Identification of Domains Conferring Ligand Binding Specificity to the Prostanoid Receptor

STUDIES ON CHIMERIC PROSTACYCLIN/PROSTAGLANDIN D RECEPTORS*

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To identify domains conferring ligand binding specificity to prostanoid receptors, we constructed a series of chimeric receptors by successively replacing the regions from the carboxyl-terminal tail of mouse prostacyclin (prostaglandin I (PGI)) receptor (mIP) with the corresponding regions of the mouse PGD receptor (mDP). The mIP receptor expressed in COS 7 cells bound [3H]PGE1, [3H]PGD2, and [3H]PGF2a, whereas the mDP receptor showed no affinity for these prostanoids. The region extending from the sixth transmembrane domain to the carboxyl terminus of the mIP receptor was next replaced with the corresponding region of the mDP receptor. This chimeric IPN/DP receptor acquired the ability to bind [3H]PGD2 and [3H]PGF2a without decreasing the affinities of the mIP receptor to [3H]iloprost and [3H]PGE1. These binding characteristics did not change when the fourth and fifth transmembrane domains of the mIP receptor were further replaced with the corresponding regions of the mDP receptor. However, when the first extracellular to second intracellular loop of the mIP receptor containing the third transmembrane domain was further replaced with those of the mDP receptor, the affinities for [3H]PGE1, [3H]PGD2, and [3H]iloprost were markedly decreased, whereas that for [3H]PGF2a was increased by about 2-fold. [3H]PGE1 showed no affinity for the mIP, mDP, and all the chimeric receptors. These results suggest that the sixth to seventh transmembrane domain of the mIP receptor confers the specificity of this receptor to bind selectively to PGE1 and not to PGE2 and that the third transmembrane domain of the mDP receptor confers the selective binding of PGD2 to this receptor.

Prostaglandins (PGs)1 contain prostanoic acid as a central structural element. PGs have two structural features in the prostanoic acid framework. First, they have functional groups on the 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. Additionally, another cyclooxygenase product, thromboxane A2, has an oxane ring instead of the cyclopentane ring. These prostanoids act on eight types and subtypes of the receptors. They are the PGD receptor (DP), the EP1, EP2, EP3, and EP4 subtypes of the PGE receptor, the PGF receptor (FP), the PGI receptor (IP), and the thromboxane A2 receptor (TP) (1–4). These receptors can recognize the structural differences of prostanoid molecules. The binding affinities of these receptors to prostanoid molecules are determined primarily by the cyclopentane ring structures of ligands. For example, the DP receptor shows the highest affinities to PGD2 and PGE1, whereas other prostanoids are at least 2 orders of magnitude less. One exception is the IP receptor, which shows the affinity to PGE1 almost comparable to PGI analogs such as iloprost. This receptor, however, can bind PGE2 with much lower affinity, suggesting that the IP receptor can discriminate a difference in the side chains.

We have cloned cDNAs for all of these types and subtypes of the mouse prostanoid receptors (5–13). These studies revealed that the prostanoid receptors belong to the G protein-coupled rhodopsin type receptor superfamily. They have several regions conserved specifically among them. These conserved regions may participate in the construction of binding domains for structures common to prostanoid molecules, whereas the other regions may confer specificity for ligand binding. For example, the arginine in the seventh transmembrane domain, which is conserved in all of the prostanoid receptors, was proposed to be the binding site for the carboxyl group of prostanoid molecules (5, 14, 15). In fact, Funk et al. (16) have shown that a point mutation at this arginine residue in the human TP receptor results in loss of ligand binding activity. However, structural domains of the prostanoid receptors conferring specificity for ligand binding are as yet unknown.

Chimeric receptors have been used to determine the regions involved in various functions of the receptors. For example, this approach was used to determine the regions involved in selective agonist and antagonist binding in adrenergic receptors (17, 18). Chimeric receptors were also used to identify the binding site of non-peptide antagonists to the neurokinin receptors (19–22) and to the angiotensin receptors (23) and the G protein activation sites of the muscarinic and β1-adrenergic receptors (24). These results show that this approach has been useful in locating functional domains of various receptors.

To identify the domains conferring the ligand binding spec-

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1 The abbreviations used are: PG(s), prostaglandin(s); G protein, heterotrimeric GTP-binding protein; PCR, polymerase chain reaction; MES, 4-morpholinethanesulfonic acid.
ificity to the prostanoid receptors, we have constructed chimeric receptors from the mIP and mDP receptors in this study. This strategy is based on the high homology of their amino acid sequences as well as common signal transduction. The prostanoid receptors can be functionally grouped into three categories: the relaxant receptors, the contractile receptors, and the

FIG. 1. A membrane topology model of the mIP receptor. The model is based on hydrophobicity analysis of the mIP receptor according to the methods of Kyte and Doolittle (38). Solid circles indicate the residues that are identical to those of the mDP receptor. Sites for replacement in chimeric receptors are shown, and restriction endonucleases used for construction are indicated (see “Experimental Procedures”).

FIG. 2. Diagrams of the mIP, mDP, and chimeric receptors (panel A) and strategy for construction of chimeric receptors (panel B). Panel A, the part of receptors derived from the mIP receptor is shown by an open box, and that from the mDP receptor is shown by a closed box. Panel B, PCR products corresponding to the mIP sequence are shown by bold lines above each box of chimeric receptor cDNA and those to the mDP sequence by bold lines below each box. Sequences of primers used in PCR are shown in Table I. Numbers in parentheses indicate nucleotide numbers of the 5′- and 3′-termini of each fragment corrected for the residue numbers in the mIP receptor cDNA. Restriction sites used for construction are indicated.
inhibitory receptor (14). The relaxant receptors, consisting of the IP, DP, EP2, and EP4 receptors, mediate calcium mobilization and induce smooth muscle contraction. The EP2 receptor is an inhibitory receptor that mediates decreases in cAMP and inhibits several biological processes such as neurotransmission, gastric acid secretion, and water resorption. Sequence homology among these functionally related receptors is higher than that among the three separate groups (25). The amino acid sequences of the mIP and mDP receptors, which belong to the same relaxant receptor group, show 58% identity in the transmembrane domains (Fig. 1), and both couple to the same G protein, Gs. Chimeric mIP/mDP receptors were expressed in the COS 7 cells, and their ligand binding properties were examined.

### EXPERIMENTAL PROCEDURES

**Materials**—PGE2, PGE1, PGE2, and PGF2α were generous gifts from Ono Pharmaceuticals Co. Ltd. (Osaka, Japan). PGE1 was obtained from Cayman Chemical Co. (Ann Arbor, MI). [5,6-3H]PGE1 (52 Ci/mmol), [5,6,8,11,12,14,15-3H]PGE2 (171 Ci/mmol), and [5,6,8,9,12,14,15-3H]PGD2 (115 Ci/mmol) were obtained from Amersham International plc, United Kingdom.

### Construction of Chimeric Receptors

The mIP and mDP cDNAs were first subcloned into pCMX expression vector (26). The BalI-Eco1001 fragment of CP302, a cDNA of mIP (11), and the Aesp78-BamH1 fragment of PC9, a cDNA of mDP (12), were subcloned into the EcoRV sites and the Asp78 and BamH1 sites of pCMX, respectively. Six types of mIP/mDP chimeric receptors were then constructed (Fig. 2A). Six restriction sites of the mIP receptor cDNA (Aesp78, PstI, PvuII, SpII, BspH, BamH1) were all inside the insert in the pCMX vector, and with those six restriction sites, the mIP and mDP cDNAs were ligated into the pCMX-IP N-V/DPVI-C. The Chimeric IP N-VII/DPIII-C Receptor—Fragments I-4 and D-4 were amplified by PCR with the primer pairs shown in Table I. In the D-4 fragment, the PvuII site was introduced (Fig. 2B). Fragment I-4 was digested with PstI and PvuII, and fragment D-4 was digested with PvuII and PstI. Both digested fragments were ligated into the PstI sites of pCMX-IP αβ/DP VI-C.

### Ligand Binding Studies

For transient expression of each prostanoid receptor, COS 7 cells cultured in 15-cm dishes were transfected with 20 μg of plasmid DNA by the lipofection method (27). After culture for 60 h, the cells were harvested, and crude membranes were prepared as described (11). Briefly, harvested COS 7 cells were homogenized using a Potter-Elvehjem homogenizer in a solution containing 25 mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM MgCl2, 1 mM EDTA, and 1% phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 800 × g for 1 min. The supernatant was collected and centrifuged at 100,000 × g for 1 h. The pellet was resuspended in 20 mM MES (pH 6.0) containing 10 mM MgCl2 and 1 mM EDTA (the suspension buffer), and used as crude membranes. Binding assays were performed essentially as described previously (11). For Scatchard analysis, 50 μg of crude membranes was incubated in the suspension buffer with various concentrations of [3H]prostacyclin, [3H]PGE2, [3H]PGE3, or [3H]PGF2α in a total volume of 200 μl at 4 °C for 2 h. In competition experiments, the crude membranes were incubated with 20 nM [3H]PGE2, or 60 nM [3H]PGD2 in the presence of various concentrations of PGE1 or PGE2. The incubation was terminated by the addition of 2 ml of the ice-cold suspension buffer, and the mixture was filtered through GF/C filters (Whatman). The filter was then washed with 5 ml of the ice-cold suspension buffer three times. The radioactivity on the filter was measured in 5 ml of Clear-Sol scintillation mixture (Nakalai Tesque, Kyoto, Japan). Nonspecific binding was determined in the presence of a 1,000-fold excess of unlabeled ligands in the incubation mixture. K, values were calculated from I50 values of radioligand binding as described previously (20).

### RESULTS

The mIP, mDP, and six chimeric receptors were expressed in COS 7 cells, and crude membranes were prepared for binding studies. Crude membranes were incubated with various concentrations of each of [3H]prostacyclin, [3H]PGE2, [3H]PGE3, [3H]PGD2, and [3H]PGF2α (Fig. 3). Saturation kinetics of these binding was obtained and subjected to Scatchard analysis. Representative analyses are shown in Fig. 4, and the results of several analyses are summarized in Table II. As shown in Fig.
the mDP receptor (Table II). Similar ligand binding specificity was exhibited by the chimeric IP\(_N\)/DP\(_{IL\cdotC}\) receptor (Fig. 4, G and h, and Table II).

The above results indicate that the mIP receptor may accommodate the cyclopentane ring structure of PGD and that it exerts its ligand binding specificity mainly by discriminating the structural difference in the \(\alpha\)-side chain. To examine this hypothesis, PGD\(_2\) binding was analyzed by competition binding studies on the mIP, mDP, and chimeric IP\(_N\)/DP\(_{VL\cdotC}\) receptors using \([\text{3H}]\text{PGF}\_2\_\alpha\) or \([\text{3H}]\text{PGD}\_2\) as a radioligand (Fig. 5). PGD\(_1\) effectively displaced \([\text{3H}]\text{PGD}\_2\) binding to the mDP receptor with a \(K_d\) value of 990 nM. PGD\(_1\) also displaced \([\text{3H}]\text{PGE}\_1\) binding to the chimeric IP\(_N\)/DP\(_{VL\cdotC}\) receptor with the \(K_d\) values of 2.5 \(\mu\)M. On the other hand, PGD\(_2\) as well as PGD\(_4\) could not displace \([\text{3H}]\text{PGE}\_1\) binding to the mIP receptor at up to a 10 \(\mu\)M concentration.

**DISCUSSION**

The present study used chimeric receptors and examined domains of the prostanooid receptors conferring the ligand binding specificity of each receptor. The receptors we used are the mIP, mDP, and chimeric mIP/DP receptors. As shown under "Results," the mIP receptor shows high affinity binding only to \([\text{3H}]\text{PGD}\_2\) and \([\text{3H}]\text{PGE}\_2\), but their affinities were too low to be analyzed by the Scatchard analysis. This ligand binding specificity of the mIP receptor is consistent with previous reports on the cloned mIP receptor (11) and on native IP receptor in various cells (28, 29). On the other hand, the mDP receptor showed a high affinity binding only to \([\text{3H}]\text{PGD}\_2\) with a \(K_d\) value of 43 ± 6 nM (Fig. 4, H and h, and Table II). This is also consistent with previous reports on the cloned mouse and human DP receptors (12, 30) and on native human DP receptor (31). We then examined the binding properties of the chimeric receptors. The carboxyl tail of the mIP receptor was first replaced with that of the mDP receptor. This chimeric IP\(_N\)/DP\(_{VL\cdotC}\) receptor showed a 12–16-fold increase in binding affinity to \([\text{3H}]\text{PGD}\_2\) and \([\text{3H}]\text{PGE}\_2\), without an appreciable increase in the binding of \([\text{3H}]\text{PGE}\_1\) and \([\text{3H}]\text{PGD}\_2\) (Fig. 4, B and b, and Table II). The sixth to seventh transmembrane domain was then further replaced. The resultant chimeric IP\(_N\)/DP\(_{VL\cdotC}\) receptor acquired the ability to bind \([\text{3H}]\text{PGD}\_2\) and \([\text{3H}]\text{PGE}\_2\) with \(K_d\) values of 69 ± 16 and 40 ± 6 nM, respectively. They bound \([\text{3H}]\text{PGF}\_2\_\alpha\) and \([\text{3H}]\text{PGE}\_2\), with affinities comparable to those of the mIP receptor with \(K_d\) values of 11 ± 1 and 17 ± 8 nM, respectively (Fig. 4, C and c, and Table II). Similar ligand binding properties were shown by the chimeric IP\(_N\)/DP\(_{VL\cdotC}\) and IP\(_N\)/DP\(_{IV\cdotC}\) receptors, which have further substitution of the fifth and fourth transmembrane domains (Fig. 4, D and d, E and e, and Table II); they bound both \([\text{3H}]\text{PGF}\_2\_\alpha\) and \([\text{3H}]\text{PGE}\_2\), with affinities similar to those of the mIP receptor. In contrast, the binding of \([\text{3H}]\text{PGF}\_2\_\alpha\), \([\text{3H}]\text{PGE}\_2\), and \([\text{3H}]\text{PGE}\_1\) was almost abolished when the first extracellular to second intracellular loop of the mIP receptor was replaced with that of the mDP receptor (Fig. 4, F and f). This chimeric IP\(_N\)/DP\(_{VI\cdotC}\) receptor, on the other hand, showed about a 2-fold increase in the binding affinity for \([\text{3H}]\text{PGD}\_2\). The \(K_d\) value of 35 ± 3 nM was close to the value of
results also suggest that the binding pocket of the mIP receptor for the cyclopentane ring of prostanoid molecules is localized in another region and can accommodate the cyclopentane rings of not only I and E but also D type, although we cannot exclude the possibility that the sixth to seventh transmembrane domain of the mDP receptor has contributed to accommodate the cyclopentane ring of D type in this chimeric receptor. Interestingly, the affinities for [3H]PGE1 and [3H]iloprost were not changed by further replacement of the fourth and fifth transmembrane domains, suggesting that the binding domain of the cyclopentane ring in the mIP receptor localizes in a region containing the first to third transmembrane domain. We have examined if the binding specificity of the mIP receptor is determined solely by recognition of the side chain structure by
analyzing the binding of PGD\textsubscript{1} to the mIP. As shown in Fig. 5, no appreciable binding of PGD\textsubscript{1} was observed in the mIP receptor, suggesting that if the mIP receptor can accommodate the cyclopentane ring of D type, the relative configuration between the cyclopentane ring and the side chains is also important in determining the ligand binding affinity. On the other hand, PGD\textsubscript{1} bound to the chimeric IPN-V/DPVI-C receptor, suggesting that the sixth to seventh transmembrane domain of the mIP receptor is also responsible for determining this binding specificity. Moreover, the facts that the affinity of PGD\textsubscript{1} for the chimeric IPN-V/DPVI-C receptor was 1 order of magnitude increased the binding affinity for PGD\textsubscript{2}. These observations suggested that this region of the mIP receptor is responsible for determining these affinities. If this is also the case for the prostanoid receptors, we can assume that the third transmembrane domain is responsible for the above selectivity. Surprisingly, the third transmembrane domain has only four amino acids different between the mIP and mDP receptors (Fig. 1). If this domain is responsible, it would be intriguing to examine if any of these four amino acids has an important influence on the recognition of the cyclopentane ring. The functional groups at the 9- and 11-positions of the cyclopentane ring of PG molecules are either oxo or hydroxy groups. One hypothesis is that these groups are involved in formation of hydrogen bonds to some amino acids of the prostanoid receptors. The vicinal hydroxyl groups of the catechol ring are shown to be involved in formation of hydrogen bonds to some amino acids has an important influence on the recognition of the cyclopentane ring of PG molecules.

This investigation has also revealed that the affinities for iloprost and PGE\textsubscript{1} of the mIP receptor are increased 12- and 34-fold by replacement of its carboxyl tail with that of the mDP receptor. There have been several reports concerning the effects of carboxyl tails of the rhodopsin-type receptors. We observed that alternative splicing of the EP3 and TP receptor in the carboxyl tail affects the specificity and efficacy of G-protein coupling (35, 36) as well as the sensitivity to agonist-induced desensitization (37). However, none of them showed a change in ligand binding properties. Whether a difference in the carboxyl tail increases the ligand binding affinity remains to be tested because our IPN-V/DP\textsubscript{C} receptor also contains the replacement of several residues of the seventh transmembrane domain (Fig. 1).
In summary, the present study has identified the domains of the mIP and mDP receptors which confer ligand binding specificities to each receptor. Continued application of molecular biology including introduction of a point mutation to the identified regions will provide a more detailed understanding of the molecular basis of ligand recognition by the prostanoid receptors and will help design more specific therapeutic agents.

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