Expression and site-directed mutagenesis of mouse prostaglandin E₂ receptor EP₃ subtype in insect cells

Chifu HUANG and Hsin-Hsiung TAI*

Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky, Lexington, KY 40536, U.S.A.

A cDNA encoding for mouse prostaglandin E₂ (PGE₂) receptor EP₃ subtype was cloned from a mouse kidney cDNA library by PCR using terminal primers derived from the known sequence of mouse lung EP₃ receptor cDNA. The cloned cDNA was confirmed by sequencing and was expressed in *Trichoplusia ni* (MG1) insect cells using a baculovirus expression system. A specific protein of 60 kDa was detected by immunoblot with antibodies generated against a unique decapeptide sequence present in the second extracellular loop of the EP₃ receptor. Specific binding of [³H]PGE₂ with a K_d of 3 nM was also found in the membrane fraction of the insect cells. Ligand binding of the receptor was further studied by site-directed mutagenesis.

Arg-309 of the receptor was separately mutated to lysine, glutamate and valine. cDNAs of the wild-type and mutant EP₃ receptors were respectively expressed and studied in MG1 insect cells. Binding studies indicated that both glutamate and valine mutant EP₃ receptors had no binding of [³H]PGE₂. On the contrary, the lysine mutant receptor exhibited an even tighter binding (K_d = 1.3 nM) than the wild-type EP₃ receptor. Immunoblot studies indicated that these receptors were expressed in a comparable amount in MG1 insect cells. These results suggest that Arg-309 of EP₃ receptor may be essential in ligand binding through ionic interaction.

INTRODUCTION

Prostaglandins (PGs) are important autocooids which have profound effects on a variety of physiological functions [1], and thus provide potential targets for pharmacological intervention in the treatment of related diseases. PGE₂ exerts its actions in diverse tissues through at least three subtypes of specific receptors, EP₁, EP₂ and EP₃ [2]. Among these subtypes, the EP₃ receptor has been the most characterized pharmacologically and has been suggested to be involved in the inhibition of neurotransmitter release, gastric acid secretion, renal sodium and water reabsorption, and adipose tissue lipolysis [3–5]. Recently, the cDNA of the mouse PGE₂ receptor EP₃ subtype was cloned, sequenced and expressed in COS-7 cells [6,7]. However, only very limited amounts of the receptor could be produced.

The baculovirus expression system has become a popular and effective means of expressing genes. Numerous eukaryotic receptor proteins have been overexpressed in insect cells using this expression system [8–10]. We have utilized this system and have successfully expressed EP₃ receptor. We started to explore the structure and function relationship of this receptor as these studies may provide valuable information for future design of antagonists for treating related diseases. Recently, Yamamoto et al. [11] employed molecular modelling to analyse the thromboxane A₂ (TXA₂) receptor–ligand interaction. This model predicts that Arg-295 from transmembrane segment VII of the TXA₂ receptor might be involved in the binding of the carboxyl group of TXA₂ [12]. Many prostaglandin receptors share structural similarity in the seventh transmembrane segment. An arginine residue corresponding to Arg-295 of the TXA₂ receptor is conserved among all the known prostaglandin receptors [13]. It follows that Arg-309 of the EP₃ receptor might also be involved in the binding of the carboxyl group of PGE₂ if the modelling prediction is correct. Accordingly, we investigated the possible role of Arg-309 of the EP₃ receptor in interacting with PGE₂ by site-directed mutagenesis. The positively charged Arg-

309 was mutated to glutamate, valine and lysine which carry negative, neutral and positive charges respectively at physiological pH. The corresponding cDNAs were expressed in *Trichoplusia ni* (MG1) insect cells and the recombinant EP₃ receptors were characterized for their binding of the ligand PGE₂. Only the lysine mutant EP₃ receptor exhibited significant binding comparable with the wild-type receptor, indicating that PGE₂ binds to its receptor through an ionic interaction.

EXPERIMENTAL

Materials

Balb/c mice were obtained from Harlan Sprague Dawley, Inc. Sequenase Version 2.0 DNA sequencing kit and restriction enzymes were from United States Biochemical Corp. Taq polymerase was from Perkin-Elmer Cetus Corp. TA-cloning kit was from Novagen. [5,6,8,11,12,14,15-³H(N)]PGE₂ (154 Ci/mmol) was obtained from Du Pont–New England Nuclear. PGE₂ and PGE₂ analogues were from Cayman Chemical Company. The EP₃-specific agonist GR63799X was a gift from Dr. P. Bhattacharjee of the University of Louisville. Immobilon-P PVDF [poly(vinylidene difluoride)] membrane was from Millipore. Anti-(rabbit IgG)–alkaline phosphatase conjugate was from Zymed Laboratories. DEAE Affi-Gel Blue column was from Bio-Rad. Epoxy-activated agarose was from Pierce. Oligonucleotide primers and peptide were synthesized by the Macromolecular Structure and Analysis Facility of the University of Kentucky. The Altered Sites *In Vitro* Mutagenesis Kit was from Promega. Grace's liquid and powdered insect medium, baculovirus transfer vector pBlueBacHis B, *Spodoptera frugiperda* (Sf9) and MG1 insect cells, and AcMNPV (*Autographa californica* multiply-enveloped nuclear polyhedrosis virus) linear DNA transfection module were purchased from Invitrogen. All other chemicals and biochemicals were obtained from various commercial sources.

Abbreviations used: Sf9, *Spodoptera frugiperda*; MG1, *Trichoplusia ni*; PGE₂, prostaglandin E₂; PGA₂, prostaglandin A₂; TXA₂, thromboxane A₂; PVDF, poly(vinylidene difluoride); MAP, multiple antigen peptide.

* To whom correspondence should be addressed.

Isolation of total RNA and mRNA

RNA was isolated according to a standard procedure [14]. Briefly, a Balb/c mouse was killed by cervical dislocation. Kidneys were collected and homogenized immediately in guanidinium isothiocyanate solution. Total RNA was isolated by ultracentrifugation and mRNA was isolated by oligo(dT) affinity chromatography. First-strand cDNA was then synthesized from mRNA by AMV reverse transcriptase using random primers.

EP₃ receptor cDNA cloning and subcloning

The EP₃ receptor cDNA was amplified from the first-strand cDNA by the PCR with synthetic oligonucleotide primers designed from the known mouse EP₃ cDNA sequence [6,7]. The sequence of the sense primer is 5'-CAAGCTGCAGTTCATA-TGGCTAGC-3' with a *Pst*I restriction site at the 5' end and that of antisense primer 5'-ATTAAGCTTTCATCTTCCAGCTGG-3' with a *Hind*III restriction site added. The PCR was conducted in a Perkin-Elmer thermal cycler for 40 cycles under the following conditions: 90 °C for 1 min, 60 °C for 2 min and 72 °C for 3 min per cycle. The PCR product was fractionated on a 1% low-melting-point agarose gel. The DNA band of the expected size of 1.1 kb was cut from the gel and purified. The purified PCR product was then ligated into the TA-cloning vector with T4 DNA ligase. The ligation mixture was used to transform Novablu competent cells. The individual white colonies were isolated and sequenced by Sanger's method [14]. The confirmed EP₃ cDNA was digested from the TA-cloning vector with *Eco*RI and *Hind*III, then subcloned into the baculovirus transfer vector pBlueBacHis B at the *Pst*I and *Hind*III sites. The recombinant transfer vector was sequenced to confirm that the EP₃ cDNA was correctly inserted.

Mutagenesis and generation of mutant EP₃ receptor

Site-directed mutagenesis on the EP₃ receptor was performed using the Altered Sites *In Vitro* Mutagenesis kit. The mutagenic oligonucleotide was a mixed primer, 5'-TTTCTAATGCAGTT(AG)(AT)GCTGGCTTCGCTGACC-3'. The above sequence corresponds to the nucleotide sequence at positions 871–1003 of the EP₃ cDNA. The base change converted Arg-309 into a lysine, valine or glutamate residue. Site-directed mutagenesis was done according to the manufacturer's instructions. After the lysine, valine and glutamate mutant EP₃ cDNAs in pSelect vector were isolated and sequenced, the mutant EP₃ cDNAs were subcloned into the baculovirus transfer vector pBlueBacHis B. Mutant EP₃ baculovirus was generated as described below. Both EP₃ and mutant EP₃ cDNAs were expressed in insect cells under the same conditions so that their activities could be compared and studied.

Cell culture

Sf9 and MG1 insect cells were grown at 27 °C in tissue-culture flasks or dishes containing insect culture medium. The insect culture medium was TNM-FH medium supplemented with 10% (v/v) fetal bovine serum as described by Summers and Smith [8]. The insect cells were subcultured whenever a monolayer of cells was formed. Only subconfluent Sf9 and MG1 insect cells were used for baculovirus infection.

Generation of wild-type EP₃ and mutant EP₃ baculovirus

Recombinant EP₃ and mutant EP₃ baculovirus were generated according to the manufacturer's instructions [15]. Briefly, recom-

binant transfer vectors containing EP₃ or mutant EP₃ cDNAs were purified by CsCl gradient ultracentrifugation. Sf9 insect cells were seeded in a 6-well tissue-culture plate. When Sf9 cells grew and covered about 50% of the surface, they were ready for viral transfection. Recombinant transfer vectors (3 µg), 1 µg of linear wild-type AcMNPV viral DNA and 20 µl of lipofectin were mixed in 1 ml of culture medium. The medium of Sf9 cells in a well was removed and the 1 ml transfection solution was added to the Sf9 cells in that well. Recombinant EP₃ and mutant EP₃ baculovirus were generated due to the *in vivo* homologous recombination between polyhedrin sequences of wild-type viral DNA and the recombinant transfer vectors in Sf9 cells. In this way, primary recombinant baculovirus was generated containing wild-type EP₃ or mutant EP₃ cDNAs. The recombinant EP₃ and mutant EP₃ baculovirus were subsequently purified by blue plaque assay from the wild-type baculovirus.

Expression of wild-type EP₃ and mutant EP₃ receptor

MG1 insect cells were used for EP₃ receptor expression. Fresh MG1 insect cells (5×10^6) were seeded in a 75 cm² tissue-culture flask in 15 ml of complete TNM-FH medium and allowed to grow to 50% confluency. Then recombinant EP₃ and mutant EP₃ baculovirus, at a multiplicity of infection of about 10, was added to the seeded MG1 cells. The MG1 insect cells were infected for about 3–4 days and were collected from the flask. The membrane fraction of the MG1 cells was prepared as previously described [16]. Briefly, the infected cells were collected and centrifuged for 10 min at 1000 *g*. The cell pellet was resuspended in PBS solution and then sonicated. The sonicated solution was centrifuged at 500 *g* for 10 min at 4 °C. The supernatant was collected and centrifuged at 100 000 *g* for 45 min at 4 °C. The membrane pellet was collected and resuspended in PBS.

Preparation of mouse kidney membrane

Mouse kidneys were collected from a dead mouse and immediately stored in solid CO₂. The kidney membrane fraction was prepared in a similar way to the insect cells.

Binding assay of wild-type EP₃ and mutant EP₃ receptors

EP₃ receptor was assayed for [³H]PGE₂ binding with a modified method as previously described [17]. Briefly, membranes were incubated with various amounts of [³H]PGE₂ in a final volume of 100 to 200 µl of binding buffer (20 mM Tris/HCl, pH 7.0, containing 10 mM MgCl₂, 1 mM EDTA and 0.1% gelatin) at 37 °C for 45 min. The membranes were collected by centrifugation at 20 000 *g* for 15 min at 4 °C. The membranes were washed with ice-cold binding buffer and again centrifuged. After washing twice with ice-cold binding buffer, the membranes were resuspended in cocktail and counted for radioactivity by liquid scintillation counting. Binding data were analysed with a computer program (radioligand binding analysis program) to calculate *K_d* and *B_{max}* from Scatchard plots [18].

Time course of EP₃ receptor expression

MG1 insect cells were seeded in 6-well plates and allowed to grow to subconfluent monolayers. Then, they were infected with recombinant EP₃ baculovirus or wild-type baculovirus. Cells were harvested at 24, 48, 72, 84, 96 and 108 h after initial infection. Cell membranes derived from the 10 000 cells were used to determine [³H]PGE₂-binding activity. About 1000 c.p.m. of [³H]PGE₂ was used for each binding assay.

Polyclonal antibodies against the EP₃ receptor peptide

A peptide N-E-T-D-P-A-R-E-P-G, which corresponds to a segment of the second extracellular loop at position 193–202 of the EP₃ receptor, was synthesized as a multiple antigen peptide (MAP) [19]. MAP (0.5 mg) was emulsified with Freund's complete adjuvant and injected into a rabbit to stimulate the production of antibodies. The rabbit was bled 1 week after each booster immunization. The serum was checked for the existence of antibodies against the MAP by ELISA. Briefly, the MAP peptide (200 ng/100 μ l per well) was coated on to a microwell plate in coating buffer (0.1 M Na₂CO₃/NaHCO₃, pH 9.6) at 4 °C overnight. After washing each well with PBS containing 0.05 % Tween-20, 200 μ l of 5 % non-fat milk solution was added to each well for 1 h at 37 °C to block the non-specific binding sites. After washing each well, 100 μ l of variously diluted rabbit serum was added to each well of the microwell plate and incubated for 1 h at room temperature. After washing each well again, 100 μ l of 1:5000 diluted anti-(rabbit IgG)–horseradish peroxidase conjugate (from a 1 mg/ml stock solution) was added to each well and incubated for 1 h at room temperature. After washing each well, 100 μ l of enzyme substrate (1.25 mM 3,3',5,5'-tetramethylbenzidine plus 6 mM H₂O₂ in 25 mM citrate buffer, pH 3.5) was added to each well and incubated for 20 min at room temperature. Finally, the microwell plate was read for absorbance at 650 nm in an ELISA reader. When a good titre of antibodies was detected, 40 ml of rabbit serum were collected from the rabbit following each booster injection.

DEAE Blue chromatography and affinity purification of antibodies

A DEAE Affi-Gel Blue column was used to isolate the IgG fraction from the rabbit serum. A sample (40 ml) of crude rabbit serum was dialysed in PBS for 5 h and loaded on to a 60 ml DEAE Affi-Gel Blue column equilibrated with PBS. The IgG fraction did not bind to the column and eluted out in the void volume. The IgG fraction was further purified with a MAP-peptide affinity column to isolate the specific anti-(EP₃ peptide) antibodies. Briefly, the MAP-peptide affinity column was prepared by mixing 2 mg of MAP-peptide to 0.2 g of epoxy-activated agarose in 1 ml of coupling buffer (0.1 M Na₂CO₃, pH 11.0) and allowed to react at 37 °C for 20 h with mixing. The mixture was then loaded on to a column and washed with PBS. The IgG fraction was loaded on to the MAP-peptide affinity column. The column was then washed extensively with washing buffer (0.1 M NaHCO₃ plus 0.5 M NaCl, pH 8.0). The affinity-purified IgG was eluted out from the column with 0.1 M glycine/HCl, pH 2.5, and neutralized immediately with 1 vol. of 1 M Tris/HCl buffer, pH 8.0. The anti-peptide antibodies were monitored for UV absorbance at 280 nm. The anti-peptide antibodies were then dialysed against PBS solution and lyophilized. The anti-peptide antibodies were dissolved in PBS.

SDS/PAGE and Immunoblotting analysis

The membrane fraction was suspended in PBS and solubilized with 1 % Tween-20 for 1 h at 4 °C. The concentration of solubilized membranes as determined by the method of Bradford [20]. A sample (50 μ g) of crude membrane proteins was fractionated by SDS/10 %-PAGE and transferred electrophoretically to an Immobilon-P PVDF membrane. The PVDF membrane was first blocked with 5 % non-fat milk solution and then incubated with 10 ml of 1:1000 diluted affinity-purified rabbit anti-(EP₃ peptide) IgG (from 50 μ g/ml stock solution) for 1 h at room temperature. After washing, the PVDF membrane was incubated with 10 ml of 1:1000 diluted anti-(rabbit IgG)–

alkaline phosphatase conjugate (from 1 mg/ml stock solution) for 1 h at room temperature. The EP₃ receptor on the membrane was visualized by using NitroBlue Tetrazolium (0.2 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.1 mg/ml) as substrate.

RESULTS

The cDNA of the EP₃ subtype was successfully expressed as an active recombinant EP₃ receptor in Sf9 and MG1 insect cells using the baculovirus system. The time course of EP₃ receptor expression in MG1 cells was monitored by PGE₂-binding assay.

Figure 1 Displacement binding assay of wild-type EP₃ and lysine mutant EP₃ receptors

MG1 insect cells were infected with baculovirus containing the wild-type EP₃ or lysine mutant EP₃ cDNAs for 84 h. Membrane proteins (35 μ g/sample) were incubated with [³H]PGE₂ (10 000 c.p.m.) plus PGA₂, EP₁-specific, EP₂-specific, and EP₃-specific agonists at the indicated concentrations. Following incubation, the membranes were washed and counted for radioactivity as described in the Experimental section. (a) The wild-type EP₃ receptor; (b) the lysine mutant EP₃ receptor. PGA₂ (○), EP₁-specific agonist 17-trinor phenyl PGE₂ (▲), EP₂-specific agonist 11-deoxyl PGE₁ (△), EP₃-specific agonist GR63799X (●).

304	F	L	I	A	V	R	L	A	S	L	N	Q	I	L	D	P	W	320	mouse	EP ₃ (<i>3a, b, c</i>)	[6, 7]
304	F	L	I	A	V	R	L	A	S	L	N	Q	I	L	D	P	W	320	rat	EP ₃ (<i>3A⁺, 3B</i>)	[30, 38]
327	F	L	I	A	V	R	L	A	S	L	N	Q	I	L	D	P	W	343	bovine	EP ₃ (<i>3A, 3B, 3C, 3D</i>)	[31]
328	F	L	I	A	V	R	L	A	S	L	N	Q	I	L	D	P	W	344	human	EP ₃ (<i>31, 311, 3111</i>)	[34]
337	F	L		A	V	R	L	A	S	W	N	Q	I	L	D	P	W	352	mouse	EP ₁	[27]
334	F	L		A	V	R	L	A	S	W	N	Q	I	L	D	P	W	349	human	EP ₁	[36]
339	D	L	Q	A	I	R	I	A	S	V	N	P	I	L	D	P	W	355	mouse	EP ₂	[28]
311	D	L	Q	A	I	R	I	A	S	V	N	P	I	L	D	P	W	327	human	EP ₂	[33]
287	L	L	I	Y	I	R	V	A	T	W	N	Q	I	L	D	P	W	303	mouse	TXA ₂	[12]
290	L	L	I	Y	L	R	V	A	T	W	N	Q	I	L	D	P	W	316	human	TXA ₂	[32]
286	T	L	F	A	L	R	M	A	T	W	N	Q	I	L	D	P	W	302	mouse	PGF _{2d}	[29]
286	T	L	F	T	L	R	M	A	T	W	N	Q	I	L	D	P	W	302	bovine	PGF _{2d}	[35]
286	T	L	F	A	L	R	M	A	T	W	N	Q	I	L	D	P	W	302	human	FP	[37]
304	L	L	A	F	R	F	N	A	F	N	P	I	L	D	P	W	319	mouse	PGI ₂	[39]	

Figure 2 Similarity of EP₃ receptor to other prostaglandin receptors at the VII transmembrane segment

The sequence shown corresponds to part of transmembrane segment VII of the prostaglandin receptors. Numbers on the left- and right-hand side of the sequence are corresponding positions of each receptor. The arrow indicates Arg-309 of the EP₃ receptor which is conserved in each of these receptors. References are indicated by italics on the right-hand side.

Table 1 Nucleotide sequence of wild-type EP₃ and mutant EP₃ receptors at residue 309

Codon	Amino acid	EP ₃ receptor	Charge of amino acid in the protein
CGC	Arginine	Wild type	+
AAG	Lysine	Mutant	+
GAG	Glutamate	Mutant	-
GTG	Valine	Mutant	0

MG1 cells, infected with recombinant EP₃ baculovirus, show high [³H]PGE₂-binding activity, whereas the MG1 cells infected with the wild-type baculovirus show no [³H]PGE₂-binding activity. When MG1 insect cells were infected by recombinant EP₃ baculovirus for 24 h, the membrane started to show detectable [³H]PGE₂ binding. Maximal binding activity was detected when MG1 insect cells were infected by recombinant baculovirus for 82 h.

To examine whether the expressed PGE₂ receptor is of the EP₃ subtype, EP₁-, EP₂- and EP₃-specific agonists were used to compete with [³H]PGE₂ for binding subtype receptor (Figure 1a). Other prostaglandins, such as prostaglandin A₂ (PGA₂), were also included in the competitive binding studies. As expected, both EP₁ agonist 17-trinor phenyl PGE₂ and EP₂-specific agonist 11-deoxyl PGE₁ showed less potency in inhibiting [³H]PGE₂-binding than the EP₃-specific agonist GR63799X [21,22]. Little inhibition of binding was observed for PGA₂. The IC₅₀ of EP₁-specific and EP₂-specific agonists are about 3 nM, while the IC₅₀ of EP₃-specific agonist is about 1 nM. These dose-response curves are comparable with that of mouse kidney membrane from which the mouse EP₃ cDNA was originally cloned. The mouse kidney membrane shows a similar competitive binding pattern for EP₁-specific agonist (IC₅₀ = 6 nM), EP₂-specific agonist (IC₅₀ = 6 nM) and EP₃-specific agonist (IC₅₀ =

Figure 3 Binding assay of wild-type EP₃ and mutant EP₃ receptors expressed in insect cells

MG1 insect cells were seeded in 75 cm² tissue-culture flasks and infected with recombinant baculovirus containing wild-type EP₃ (arginine), mutant EP₃ (glutamate, lysine or valine) cDNAs for 84 h. The membrane fractions were prepared as described in the Experimental section. Membranes (30 μg of protein/sample) were incubated with various concentrations of [³H]PGE₂ and counted for radioactivity. Receptors: wild-type EP₃ (●), lysine mutant EP₃ (○), valine mutant EP₃ (×), glutamate mutant EP₃ (△), control (□).

3 nM). This result confirmed that the expressed PGE₂ receptor is indeed a specific EP₃ subtype.

Site-directed mutagenesis was carried out on residue Arg-309 in transmembrane domain VII of the EP₃ receptor. This residue was found to be conserved in transmembrane domain VII of all the known cDNAs of prostaglandin receptors (Figure 2). As discussed above, molecular modelling studies of the TXA₂

Figure 4 Western-blot analysis of wild-type EP₃ and mutant EP₃ receptors expressed in insect cells

MG1 insect cells were seeded in 75 cm² tissue-culture flasks and were infected with baculovirus containing EP₃ (arginine) and mutant EP₃ cDNAs (glutamate, valine or lysine) for 84 h. Membrane fractions were prepared as described in the Experimental section. Membranes (50 μg/sample) were fractionated by an SDS/10%-PAGE and then transferred to Immobilon-P PVDF membrane. The PVDF membrane was incubated with antibodies and developed as described in the Experimental section. Val mutant EP₃ (lane 1); Glu mutant EP₃ (lane 2); control (lane 3); wild-type EP₃ with Arg-309 (lane 4); Lys mutant EP₃ (lane 5).

Figure 5 Scatchard analysis of PGE₂ binding of wild-type EP₃ and lysine mutant EP₃ receptors

MG1 insect cells were infected by recombinant baculovirus containing wild-type EP₃ or lysine mutant EP₃ cDNA for 84 h. Membrane fractions (50 µg of protein/sample) were incubated with various concentrations of [³H]PGE₂. The membranes were washed and counted for radioactivity as described in the Experimental section. Non-specific binding was taken as radioactivity counted on the membrane of MG1 cells infected only by the wild-type baculovirus. (a) Scatchard plot of lysine mutant EP₃ receptor. (b) Scatchard plot of wild-type EP₃ receptor.

receptor suggested that the positively charged Arg-295 is possibly involved in ligand binding [11]. We expect the same interaction might be true for the PGE₂ receptor. We anticipated that the positively charged guanidyl group of Arg-309 might interact with the negatively charged carboxyl group of PGE₂. To test this hypothesis, Arg-309 of the EP₃ receptor was mutated into positively charged lysine, negatively charged glutamate and uncharged valine at physiological pH (Table 1).

The cDNAs of EP₃ and mutant EP₃ receptors were expressed in MG1 insect cells and were studied for PGE₂-binding activity.

Binding assays indicated that both the wild-type EP₃ and lysine mutant EP₃ receptors carrying a positively charged amino acid had high [³H]PGE₂-binding activity, whereas the negatively charged glutamate and uncharged valine mutant EP₃ receptors showed no [³H]PGE₂-binding activity (Figure 3). This result clearly suggests that the positive charge of the guanidyl group of Arg-309 is essential for interacting with PGE₂.

Immunoblotting analysis indicated that the wild-type EP₃ and the mutant EP₃ receptors were expressed in comparable amounts in the MG1 cells (Figure 4). A specific protein of 60 kDa was detected on the immunoblot membrane. This 60 kDa specific protein was detected only when MG1 cells were infected by the wild-type EP₃ and the mutant EP₃ recombinant baculovirus. However, it was not detected when MG1 cells were infected by the wild-type baculovirus. The presence of the 60 kDa protein apparently showed that the lack of [³H]PGE₂ binding by the glutamate and valine mutant EP₃ receptors was not due to the failed expression of mutant receptors, but rather due to the fact that both mutants lost their PGE₂-binding activity when the positively charged Arg-309 was changed to a negatively charged or uncharged amino acid residue.

The recombinant wild-type EP₃ and lysine mutant EP₃ receptors were further characterized and studied for their PGE₂-binding activity. Scatchard analysis indicated that the lysine mutant EP₃ receptor had an even higher binding affinity than that of the wild-type EP₃ receptor (Figure 5). The recombinant wild-type EP₃ receptor had a K_d value of 3 nM and B_{max} of 7.20×10^{-10} M, while the lysine mutant EP₃ receptor had a K_d of 1.3 nM and a B_{max} of 7.47×10^{-10} M. The lysine mutant EP₃ receptor had a lower K_d than that of the EP₃ receptor, but their B_{max} values were about the same. The subtype specificity of lysine mutant receptor was also examined. Similar to Figure 2(a), EP₃ agonist was the most potent in inhibiting [³H]PGE₂ binding (Figure 1b). But EP₂-specific agonist became more potent than the EP₁-specific agonist in inhibiting [³H]PGE₂ binding of lysine mutant EP₃ receptor.

DISCUSSION

Prostaglandin receptors have not been expressed in a baculovirus system even though some of them were expressed in mammalian cells, such as COS-7 cells and Chinese hamster ovary cells [6,23]. In this study, we report that the cDNA of the EP₃ receptor was successfully expressed as a functional receptor in insect cells using a baculovirus expression system. The recombinant EP₃ receptor had a high-affinity binding specific for PGE₂ with a K_d of 3 nM, a value very similar to that of the native EP₃ receptor [24]. Several reports have demonstrated that various receptors expressed in insect cells and the native receptors have comparable binding affinities [8–10]. We also attempted to express the cDNA of the EP₃ receptor in *Escherichia coli*, but no PGE₂-binding activity was detected in the membrane fraction even though a specific protein was observed in an SDS/10 %-PAGE experiment. This is consistent with the previous observation that the *E. coli* expression system is not suitable for expression of some membrane proteins [25].

Antibodies against the EP₃ receptor and other subtypes are not available. Therefore, specific anti-peptide antibodies against the EP₃ receptor were raised in this study. We selected an EP₃-specific peptide sequence in the second extracellular loop to produce specific anti-peptide antibodies. A specific protein of 60 kDa was detected in Western blots and it was the only band shown on the membrane. This suggests that the EP₃ receptor has an estimated molecular mass of 60 kDa. This is the first report showing that the EP₃ receptor has an estimated molecular mass

of 60 kDa. This conclusion was also indirectly supported by other studies. Negishi et al. [23] incubated [^3H]PGE₂ with the EP₃ receptor and were able to elute out a [^3H]PGE₂-bound protein with a molecular mass of 60 kDa in gel filtration. The TXA₂ receptor has about 10 fewer amino acids than that of EP₃ receptor and has an estimated molecular mass of 55–58 kDa [26]. Therefore, the estimated molecular mass in our study should be about correct for the EP₃ receptor.

This is the first site-directed mutagenesis study that was carried out on the cDNA of the EP₃ receptor. The principal conclusion derived from our site-directed mutagenesis study is that Arg-309 of the EP₃ receptor is a very important residue for PGE₂-binding activity. The significance of Arg-309 of the EP₃ receptor is apparently due to its positive charge on the guanidinyll group. Once the positive charge at position 309 was removed by mutation to an uncharged valine, the PGE₂-binding activity of the EP₃ receptor was subsequently abolished. This conclusion was further supported by the fact that the presence of a negatively charged glutamate at position 309 also abolished its PGE₂-binding activity. However, when Arg-309 was substituted by a positively charged lysine residue, the PGE₂-binding activity was maintained, indicating that the ionic interaction is required for binding.

To investigate the possible effect of the change to lysine at position 309, recombinant EP₃ and the lysine mutant EP₃ receptors were compared and studied for affinity and specificity of their PGE₂-binding activity. The lysine residue did not change the specificity of the EP₃ receptor. However, the lysine mutant EP₃ receptor shows a higher binding affinity than that of the wild-type EP₃ receptor, even though it shares a similar B_{max} value. EP₂-specific agonist also became much more potent in inhibiting [^3H]PGE₂ binding than EP₁-specific agonist in lysine-mutant EP₃ receptor. The binding affinity and specificity shift could be due to the different strength of interaction between their side chains and the carboxyl group of PGE₂ because of the conformational difference of the positively charged side chain between the arginine and lysine residues. It is conceivable that the positively charged side chain of lysine could be slightly more favourable and stronger than that of the arginine side chain in interacting with the carboxyl group of PGE₂.

The important role of Arg-309 of the EP₃ receptor was consistent with the prediction by molecular modelling of the TXA₂ receptor [11]. Prostaglandin receptors share many homologous regions in their amino acid sequences, especially at the transmembrane domains [13]. Prostaglandins also have very similar structures with a carboxyl tail. Therefore, the corresponding arginine in transmembrane segment VII of the prostaglandin receptors may have a similar function in interacting with their respective ligands. Our study could be helpful for a similar future study on other prostaglandin receptors. Site-directed mutagenesis is being carried out to identify other important residues of the EP₃ receptor which might also interact with the PGE₂ ligand.

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