{84}$(mDP), and Gln$^{90}$(mDP) for Phe$^{96}$(mIP), Ala$^{100}$(mIP), Val$^{103}$(mIP), and Ser$^{109}$(mIP) showed an affinity to PGD$_2$ almost comparable to that of CRT94K. As for iloprost binding, one mutant receptor, CRT94K/F96L showed
about the same affinity as CR T94K itself (Fig. 7B, Table IV). On the other hand, each of the CR T94K/DPII-C, CR T94K/DPIII-C, and CR T94K/DPII-C chimeras showed the lower affinity to iloprost than CR T94K, albeit about the 1.4–2-fold difference (Fig. 7 C, E, and F, Table IV). A more marked reduction in the binding affinity to iloprost was observed when Leu83(mDP) was substituted for Phe102(mIP). The resultant CR T94K/F102L receptor showed about a 5-fold decrease in the binding affinity to iloprost in comparison to CR T94K without an appreciable decrease in the binding of PGD2 (Fig. 7D, Table IV).

**DISCUSSION**

Clarification of the ligand binding domain of receptors is important in understanding how each receptor responds selectively to its agonist, and ultimately contributes to development of more selective agonists and antagonists. The cyclopentane ring structures of each PG molecules are the primary determinant of the ligand binding affinity and specificity of each PG receptor. In this study, to identify the domains and amino acid residues responsible for recognition of each PG, we started with the high homology of the amino acid sequences. We constructed a series of mutant receptors from mIP and mDP based on their high homology in the first and second transmembrane regions and their flanking regions of four Gs-linked mouse prostanoid receptors. The amino acid sequences of the mouse IP, DP, EP, and EP4 receptors are aligned. The putative transmembrane domains are indicated by _italics_ above the sequences. Amino acid residues, which are discussed in the text, are highlighted.

This finding is to correct our previous proposal that the third transmembrane domain is exchangeable. This domain was interpreted as the ring recognition domain for the cyclopentane ring structures of each PG molecules are the primary determinant of the ligand binding affinity and specificity of each PG receptor. In this study, to identify the domains and amino acid residues responsible for recognition of each PG, we started with the high homology of the amino acid sequences. We constructed a series of mutant receptors from mIP and mDP based on their high homology in the first and second transmembrane regions and their flanking regions of four Gs-linked mouse prostanoid receptors. The amino acid sequences of the mouse IP, DP, EP, and EP4 receptors are aligned. The putative transmembrane domains are indicated by _italics_ above the sequences. Amino acid residues, which are discussed in the text, are highlighted.

because this domain plays an important role in recognition of ligands in the receptors for various biogenic amines with an Asp in this domain working as a counterion for the amine group of the ligands (23). Whether this finding is also applicable to other prostanoid receptors such as EP, FP, and TP can be tested by replacing the above region of one receptor with the corresponding region of the other receptor. The next finding obtained by the present chimera analysis was that the replacement of a region containing the second transmembrane domain and the first extracellular loop of DP with that of IP conferred the iloprost binding without binding of the D and E types of PGs. IP and DP are believed to have been evolved with EP2 and EP4 receptors, respectively, in the absence of the iloprost binding without losing the PGE binding, mainly by mutations in the region from the second transmembrane domain to the first extracellular loop. Similarly, the acquisition of PGD2 binding by DP appears to have been made also by mutations in this region with, in this case, a concomitant loss of PGE binding, because IP N/DPII-C showed the selective binding to PGD2. Exclusion of PGE binding in DP appears to be exerted also by mutation(s) in the first transmembrane domain, for DP N/IP II/DP III/C did not show the PGE binding. Indeed, a mutation of Gly in the first transmembrane domain of this receptor to the corresponding amino acid, Ser, in IP and EP3 recovered the PGE binding in the receptor.

In search for amino acid residues responsible for the identified effects of replacing the above mentioned domains between IP and DP, we engineered a series of mutant receptors in which amino acid residues derived from one receptor were mutagenized to the corresponding amino acids of the other receptor in DP N/IP II/DP III/C. By this procedure, we identified Ser50 of IP responsible for conferring the PGE as well as PGD binding back in the chimeric receptor as mentioned above, and Lys87 in the second transmembrane domain of DP required for the high affinity PGD2 binding. Interestingly, FP has a His residue at the corresponding position. Rehwald et al. (24) recently mutagenized this His to various amino acids, and found the decrease in binding of PGF2α to the FP receptor. Based on the pH effects on
the PG binding to the mutant receptors, they indicated that His at this position may work in the PGF_{2a} binding as a hydrogen bond donor. Our findings that Lys at this position of DP works to recognize the ring structure of the D type of PGs indicate that amino acid at this position may form a hydrogen bond with a functional group attached to the ring and not with a carboxyl group as suggested by these authors. The above identifications are consistent with the findings on the chimeric receptors described above. Also consistent is the identification of amino acid residues preventing iloprost binding in the DP receptor. The chimera study using IP_{II-Val101/II-Leu83-C} suggested that they localize in the COOH-terminal end of the second transmembrane domain and the first extracellular loop. The following point mutation study identified four amino acid residues, Met^{81}, Leu^{93}, Ala^{94}, and Gln^{95}, of DP, attenuating the binding of iloprost to DP, three of which localize in the above defined region. Although each of these mutations decreases the iloprost binding to these mutants by only 1.5–5-fold, in combination, as

**TABLE III**

Summary of binding studies on the DP_{N-I/II-Ex1/III-C} receptors with single mutations

| Compounds | $K_i$ (nM) | DP_{N-I/II-Ex1/III-C} | CRA19P | CRG22S | CRT94K | CRJ907Q |
|-----------|-----------|-----------------------|--------|--------|--------|--------|
| Iloprost  | 389 ± 76 (3) | 444 ± 67 (3) | 21 ± 7 (3) | 115 ± 35 (3) | 295 ± 57 (4) |
| PGE₁     | >1000     | >1000                        | 65 ± 12 (3) | >1000                        | >1000                        |
| PGE₂     | >1000     | >1000                        | 75 ± 18 (3) | >1000                        | >1000                        |
| PGD₂     | >1000     | >1000                        | 503 ± 73 (3) | >1000                        | >1000                        |
| PGF₂₅₀  | >1000     | >1000                        | >1000                        | >1000                        | >1000                        |
| $B_{max}$ (fmol/1×10⁶ cells) | 207 ± 50 (3) | 487 ± 168 (3) | 212 ± 103 (3) | 323 ± 75 (3) | 151 ± 27 (4) |

*Mean ± S.E., with the number of independent determinations indicated in parentheses.*

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**FIG. 6. Effects of point mutations on binding properties of the DP_{N-I/II-Ex1/III-C} receptor.** Binding of [³H]iloprost to the DP_{N-I/II-Ex1/III-C} (A), CRA19P (B), CRG22S (C), CRJ907Q (D), and CRJ907 (E) receptor was measured in the presence of various concentrations of PGD₂ (○), iloprost (●), PGE₁ (□), PGE₂ (▲), and PGF₂₅₀ (□) and expressed as percentage of binding compared with the control without a competitor.
seen in DP, they could be sufficient to exclude iloprost as observed in native DP. Interestingly, three of them are present in the COOH-terminal end of the second transmembrane domain, and only one is in the extracellular loop, suggesting that the iloprost exclusion is carried out mainly by the transmembrane domains and not as a consequence of filter action of the extracellular loops as recently shown for the EP3 receptor (25).

Thus, we identified several amino acids in the ligand binding domains of IP and DP conferring the binding properties of each receptor. However, it should be mentioned that this mutation analysis was carried out based on the DP1-IP3E-DP3E chimera receptor. The converse analysis should be performed on IP1-DP3 to identify amino acid residues of DP in the first transmembrane domain to increase the affinity for PGD2 binding, and, similarly, IP residues in the second transmembrane domain to enable iloprost binding. These analyses combined together will tell us exactly which amino acid residues in IP and DP determine the binding selectivity of each receptor, and such knowledge may help to define the ligand binding domains of other prostanoid receptors such as EP1, EP2, EP3, EP4, FP, and DP.

**Table IV**

Summary of binding studies on the CRT94K receptors with additional mutations

| Compounds | CRT94K | CRT94K/F96L | CRT94K/A100M | CRT94K/F102L | CRT94K/V103A | CRT94K/S109Q |
|-----------|--------|------------|-------------|-------------|-------------|-------------|
| Iloprost  | 93 ± 11 (3) | 89 ± 19 (3) | 219 ± 53 (3) | 443 ± 58 (4) | 126 ± 11 (3) | 176 ± 9 (3) |
| PGE1      | >1000  | 707 ± 103 (3) | >1000       | >1000       | >1000       | >1000       |
| PGE2      | >1000  | 377 ± 101 (3) | >1000       | >1000       | 665 ± 158 (3) | >1000 |
| PGD2      | 7 ± 1 (3) | 7 ± 2 (3) | 13 ± 3 (3) | 11 ± 4 (4) | 6 ± 1 (3) | 21 ± 6 (3) |
| PGF2a     | >1000  | >1000       | >1000       | >1000       | >1000       | >1000       |
| Bmax (fmol/1 × 10^6 cells) | 99 ± 25 (3) | 440 ± 49 (3) | 1633 ± 366 (3) | 269 ± 60 (4) | 403 ± 69 (3) | 620 ± 100 (3) |

* Mean ± S.E., with the number of independent determinations indicated in parentheses.

**Fig. 7.** Effects of additional mutations on binding properties of CRT94K. Binding of [3H]PGD2 to the CRT94K (A), CRT94K/F96L (B), CRT94K/A100M (C), CRT94K/F102L (D), CRT94K/V103A (E), and CRT94K/S109Q (F) receptors was measured in the presence of various concentrations of PGD2 (●), iloprost (○), PGE1 (△), PGE2 (▲), and PGF2a (□) and expressed as percentage of binding compared with the control without a competitor.
and TP.

PGs have two structural features, a cyclopentane ring and the side chains, and the receptors are supposed to recognize both of these structures and stabilize the ligand binding. Already much evidence has accumulated to suggest that the Arg residue conserved in the seventh transmembrane domain makes a hydrogen bond with the carboxyl group of prostanoid molecules (reviewed in Ref. 13). We previously used the IP/DP chimeras and suggested that the recognition of the side chain of iloprost and PGE1 takes place in the sixth and/or seventh transmembrane domains (15). Recently, Kedzie et al. (26) introduced the Leu$^{304}$ → Tyr substitution in the seventh transmembrane domain of the EP$_2$ receptor and found that this mutation caused an approximately 100-fold increase in the affinity to iloprost. These results taken together with our previous and present studies suggest that the ligand binding pocket of the prostanoid receptors are formed mainly by the first, second and seventh transmembrane domains, of which the former two are involved in the recognition of the ring structure and the latter in that of the side chains.

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REFERENCES
1. Samuelsson, B., Goldyne, M., Granstro¨m, E., Hamberg, M., Hammarstom, S., and Malmsten, C. (1978) Annu. Rev. Biochem. 47, 997–1029
2. Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S., and Narumiya, S. (1991) Nature 349, 617–620
3. Namba, T., Sugimoto, Y., Hirata, M., Hayashi, Y., Honda, A., Watabe, A., Negishi, M., Ichikawa, A., and Narumiya, S. (1992) Biochem. Biophys. Res. Commun. 184, 1197–1203
4. Sugimoto, Y., Namba, T., Honda, A., Hayashi, Y., Negishi, M., Ichikawa, A., and Narumiya, S. (1992) J. Biol. Chem. 267, 6463–6466
5. Honda, A., Sugimoto, Y., Namba, T., Watabe, A., Irie, A., Negishi, M., Narumiya, S., and Ichikawa, A. (1993) J. Biol. Chem. 268, 7759–7762
6. Watabe, A., Sugimoto, Y., Honda, A., Irie, A., Namba, T., Negishi, M., Itu, S., Narumiya, S., and Ichikawa, A. (1993) J. Biol. Chem. 268, 20175–20178
7. Sugimoto, Y., Hasumoto, K., Namba, T., Irie, A., Katsuyuka, M., Negishi, M., Kakizuka, A., Narumiya, S., and Ichikawa, A. (1994) J. Biol. Chem. 269, 1356–1360
8. Namba, T., Oida, H., Sugimoto, Y., Kakizuka, A., Negishi, M., Ichikawa, A., and Narumiya, S. (1994) J. Biol. Chem. 269, 9986–9992
9. Hirata, M., Kakizuka, A., Azawa, M., Ushikubi, F., and Narumiya, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11192–11196
10. Katsuyama, N., Nishigaki, N., Sugimoto, Y., Morimoto, K., Negishi, M., Narumiya, S., and Ichikawa, A. (1995) FEBS Lett. 372, 151–156
11. Regan, J. W., Bailey, T. J., Pepperl, D. J., Pierce, K. L., Bagordus, A. M., Donello, J. E., Fairbairn, C. E., Kedzie, K. M., Woodward, D. F., and Gil, D. W. (1994) Mol. Pharmacol. 46, 213–220
12. Wise, H., and Jones, R. L. (2000) Prostacyclin and Its Receptors, 1st Ed., Kluwer Academic/Plenum Publishers, New York
13. Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999) Physiol. Rev. 79, 1193–1226
14. Toh, H., Ichikawa, A., and Narumiya, S. (1995) FEBS Lett. 361, 17–21
15. Kohayashi, T., Kuriyama, M., Hirata, M., Hirata, M., Ushikubi, F., and Narumiya, S. (1997) J. Biol. Chem. 272, 15154–15160
16. Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) Cell 65, 1255–1266
17. Feltner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7413–7417
18. Gether, U., Yokota, Y., Enmonds-Alt, X., Breliere, J.-C., Lowe, J. A., III, Snider, R. M., Nakanishi, S., and Schwartz, T. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6194–6198
19. Tsai, A.-L., Hsu, M.-J., Vijjeswarapu, H., and Wu, K. K. (1989) J. Biol. Chem. 264, 61–67
20. Leigh, P. J., Cramp, W. A., and MacDermot, J. (1984) J. Biol. Chem. 259, 12431–12436
21. Boie, Y., Sawyer, N., Slipetz, D. M., Metters, K. M., and Abramovitz, M. (1995) J. Biol. Chem. 270, 18910–18916
22. Town, M.-H., Casals-Stenzel, J., and Schillinger, E. (1983) Prostaglandins 23, 13–28
23. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. (1994) Annu. Rev. Biochem. 63, 101–132
24. Rehwald, M., Neuscha¨fer-Rube, F., de Vries, C., Puschel, G. P. (1999) FEBS Lett. 443, 357–362
25. Audoly, L., and Breyer, R. M. (1997) Annu. Rev. Biochem. 66, 13–28
26. Audoly, L., and Breyer, R. M. (1997) Annu. Rev. Biochem. 66, 13–28
27. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132