Functional role of carboxyl-terminal tail of prostaglandin EP3 receptor in Gi coupling

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Abstract We recently demonstrated that the mouse EP3β receptor and its carboxyl-terminal tail-truncated receptor showed agonist-dependent and full constitutive Gi activities, respectively (Hasegawa, H., Negishi, M. and Ichikawa, A. (1996) J. Biol. Chem. 271, 1857–1860). To assess the role of the carboxyl-terminal tail in the EP3β receptor Gi coupling, we constructed a series of mutant receptors with progressively truncated carboxyl-termini. The truncated receptors displayed constitutive Gi activities, the degree of constitutive activity basically correlating with the inverse of the length of the carboxyl-terminal tail, but the sequence between Leu340 and Val347 was mainly contributed to the constitutive activity. Thus, the carboxyl-terminal tail plays an important role in the constraint of the EP3 receptor in its inactive conformation.

Key words: Prostaglandin E_2; G protein; Constitutive activity

1. Introduction

The interaction of cell surface hormone receptors with heterotrimeric G proteins is crucial for hormonal actions [1]. Most G protein-coupled rhodopsin-type receptors share conserved structural features, consisting of seven transmembrane-spanning domains and three intracellular loops and one COOH-terminal tail [2]. The domains of the receptors which interact with and activate G proteins have been extensively studied, and specific regions in the second and third intracellular loops were shown to function in G protein coupling [3]. However, precise molecular mechanisms for regulation of the receptor-induced G protein activation are still poorly understood.

We have cloned the mouse prostaglandin (PG) EP3 receptor and demonstrated that this receptor is a G protein-coupled rhodopsin-type receptor that engages in inhibition of adenylate cyclase [4]. Furthermore, we identified the three isoforms of the mouse EP3 receptor, EP3α, β, and γ, with different COOH-terminal tail, which were produced through alternative splicing [5,6] and differed in agonist-independent constitutive Gi activity; the EP3α receptor showed marked agonist-independent Gi activity, the EP3β receptor had no constitutive activity, and the EP3γ receptor showed mostly full constitutive activity [7,8]. In addition, the COOH-terminal tail-truncated receptor showed only agonist-independent constitutive Gi activity, suggesting that the core of the EP3 receptor has ability to associate with and activate Gi and the COOH-terminal tails after the alternative splicing site suppress the activation of Gi by the EP3 receptor [7]. This idea was recently supported by the finding that the human EP3 receptor isoforms showed constitutive Gi activity in various degrees and truncation of the COOH-terminal tail induced complete constitutive activity [9]. Among three isoforms, the EP3β receptor showed no constitutive activity, indicating that the COOH-terminal tail of the EP3β receptor has the strongest ability to suppress the Gi activation by the EP3 receptor. To assess the role of the COOH-terminal tail of the EP3β receptor in Gi coupling, we constructed a series of mutant receptors with progressively truncated COOH-termini, and examined structural determinant of the tail for the suppression of the receptor-mediated Gi activation.

2. Materials and methods

2.1. Materials

M&B 28767 was a generous gift from Dr. M.P.L. Caton of Rhone-Poulenc Ltd. [5,6,8,11,12,14,15-^{3}H]PGE_2 (181 Ci/mmol) and a ^{125}I-labeled cAMP assay system were obtained from Amersham Corp.; GTPγS was from Boehringer Mannheim; and forskolin was from Sigma.

2.2. Construction and stable expression of the mutant receptors

Progressively truncated receptor cDNAs were constructed by means of a polymerase chain reaction-mediated mutagenesis technique [10], using cDNA for EP3β [5] as a template. cDNAs for mutated receptors, T339, T347, and T355, which encode receptors that terminate after Asn339, Val347, and Ile355, respectively, were prepared by creating a stop codon immediately after the codon encoding respective residues. The mutated regions in the cDNAs were sequenced [11]. cDNAs were transfected into Chinese hamster ovary (CHO) cells and stable transformants were cloned as described previously [5].

2.3. Measurement of cAMP formation

Cyclic AMP levels in CHO cells were determined as reported previously [12]. The receptor-expressing CHO cells cultured in 24-well plates (5×10^5 cells/well) were washed with HEPES-buffered saline containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, and 15 mM HEPES, pH 7.4, and preincubated for 10 min. Reactions were started by the addition of test agents 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 an Amersham cAMP assay system.

2.4. PGE_2-binding assay

The harvested CHO cells expressing each receptor were homogenized using a Potter-Elvehjem homogenizer in 20 mM Tris-HCl (pH 7.5), containing 10 mM $MgCl_2$, 1 mM EDTA, 20 μM indomethacin and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation at 250 000×*g* for 20 min, the pellet was washed, suspended in 20 mM

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Abbreviations: PG, prostaglandin; G protein, heterotrimeric GTP-binding protein; CHO, Chinese hamster ovary; COOH, carboxyl; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate)

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Mes-NaOH (pH 6.0) containing 10 mM $MgCl_2$ and 1 mM EDTA, and was used for the [^{3}H]PGE_2-binding assay. The membrane (50 μg) was incubated with various concentrations of [^{3}H]PGE_2 in the presence or absence of 100 μM GTPγS at 30°C for 1 h, and [^{3}H]PGE_2 binding to the membrane was determined as described previously [13]. Nonspecific binding was determined using a 1000-fold excess of unlabeled PGE_2 in the incubation mixture. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

3. Results and discussion

A series of mutant EP3 receptors were constructed in which stop codons were inserted at similar spaced intervals throughout the COOH-terminal tail after the splicing site (Fig. 1), and we chose the CHO cell lines stably expressing each mutant receptor, T339, T347, T355, showing receptor numbers similar to those of the EP3β receptor and T335, which had been established previously [5,14] (EP3β, 365 ± 8.3 fmol/mg; T355, 408 ± 1.1 fmol/mg; T347, 505 ± 6.0 fmol/mg; T339, 386 ± 3.1 fmol/mg; T335, 362 ± 13 fmol/mg). We tested the ability of the wild-type and mutant EP3β receptors to inhibit the forskolin-activated adenylate cyclase in the presence or absence of an EP3 agonist, M&B 28767, and compared their abilities (Fig. 2). In the absence of the agonist, forskolin increased the intracellular cAMP levels in the order of EP3β > T347 ≥ T355 > T339 > T335. Increasing concentrations of M&B 28767 caused a decrease in the forskolin-stimulated cAMP formation in the EP3β receptor, T347, T355, and T339, but this decrease was not observed in T335. The cAMP level of T335 in the presence of the agonist was significantly higher than the levels maximally reduced by the agonist in other mutant receptors and EP3β receptor. Similar result has been observed with the COOH-terminal tail-truncated human EP3 receptor [9]. These results indicate that the EP3β receptor has no constitutive activity, T347 and T355 are partially constitutive, T339 is mostly constitutive and T335 is completely constitutive in Gi activity. We examined several clones of T335 with different expression levels, but they showed fully constitutive Gi activity (data not shown). We transiently transfected HEK293 cells with the EP3β and T335 receptors, and they showed agonist-dependent and constitutive Gi activity in HEK293 cells, respectively (data not shown). Thus, the degree of constitutive activity basically correlated with the inverse of the length of the COOH-terminal tail. However, the sequence from Leu340 to Val347, within the COOH-terminal tail, is strongly contributed to the COOH-terminal tail-induced suppression of the core of the EP3 receptor-mediated Gi activation, because the truncation from Val347 to Leu340 caused marked constitutive Gi activation.

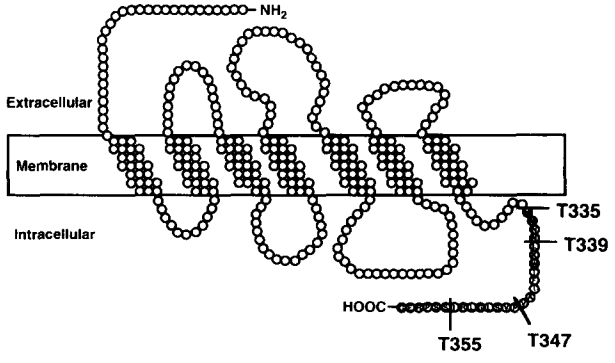

Fig. 1. Schematic depiction of structure of truncated EP3β receptors. The COOH-terminal tail of EP3β receptor after the splicing site was shown by the single-letter code for amino acid residues. The truncation sites are shown as black bars.

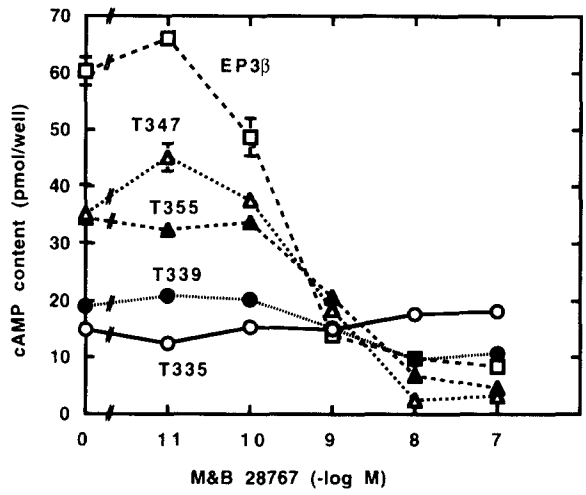

Fig. 2. Inhibition of adenylate cyclase by the EP3β receptor and four mutant receptors. CHO cells expressing EP3β (□), T347 (△), T355 (▲), T339 (●), or T335 receptor (○) were incubated at 37°C for 10 min with 10 μM forskolin in the presence or absence of the indicated concentrations of M&B 28767, then cAMP contents were determined as described in Section 2. The results shown are the means ± S.E.M. for triplicate determinations.

The interaction of receptors and G proteins can be examined by investigating modulation of the binding affinity of receptors by guanine nucleotides [15]. In contrast to intact cells, in the broken cell membranes, receptors with potentially active conformation in a basal condition can associate with G proteins and promote the release of GDP from the G proteins and form the stable complex of agonist-receptor-nucleotide-free G protein in the absence of GTP. When high concentration of nonhydrolyzable GTP, GTPγS, is added, GTPγS strongly binds to G proteins and destabilizes the complex, promoting the release of G protein from the complex, then all receptors show G protein-free form [16]. In in vitro membrane experiments, receptors cannot turn activation cycle of G proteins in the absence of GTP, and thus the experimental results show the receptors with an initial transient conformation. In intact cells, agonists increase the percentage of active form of receptors. We then examined the effects of GTPγS on PGE_2-binding affinities of the EP3β receptor (agonist dependently active receptor) and T335 (constitutively active receptor). Fig. 3 shows the Scatchard analysis of PGE_2 binding to membrane expressing each receptor in the presence or absence of GTPγS. The EP3β receptor had a single high-affinity binding site in the absence of GTPγS (K_d = 2.5 nM; B_{max} = 365 fmol/mg). GTPγS did not affect the affinity of the receptor (K_d = 2.5 nM; B_{max} = 355 fmol/mg). On the other hand, the PGE_2-binding affinity of T335 showed high-affinity (K_{dH} = 1.0 nM; B_{maxH} = 65.0 fmol/mg) and low-affinity states (K_{dL} = 61 nM; B_{maxL} = 266 fmol/mg) and the majority of T335 was the low-affinity state, as reported previously [14]. The addition of GTPγS increased the number of T335 in the high-affinity state (K_{dH} = 0.83 nM; B_{maxH} = 220 fmol/mg) and decreased the number in the low-affinity state (K_{dL} = 82 nM; B_{maxL} = 82.5 fmol/mg) without any change in the total number

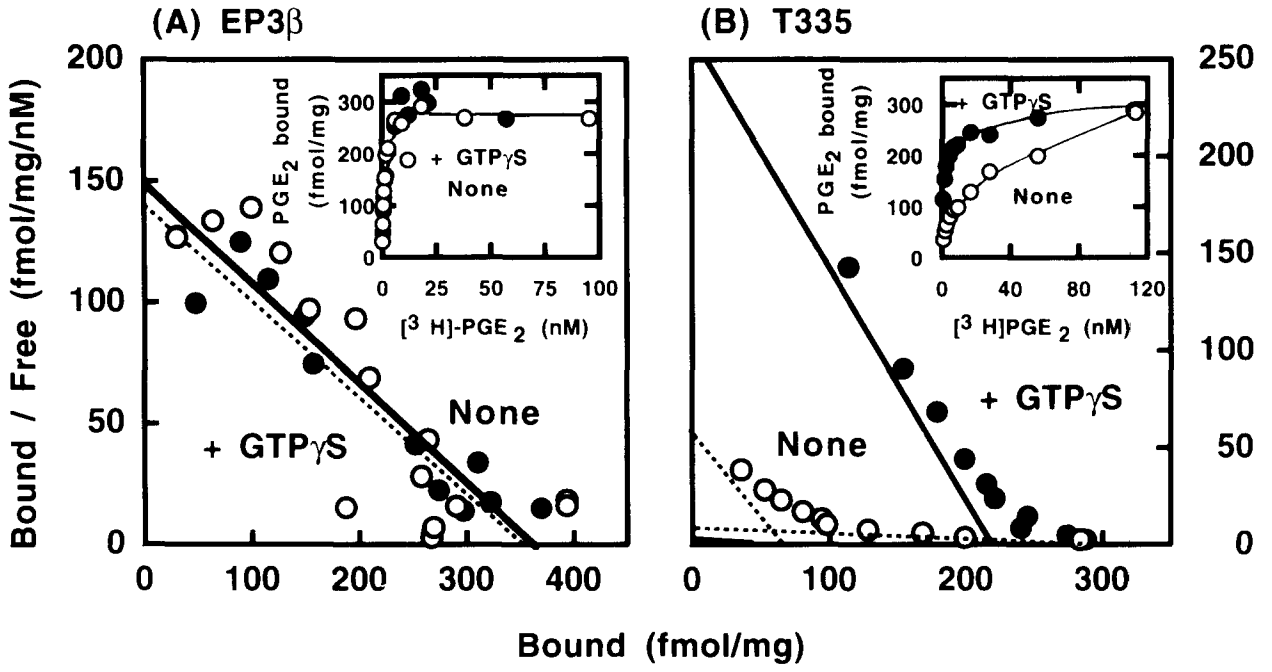

Fig. 3. Scatchard analysis of PGE_2 binding to the membrane of CHO cells expressing the EP3β or T335 receptor. The membrane fraction of CHO cells expressing the EP3β receptor (A) or T335 receptor (B) was incubated for 60 min at 30°C with increasing concentrations of [^{3}H]PGE_2 in the presence (●) or absence (○) of 100 μM GTPγS. The specific binding of [^{3}H]PGE_2 was determined as described in Section 2. The Scatchard plot was transformed from the values of specific [^{3}H]PGE_2 binding (inset). The results shown are the means for triplicate determinations.

of receptor or in the K_d values of the high-affinity and low-affinity states, indicating that GTPγS converted the receptor from its low-affinity state to the high-affinity state. Most hormone receptors show high affinity in a G protein-coupled form and low affinity in a form not associated with G proteins, and guanine nucleotides cause the dissociation of the G proteins from the receptor G protein complex, leading to the transition of the receptors from a high-affinity state to a low-affinity state [15]. In contrast with most receptors, GTPγS induced the inverse transition for T335. This means that T335 shows low affinity in the complex form with the G protein but shows high affinity in the form not associated with the G protein. Since T335 shows the constitutive Gi activity and is mainly low-affinity state, most of the T335 receptor constitutively associates with Gi. In intact cells, T335 receptor promotes the activation cycle of Gi in the absence of agonists. On the other hand, the EP3β receptor shows the agonist-dependent Gi activity and high-affinity state. Thus, it is assumed that the EP3β receptor is the form not associated with Gi and thus is insensitive to GTPγS.

Considering these findings, the EP3 receptor has a unique feature in guanine nucleotide sensitivity: the EP3 receptor shows a high affinity in the G protein-free form and guanine nucleotides increase the agonist-binding affinity. Therefore, the ratio of the G protein-associated and G protein-free EP3 receptors can be inferred from the sensitivity to guanine nucleotides. We next examined the effects of GTPγS on the PGE_2 binding to wild-type and mutant EP3 receptors. As shown in Fig. 4, GTPγS did not affect the PGE_2 binding to T355 as well as the EP3β receptor, but GTPγS slightly increased the binding to T347. On the other hand, GTPγS markedly enhanced the binding to T339 and T335. This result indicates that the progressive truncation of the COOH-terminal tail of the EP3β receptor increases the percentage of the receptor constitutively associated with the G protein with a low-binding affinity for PGE_2 but the truncation up to Leu340 induces a marked increase in this percentage. This pattern of GTPγS sensitivity is consistent with the levels of constitutive Gi activity of the progressively truncated receptors.

Lefkowitz and co-workers have proposed a two-state model in which receptors are in equilibrium between the inactive form and the active form that can associate with and activate G protein [17]. Gi activity of the EP3β receptor is completely agonist dependent, and the EP3β receptor shows high-affinity state, which does not associate with G protein (Figs. 2 and 3). Therefore, the equilibrium of the EP3β receptor basically shifts toward the inactive conformation in the absence of agonists, and the COOH-terminal tail after the alternative splicing site suppresses activation of Gi by the EP3β receptor and has strong potency in the constraint of the receptor in its inactive conformation. In intact cells, agonists move the equilibrium to the rightward direction and increase the percentage of active conformation. When the COOH-terminal tail is truncated, the equilibrium of the receptor shifts toward the active conformation, showing the G protein-associated low-affinity state, which displays constitutive activity. The COOH-terminal tail may regulate the equilibrium between the inactive and active conformations. Since the degree of constitutive activity basically correlated with the inverse of the length of the COOH-terminal tail, the length of the tail is one of the important determinants for the constraint of the receptor in the inactive form. In addition, the region between Leu340 and Val347 may play the most important role in the suppression, because marked increases in constitutive Gi activity and GTPγS sensitivity were observed with truncation of this region.

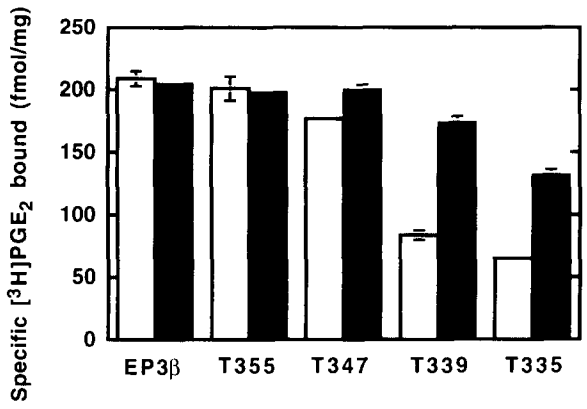

Fig. 4. Effect of GTPγS on [^{3}H]PGE_2 binding to the EP3β receptor and four mutant receptors. The membrane of CHO cells expressing the EP3β, T355, T347, T339, or T335 receptor was incubated with 4 nM [^{3}H]PGE_2 in the presence (■) or absence (□) of 100 μM GTPγS. Specific [^{3}H]PGE_2 binding was determined as described in Section 2. The results shown are the means ± S.E.M. for triplicate determinations.

In summary, we here demonstrated that inhibition of the EP3β receptor-mediated Gi activity by the COOH-terminal tail is basically regulated by the length of the tail but the region from Leu340 to Val347 plays the most important role in this inhibition. This study will help to elucidate the molecular mechanism of G protein activation induced by receptors.

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