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The Key Amino Acid Residue of Prostaglandin EP3 Receptor for Governing G Protein Association and Activation Steps

Shuji Satoh, Chang-sheng Chang,[1] Hironori Katoh, Hiroshi Hasegawa, Kazuhiro Nakamura, Junko Aoki, Hirotada Fujita, Atsushi Ichikawa,* and Manabu Negishi[2]

*Department of Molecular Neurobiology and *Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*

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To assess the role of the conserved DPWXY motif of the seventh transmembrane domain in prostanoid receptor-mediated G protein activation, we have mutated the negatively charged Asp-318 in this motif of the Gi-coupled mouse prostaglandin EP3 receptor to uncharged but polar Asn (EP3-D318N) and to the non-polar Leu (EP3-D318L). The EP3 agonist and antagonist showed similar binding affinities for the wild-type and two mutant receptors. The wild-type and EP3-D318N receptors but not EP3-D318L receptor associated with Gi in guanine nucleotide- and pertussis toxin-sensitive manners. On the other hand, the wild-type receptor but not two mutant receptors had the ability to stimulate GTPase activity and to inhibit the adenylate cyclase. These findings demonstrate that the chemical nature of the amino acid residue at position 318 of the seventh transmembrane domain of the EP3 receptor dissociates the step of Gi association from that of subsequent Gi activation in the process of the EP3 receptor-Gi coupling. © 1999 Academic Press

The interaction of cell-surface receptors with heterotrimeric GTP-binding proteins (G proteins) is crucial for hormonal action (1). An agonist-bound receptor associates with a G protein, forming an intermediary ternary complex of the agonist, receptor and G protein, and then activates the G protein to a state in which it can regulate effectors (1). A growing number of studies have identified regions or motifs in G protein-coupled receptors that participate in the processes of receptor-mediated G protein activation (2). Among the most conserved regions of receptors is a sequence of amino acid residues, D/N-P-$(X)_{2,3}$-Y, found in the seventh transmembrane domain, which has been suggested to be involved in receptor-G protein coupling, receptor sequestration or internalization (3, 4). However, it has not yet been fully elucidated how this motif participates in the receptor functions.

Prostanoids exhibit a broad range of biological actions in diverse tissues through their binding to specific receptors on the plasma membrane (5). We and other laboratories have revealed the primary structures of eight types of prostanoid receptors and demonstrated that they belong to the G protein-coupled rhodopsin-type receptor superfamily (6). They have several features in common with other rhodopsin-type receptors, and the above-mentioned motif, D/N-P-$(X)_{2,3}$-Y, was also found in all prostanoid receptors, but this consensus sequence was DPWXY. To assess the role of Asp of this motif in prostanoid receptor-mediated G protein activation, we mutated the negatively charged Asp-318 in this motif of the mouse prostaglandin (PG) EP3 receptor to the uncharged but polar Asn residue and to the non-polar Leu residue. Here we report that chemical nature of this residue dissociates the step of G protein association from that of subsequent activation in the process of the EP3 receptor-Gi coupling.

MATERIALS AND METHODS

Materials. M&B28767, AH23848B and sulprostone were generous gifts from Drs. M. P. L. Caton of Rhone-Poulenc Ltd., R. A. Coleman of Glaxo Group Research Ltd. and K.-H. Thierauch of Schering, respectively. [5,6,8,11,12,14,15-^{3}H]PGE_2 (179 Ci/mmol) was obtained from Amersham Corp; PGE_2 was from Cayman Chemical (Ann Arbor, MI); pertussis toxin (PT) was from Seikagaku Kogyo (Tokyo, Japan); forskolin was from Sigma; and guanosine 5′-[γ-thio]triphosphate (GTPγS) was from Boehringer Mannheim.

[1] Present address: Department of Molecular Cardiology, The Lerner Research Institute, Cleveland Clinic Foundation, Cleveland OH, 44195.

[2] To whom correspondence should be addressed. Fax: 81-75-753-4557. E-mail: mnegishi@pharm.kyoto-u.ac.jp.

Abbreviations used: G protein, heterotrimeric GTP-binding protein; PG, prostaglandin; PT, pertussis toxin; GTPγS, guanosine 5′-[γ-thio]triphosphate; CHO, Chinese hamster ovary.

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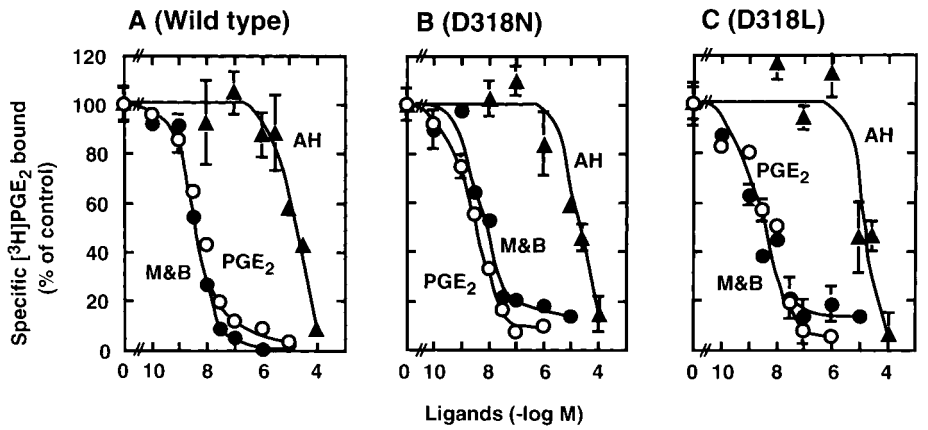

FIG. 1. Displacement of $[^3H]PGE_2$ binding by EP3 agonist and antagonist in the membrane of CHO cells expressing the wild-type or mutant EP3 receptors. The membrane of CHO cells expressing the wild-type (A), EP3-D318N (B) or EP3-D318L (C) receptor was incubated with 4 nM $[^3H]PGE_2$ and the indicated concentrations of PGE_2 (E), M&B28767 (J) or AH23848B (H). Specific $[^3H]PGE_2$ binding was determined as described in Materials and Methods. The values are the means ± S.E.M. for triplicate experiments and are expressed as percentages of controls obtained with membranes in the absence of ligands.

Construction and stable expression of the mutant receptors. PCR-mediated mutagenesis (7) was employed to replace Asp-318 of mouse EP3α cDNA with Asn and Leu, and the cDNAs for the respective mutant receptors (EP3-D318N and EP3-D318L) were constructed. Stable cDNA transfection into Chinese hamster ovary (CHO) cells was performed by lipofection, essentially as described previously (8), and clonal cell lines were obtained by single-cell cloning and were screened by $[^3H]PGE_2$ binding and RNA blotting.

$[^3H]PGE_2$ binding assay. The harvested CHO cells expressing each receptor were homogenized with a Potter-Elvehjem homogenizer in 20 mM Mes/NaOH, pH 6.0, containing 10 mM $MgCl_2$, 1 mM EDTA, 20 μM indomethacin and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation of the homogenate at 250, 000 × g for 10 min, the pellet was washed and suspended in the same buffer. The membrane fraction (80 μg) was incubated with 4 nM (displacement experiment) or various concentrations (Scatchard analysis) of $[^3H]PGE_2$ at 30°C for 1 h, and $[^3H]PGE_2$ bound to the membrane fraction was determined as described previously (9). Non-specific binding was determined by using a 1000-fold excess of unlabeled PGE_2 in the incubation mixture. Specific binding was calculated by subtracting the non-specific binding from the total binding. K_d values for $[^3H]PGE_2$ were obtained from Scatchard plots transformed from the values of specific $[^3H]PGE_2$ binding, and K_i values for ligands were calculated with the Cheng-Prusoff equation.

GTPase activity. The membrane pellet prepared from the cells was suspended in 20 mM Hepes/NaOH, pH 7.5, containing 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 M NaCl (assay buffer). GTPase activity was assayed by incubating the membrane (15 μg) at 37°C for 5 min in 100 μl of assay buffer, containing 1 mM AppNHp, 0.2 mM ATP, and 0.1 μM [γ-^{32}P]GTP (0.5 μCi), as described previously (10). Non-specific GTP hydrolysis was determined using a 1000-fold excess of unlabeled GTP, and the specific low K_m GTPase activity was calculated by subtracting the non-specific hydrolysis from the total hydrolysis.

Measurement of cAMP formation. The cAMP contents of CHO cells expressing the wild-type and mutant receptors were determined as reported previously (11). After 10 min of incubation at 37°C with 10 μM forskolin and 100 μM Ro-20-1724, a phosphodiesterase inhibitor, the reaction was terminated by the addition of 10% trichloroacetic acid. The cAMP contents of the cells were measured by radioimmunoassay with an Amersham cAMP assay system.

RESULTS

EP3 receptor is a Gi-coupled receptor, inhibiting the adenylate cyclase activity (6). We first examined the binding affinities of the wild-type and mutant receptors for PGE_2 (natural agonist), M&B28767 (EP3 agonist) and AH23848B (EP3 antagonist) by assessing the displacement of $[^3H]PGE_2$ binding to the receptors. As shown in Fig. 1, these ligands inhibited the $[^3H]PGE_2$ binding to the wild-type, EP3-D318N and EP3-D318L receptors in similar displacement profiles in the order of M&B28767 = PGE_2 ⩾ AH23848B. To obtain the K_d and K_i values of the receptors for these ligands, we performed the Scatchard analyses of the $[^3H]PGE_2$ binding to the receptors and calculated the K_d values for $[^3H]PGE_2$ and the K_i values for M&B28767 and AH23848B. As shown in Table 1, the wild-type and mutant receptors showed similar affinities for both agonist and antagonist.

TABLE 1

Binding Affinities of the Wild-Type and Mutant EP3 Receptors for PGE_2, M&B28767 and AH23848B

Receptors	PGE_2	M&B28767	AH23848B
	K_d (nM)	K_i (nM)	K_i (nM)
Wild type	5.6 ± 0.1	4.3 ± 0.04	9300 ± 83
D318N	11 ± 0.7	3.1 ± 0.05	4900 ± 49
D318L	3.8 ± 0.2	3.0 ± 0.08	4600 ± 109

Note. K_d values for PGE_2 were obtained from Scatchard plots of specific $[^3H]PGE_2$ binding to the wild-type, EP3-D318N and EP3-D318L receptors, as described in Materials and Methods. K_i values for M&B28767 and AH23848B were obtained from IC_{50} values of 4 nM $[^3H]PGE_2$ binding displacement by both ligands (Fig. 1) with the use of the Cheng-Prusoff equation. Results are the means ± S.E.M. for three independent experiments.

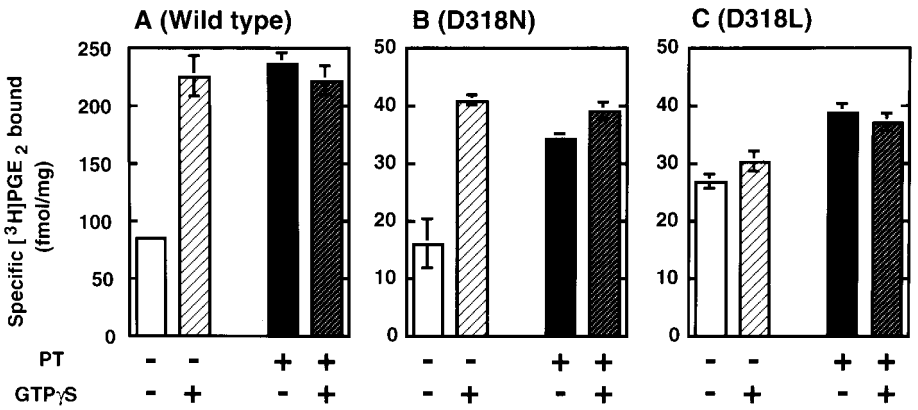

FIG. 2. Effects of GTPγS and PT treatment on [^{3}H]PGE_2 binding to the wild-type and mutant receptors. After CHO cells expressing the wild-type (A), EP3-D318N (B) or EP3-D318L(C) receptor had been treated for 12 h with vehicle (PT; −) or 100 ng/ml of PT (PT; +), the membranes prepared from the cells were incubated with 4 nM [^{3}H]PGE_2 in the presence (GTPγS; +) or absence (GTPγS; −) of 100 μM GTPγS. Specific [^{3}H]PGE_2 binding was determined as described in Materials and Methods. The values are the means ± S.E.M. for triplicate experiments.

The association of receptors with G proteins can be evaluated by modulation of the agonist-binding affinity of the receptors by guanine nucleotides (12). In contrast with most hormone receptors, in which guanine nucleotides promote the dissociation of a ligand-receptor-G protein ternary complex and decrease the agonist-binding affinity of receptors, the PGE_2-binding affinity of EP3 receptor was inversely increased by guanine nucleotides (13). We then examined the effect of GTPγS on the PGE_2 binding to the wild-type and mutant receptors. GTPγS markedly increased the PGE_2 binding to the wild-type receptor as expected, and also increased the binding to EP3-D318N receptor, while GTPγS did not significantly affect the binding to EP3-D318L receptor (Fig. 2). EP3 receptor is coupled to Gi and PT treatment induces decoupling of the receptor and Gi, resulting in the increase in [^{3}H]PGE_2 binding to the receptor as observed with the guanine nucleotide treatment (14). Thus, we examined the effect of PT treatment on the PGE_2 binding to the receptors. PT treatment also increased the PGE_2 binding to the wild-type and EP3-D318N receptors but not to EP3-D318L receptor, and GTPγS did not further increase the binding to the wild-type and EP3-D318N receptors. Therefore, these results indicate that the wild-type and EP3-D318N receptors associate with Gi, while EP3-D318L receptor does not.

To assess the functional interaction of EP3 receptor and Gi, we examined the ability of the wild-type and mutant receptors to stimulate the GTPase activity of Gi and to inhibit the adenylate cyclase. Fig. 3 shows the effect of PGE_2 on the GTPase activity in the membrane of each receptor-expressing cells. PGE_2 concentration-dependently stimulated the GTPase activity in the wild-type receptor, while PGE_2 did not affect the activity in both mutant receptors. Fig. 4 shows the effect of sulprostone, an EP3 agonist, on the forskolin-stimulated cAMP formation. Sulprostone strongly inhibited the forskolin-stimulated cAMP formation in the wild-type receptor in a PT-sensitive manner. We further examined the antagonist activity of AH23848B for the EP3 receptor, and AH23848B shifted the inhibition curve of the agonist toward the right in the wild-type receptor, indicating that AH23848B acts as an antagonist for EP3 receptor and the wild-type EP3 receptor shows functional response to agonist and antagonist (data not shown). On the other hand, the PT-sensitive inhibition induced by sulprostone was very weak in EP3-D318N receptor, and no inhibition was observed in EP3-D318L receptor.

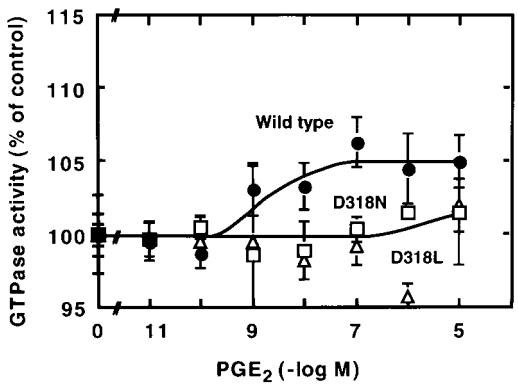

FIG. 3. Effect of PGE_2 on GTPase activity in the membrane of CHO cells expressing the wild-type or mutant EP3 receptors. The membrane of CHO cells expressing the wild-type (J), EP3-D318N (G) or EP3-D318L (C) receptor was assayed for GTPase activity with the indicated concentrations of PGE_2, as described in Materials and Methods. The values are the means ± S.E.M. for triplicate experiments and are expressed as percentages of the GTPase activity without the agonist in the respective membranes.

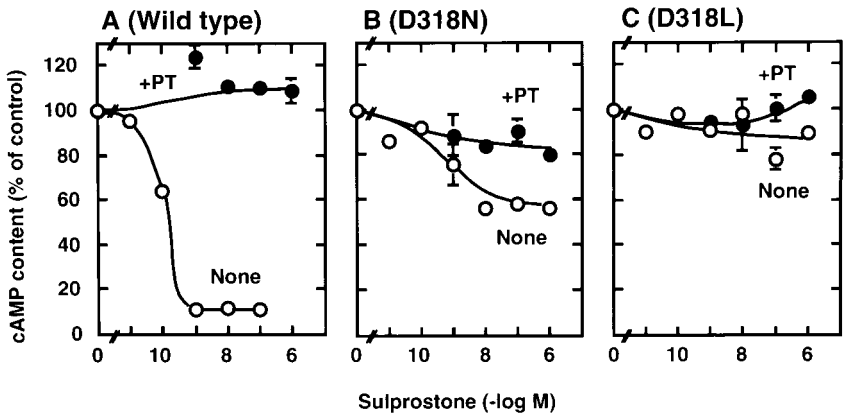

FIG. 4. Effect of EP3 agonist on forskolin-induced cAMP formation in CHO cells expressing the wild-type or mutant EP3 receptors. After CHO cells expressing the wild-type (A), EP3-D318N (B) or EP3-D318L (C) receptor had been treated for 12 h with vehicle (E) or 100 ng/ml of PT (J), they were incubated for 10 min at 37°C with the indicated concentrations of sulprostone in the presence of 10 μM forskolin and 100 μM Ro-20-1724. The cAMP content was measured as described in Materials and Methods. The results shown are the means ± S.E.M. for triplicate experiments and are expressed as percentages of controls obtained with the cells in the absence of agonist.

DISCUSSION

In the present study, we examined the functional role of Asp residue in DPWXY motif of the seventh transmembrane domain of the EP3 receptor in the process of the EP3 receptor-mediated Gi activation. The wild-type and two Asp-mutated receptors showed similar affinities for both agonist and antagonist, indicating that the Asp-318 is not a determinant for the ligand binding to the receptor. The wild-type EP3 receptor, having negatively charged Asp-318 in the motif, could associate with and subsequently activate Gi. In contrast, EP3-D318N receptor, having uncharged but polar Asn-318, was able to associate with Gi but lost the ability to activate the G protein. EP3-D318L receptor, having non-polar Leu-318, could neither associate with nor activate Gi. Thus, the association of EP3 receptor with Gi is prerequisite to the activation of Gi, and the association with Gi and formation of a ternary complex are not sufficient for Gi activation. This means that the association of EP3 receptor with Gi and activation of Gi are different steps, and both the association with Gi and its activation are somehow regulated by different mechanisms.

Asp-318 in DPWXY motif is the key amino acid residue for both G protein association and activation steps, and the chemical nature of this residue appears to determine the transition of the receptor to these steps. Asp can not only form an ionic interaction but also form hydrogen bonding interaction (15). In contrast, Asn residue substituted for Asp-318 can only form hydrogen bonding interaction, but non-polar Leu residue can form neither a hydrogen bond nor an ionic bond. Considering these findings, it is inferred that the hydrogen bonding interaction of Asn residue is sufficient for the association of the EP3 receptor with Gi but the ionic interaction of Asp residue is essential for the subsequent activation of Gi. Which amino acid residue within the EP3 receptor structure interacts with Asp-318? As for this issue, the Asp residue in DPXXY motif of gonadotropin-releasing hormone receptor has been proposed to interact with the Arg residue in highly conserved DRY motif, located in cytoplasmic side of third transmembrane domain, in the active state of the receptor (16). This Arg residue in DRY motif is also conserved in all prostanoid receptors (6). Therefore, the interhelical salt bridge formation between Arg residue of DRY motif and Asp residue of DPWXY motif may be essential for the EP3 receptor-mediated Gi activation, while the hydrogen bonding interaction of Arg and Asn may support only association of the receptor with Gi. Since an ionic bond is apparently stronger than a hydrogen bond, the selective ability of Asp to activate Gi may reflect the different bond strengths of hydrogen and ionic bonds.

Recently, we demonstrated that the truncation of the carboxyl-terminal tail of the EP3 receptor caused agonist-independent constitutive Gi activity, suggesting that the carboxyl-terminal tail suppresses the receptor-induced Gi activation and agonist binding induces the conformational change of the receptor, which releases the constraint, allowing the receptor to associate with and activate Gi (17). The seventh transmembrane domain is directly connected with the carboxyl-terminal tail. Thus, the interaction of Asp of DPWXY motif and Arg of DRY motif may induce a confomational change of the seventh transmembrane domain and the carboxyl-terminal tail, leading to the association with Gi and subsequent Gi activation, and the chemical nature of this interaction may regulate the conformational changes, determining two steps, G protein association and activation.

In conclusion, we here described that the EP3 receptor signal to Gi proceeds to two steps, Gi association and subsequent activation and that the chemical nature of the residue at position 318 of the receptor is the key element in the process of EP3 receptor-induced Gi activation. This study contributes to our understanding of G protein-coupled rhodopsin-type receptor-mediated G protein activation mechanism.

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