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A Single Amino-Acid Substitution in the EP_2 Prostaglandin Receptor Confers Responsiveness to Prostacyclin Analogs

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ABSTRACT

A high degree of homology between the four G_s-coupled prostaglandin (PG) receptors [EP_2, EP_4, prostacyclin (IP), PGD_2 (DP)] and the four G_q/G_i-coupled receptors [EP_1, EP_3, $PGF_{2\alpha}$ (FP), thromboxane A_2 (TP)] suggests that prostaglandin receptors evolved functionally from an ancestral EP receptor before the development of distinct binding epitopes. If so, ligand selectivity should be determined by a limited number of amino acids. EP_2 receptor transmembrane domain residues that are similar to those in the EP_4 receptor but differ from those in the IP receptor were mutated to the corresponding IP receptor residue. Activation of the mutant receptors by PGE_2 (EP_2 ligand), iloprost (stable prostacyclin analog), and PGE_1 (EP_2/IP ligand) was determined using a cAMP-dependent reporter gene assay. A Leu304-to-tyrosine substitution in the seventh transmembrane domain enhanced iloprost potency approximately 100-fold. A glycine substitution at Ser120 in the third transmembrane domain had no effect on drug potency but improved the response of the Tyr304 mutant. The potency of the natural prostaglandins $PGF_{2\alpha}$ and PGD_2 was not enhanced by the mutations. In contrast, the potency of all prostaglandins was reduced 10- to 100-fold when arginine 302, which is thought to be a counterion for the prostaglandin carboxylic acid, was mutated. Thus, a single amino acid change resulted in a selective gain of function for iloprost, which is consistent with the proposed phylogeny of the prostaglandin receptors.

The molecular identification of eight G protein-coupled membrane receptors that mediate the actions of the five primary prostaglandins has helped to explain the myriad of biological effects of these arachidonic acid metabolites. In particular, PGE_2 affects almost every tissue in the body (often in opposing ways), including smooth muscle contraction and relaxation and pro-inflammatory and anti-inflammatory actions. Four subtypes of the PGE_2 receptor, termed EP_1, EP_2, EP_3, and EP_4, have been cloned (Pierce *et al.*, 1995) and shown to couple to different signaling systems. The EP_1 receptor couples preferentially to G_q, the EP_2 and EP_4 receptors couple to G_s, and the six EP_3 receptor carboxyl tail splice variants couple to G_i. Thus, these four receptors respond quite differently to the same physiological ligand.

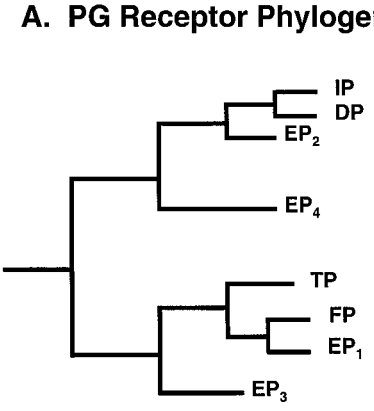
Surprisingly, when the deduced amino acid sequences for the four EP receptors are aligned and compared, they demonstrate an unexpected degree of divergence. Alignments of all the prostaglandin receptors showed that the EP_2 receptor is more similar to the IP receptor and the DP receptor than to the other three EP receptors. Phylogenetic analysis (Regan *et al.*, 1994; Toh *et al.*, 1995) of receptor sequences led to the conclusion that the prostaglandin receptors evolved from a precursor EP receptor into two subfamilies that differ with respect to their G protein coupling (Fig. 1A). Receptors that preferentially interact with the major endogenous prostaglandins other than PGE_2 must have evolved following the functional division of the EP receptors. Thus, the IP and DP receptors evolved from the EP_2 receptor, and the FP and TP receptors evolved from the EP_3 or EP_1 receptor. If this hypothesis is correct, a relatively small number of amino acids may determine the selective interactions of the prostaglandins with their receptors.

The EP_2 and the IP receptors represent an ideal receptor pair for studying the determinants of ligand selectivity. Their seven TMs are more than 60% identical at the amino acid level and they share some common ligands, such as PGE_1. However, PGE_2 is more than 1000-fold selective for the EP_2 receptor, and the stable prostacyclin analog iloprost is more than 1000-fold selective for the IP receptor. We identified approximately 15 residues that were similar in the EP_2 and EP_4 receptor but differed in the IP receptor. To determine whether these amino acid residues were responsible for the preferential activities of PGE_2 and iloprost, the EP_2 receptor sequence was changed at these positions to the correspond-

ABBREVIATIONS: PG, prostaglandin; EP_2, PGE_2 receptor subtype 2; EP_4, PGE_2 receptor subtype 4; IP, prostacyclin receptor; DP, PGD_2 receptor; EP_1, PGE_2 receptor subtype 1; EP_3, PGE_2 receptor subtype 3; TP, thromboxane A_2 receptor; FP, $PGF_{2\alpha}$ receptor; TM, transmembrane domain; CRE, cAMP response element; CAT, chloramphenicol acetyl transferase.

ing IP receptor amino acid (Fig. 1B) by site-directed mutagenesis and the effect on activation by PGE_2 and iloprost determined in a reporter gene assay. PGE_1, a ligand at both the EP_2 and IP receptors, was used to confirm that the mutant receptors were functionally expressed.

A single amino acid change in TM7 enables iloprost to activate the EP_2 receptor. This mutant receptor is activated by both PGE_2 and iloprost, but not by PGD_2 or $PGF_{2\alpha}$. This represents the first report of a single amino acid change in a prostaglandin receptor resulting in a gain of function for prostaglandin ligands and is consistent with the proposed phylogeny of the prostaglandin receptors.



B.

	EP_2	^{26}A I S S V M F S A G V L G N L I A L A L L A
TM1	IP	^{18}A T S T L M F V A G V V G N G L A L G I L S
	EP_4	^{22}T I P A V M F I F G V V G N L V A I V V L C
	EP_2	^{69}V L V T E L V F T D L L G T C L I S P V V L A
TM2	IP	^{51}V L V T G L A A T D L L G T S F L S P A V F V
	EP_4	^{56}T L V C G L A V T D L L G T L L V S P V T I A
	EP_2	^{93}Y A R N Q T L V A L A P E S
1EX	IP	^{75}Y A R N S S L L G L A R G G
	EP_4	^{80}Y M K G Q W - - - - - P G G
	EP_2	^{111}Y F A F A M T F F S L A T M L M L F A M A L
TM3	IP	^{94}A F A F A M T F F G L A S M L I L F A M A V
	EP_4	^{94}Y S T F I L L F F S L S G L S I I C A M S V
	EP_2	^{156}V L P V I Y A V S L L F C S L P L L D Y G
TM4	IP	^{139}A L P A I Y A F C V L F C A L P L L G L G
	EP_4	^{39}T L F A V Y A S N V L F C A L P N M G L G
	EP_2	^{177}Q Y V Q Y C P G T W C F I R H
2EX	IP	^{160}Q H Q Q Y C P G S W C F L R M
	EP_4	^{161}S R L Q Y - P D T W C F I D W
	EP_2	^{199}L Y A T L L L L L I V S V L A C N F S V I
TM5	IP	^{187}A Y A G L V A L L V A A I F L C N G S V T
	EP_4	^{187}M Y A G F S S F L I L A T V L C N V L V C
	EP_2	^{263}L I L L A I M T I T F A V C S L P F T I
TM6	IP	^{238}L I L L A L M T V V M A V C S L P L T I
	EP_4	^{271}V I L L I A T S L V V L I C S I P L V V
	EP_2	^{299}Q A L R F L S I N S I I D P W V F A I L
TM7	IP	^{276}L A F R F Y A F N P I L D P W V F I L F
	EP_4	^{313}Q A I R I A S V N P I L D P W I Y I L L

Fig. 1. Phylogeny of human prostaglandin receptors. A, Alignment of deduced amino acid sequences of eight cloned human prostaglandin receptors was used to generate the phylogenetic tree (Regan *et al.* 1994). Receptors linked to G_s are clustered, as are those that are linked to G_q and/or G_i. B, Deduced amino acid sequences of three G_s-linked receptors (EP_2, IP, and EP_4) were aligned using the University of Wisconsin GCG program. The most highly conserved regions are shown. *Underlined* residues in the EP_2 receptor are those that were mutated, usually to the corresponding residue from the IP receptor. *EX*, extracellular loop.

Experimental Procedures

Materials. Iloprost and [^{3}H]acetyl Coenzyme A were purchased from Amersham Life Sciences (Arlington Heights, IL). All other prostaglandin compounds were purchased from Cayman Chemical (Ann Arbor, MI). LipofectAMINE, Opti-MEM, and other tissue culture media and serum were purchased from Life Technologies (Gaithersburg, MD). Stripped fetal bovine serum was obtained from Gemini Products (Calabasas, CA). Acetyl coenzyme A, chloramphenicol, and DNase I were purchased from Sigma Chemical (St. Louis, MO).

Site-directed mutagenesis. Missense mutations were introduced by the Kunkel method (Kunkel, 1985) using a Muta-Gene kit purchased from BioRad (Richmond, CA). The human EP_2 receptor cDNA (Regan *et al.*, 1994) was placed in pcDNA3 (InVitrogen, Carlsbad, CA) for all mutagenesis and expression studies. Oligonucleotides were purchased from Genosys (The Woodlands, TX) or synthesized in-house on an Oligo1000 oligonucleotide synthesizer (Beckman Instruments, Fullerton, CA). Mutations were verified by DNA sequence analysis using a Sequenase kit (Amersham Life Sciences, Arlington Heights, IL). The double mutant EP_2 S120G L304Y was constructed from two single mutants by using the internal *Apa*I restriction site and standard protocols.

Expression of EP_2 cDNAs in cell culture. CV-1 cells were transiently transfected with plasmids carrying the wild-type or mutant EP_2 cDNA using lipofectamine. The CRE-CAT reporter plasmid TES*blg*IICRE(+)ΔNHSE was obtained from Dr. Pamela Mellon of The Salk Institute (La Jolla, CA). This construct contains an 18-base-pair CRE from the promoter of the α subunit gene for the human glycoprotein hormone linked to the herpes simplex virus thymidine kinase promoter, which drives bacterial CAT gene expression (Delegeane *et al.*, 1987). Thus, CAT enzyme activity is dependent on and proportional to cAMP levels in the cells. Fifty thousand cells were plated in wells of a 24-well plate and transfected with 125 ng of receptor plasmid, 250 ng of reporter plasmid, and 6 μg of lipofectamine per well. Cells were fed with Dulbecco's modified Eagle's medium containing 20% stripped fetal bovine serum at approximately 5 hr. Cells were dosed with drug approximately 24 hr after feeding, and assayed for CAT activity approximately 18 hr after dosing.

CAT assay. Medium was removed by aspiration and the cells washed twice with ice-cold phosphate-buffered saline (1× = 0.02% KCl, 0.02% KH_2PO_4, 0.8% NaCl, 0.216% $Na_2HPO_4{\cdot}7H_2O$) without calcium or magnesium. Lysis buffer (50 μl) containing 1% Triton X-100, 1 mM Tris·HCl, pH 7.8, and 2 mM EDTA, pH 8.0, and 0.4 mg/ml DNase I was added and cells lysed on ice with periodic shaking for 45 min. Reaction mix (50 μl) containing 40 μM [^{3}H]acetyl coenzyme A, 60 μM acetyl coenzyme A, 30 μM HCl, 2 mM chloramphenicol, 200 mM Tris·HCl, pH 7.8, and 4 mM EDTA, pH 8.0, was added to the lysate and the mixture incubated for 90 min at 37°. The reaction was stopped with 100 μl of 7 M urea and the entire volume (200 μl) transferred to scintillation vials. One milliliter of scintillant (0.8% 2,5-diphenyloxazole in toluene) was added and the vials shaken to mix the phases. The phases were allowed to separate for 15 min before reading in a scintillation counter, to allow the [^{3}H]acetylated chloramphenicol product to partition into the nonpolar phase (Nielsen *et al.*, 1989). Samples were assayed in triplicate and average dpm values were obtained.

Data analysis. Because PGE_1 is the common ligand for EP_2 and IP receptors, all values were expressed as a percentage of the maximum PGE_1 value. The basal value for each dose-response curve was subtracted from all dpm values. The highest PGE_1 value for each receptor on each assay day was considered 100%. Dose-response curves were generated using KaleidaGraph (Abelbeck/Synergy Software, Reading, PA) by least-squares fits to this equation: response = maximum response + (minimum response − maximum response)/[1 + (concentration of ligand/EC_{50})]. The data are reported as mean ± standard error of three to 12 independent experiments.

Results

To identify key residues for ligand discrimination, mutants were screened for CAT activity in a CRE-CAT reporter gene assay, using 0.1, 10, and 100 nM concentrations of PGE_1, PGE_2, and iloprost (data not shown). Because PGE_1 activity should not be affected by mutations that alter ligand selectivity between the EP_2 and IP receptors, it was used to assess the functionality of the mutant receptors. Mutants that were not activated by PGE_1 were assumed to be inappropriately expressed or improperly assembled and were not pursued further. The majority of mutants were unremarkable, in that they retained the ability to signal in response to PGE_1 and PGE_2 and did not gain the ability to signal in response to iloprost (Table 1). Active mutants were assayed for CAT activity over a complete range of doses from 1 nM to 10 μM.

EP_2 activity. The EP_2 receptor demonstrated function, as determined by CAT activity, in response to PGE_1 and PGE_2 (Fig. 2A). EC_{50} values of 34.0 ± 11.0 nM for PGE_1 and 25.5 ± 6.6 nM for PGE_2 are in the range of previously published values for the EP_2 receptor (Regan *et al.*, 1994; Woodward *et al.*, 1995; Nishigaki *et al.*, 1996). The EP_2 receptor responds to iloprost (Fig. 2A), carbacyclin (Fig. 3), PGD_2, and $PGF_{2\alpha}$ (Fig. 2E) only at micromolar concentrations, as previously reported.

Residue 304 is a key determinant of selective ligand interaction. The mutant receptor EP_2 L304Y was activated in response to PGE_1, PGE_2, and iloprost (Fig. 2B). The EC_{50} values are 99.9 ± 29.5 nM for PGE_1, 33.3 ± 11.2 nM for PGE_2, and 128.9 ± 38.3 nM for iloprost. The potency of iloprost is increased at least 50-fold because of the mutation at position 304.

Computer modeling (data not shown) suggested that residue 120 in TM3 participates in interactions with residue 304 in TM7. For this reason, mutant EP_2 S120G and the double mutant EP_2 S120G L304Y were generated and analyzed. Mutant EP_2 S120G retains the ability to respond to PGE_1 and PGE_2 (EC_{50} values of 47.4 ± 21.1 nM and 33.0 ± 11.7 nM, respectively) without responding to iloprost except at micromolar concentrations (Fig. 2C). It seems to respond much as

TABLE 1

Summary of Pharmacology of EP_2 single mutants

CV-1 cells transiently transfected with mutant EP_2 receptor cDNA and reporter cDNA were evaluated in the CRE-CAT assay. "Yes" signifies a functional response to agonist at concentrations <1 μM. Each mutant was tested in the indicated number of independent experiments (n), with each assay carried out in triplicate.

Mutant	Location	*n*	PGE_1	PGE_2	Iloprost
L84F	2 TM	2	yes	yes	no
L90F	2 TM	2	yes	yes	no
Q97S	1 EX[a]	1	no	no	no
P104R	1 EX	1	no	no	no
Y111A	3 TM	3	yes	yes	no
S120G	3 TM	4	yes	yes	no
ΔC182	2 EX	1	no	no	no
R190N	2 EX	4	yes	yes	no
H226Y	3 IN[b]	3	yes	yes	no
Q299L	7 TM	3	yes	yes	no
L301F	7 TM	3	yes	yes	no
R302E	7 TM	3	yes	yes	no
R302Q	7 TM	3	yes	yes	no
L304Y	7 TM	7	yes	yes	yes
S305A	7 TM	3	yes	yes	no
F315Y	7 TM	1	no	no	no

[a] extracellular loop.
[b] intracellular loop.

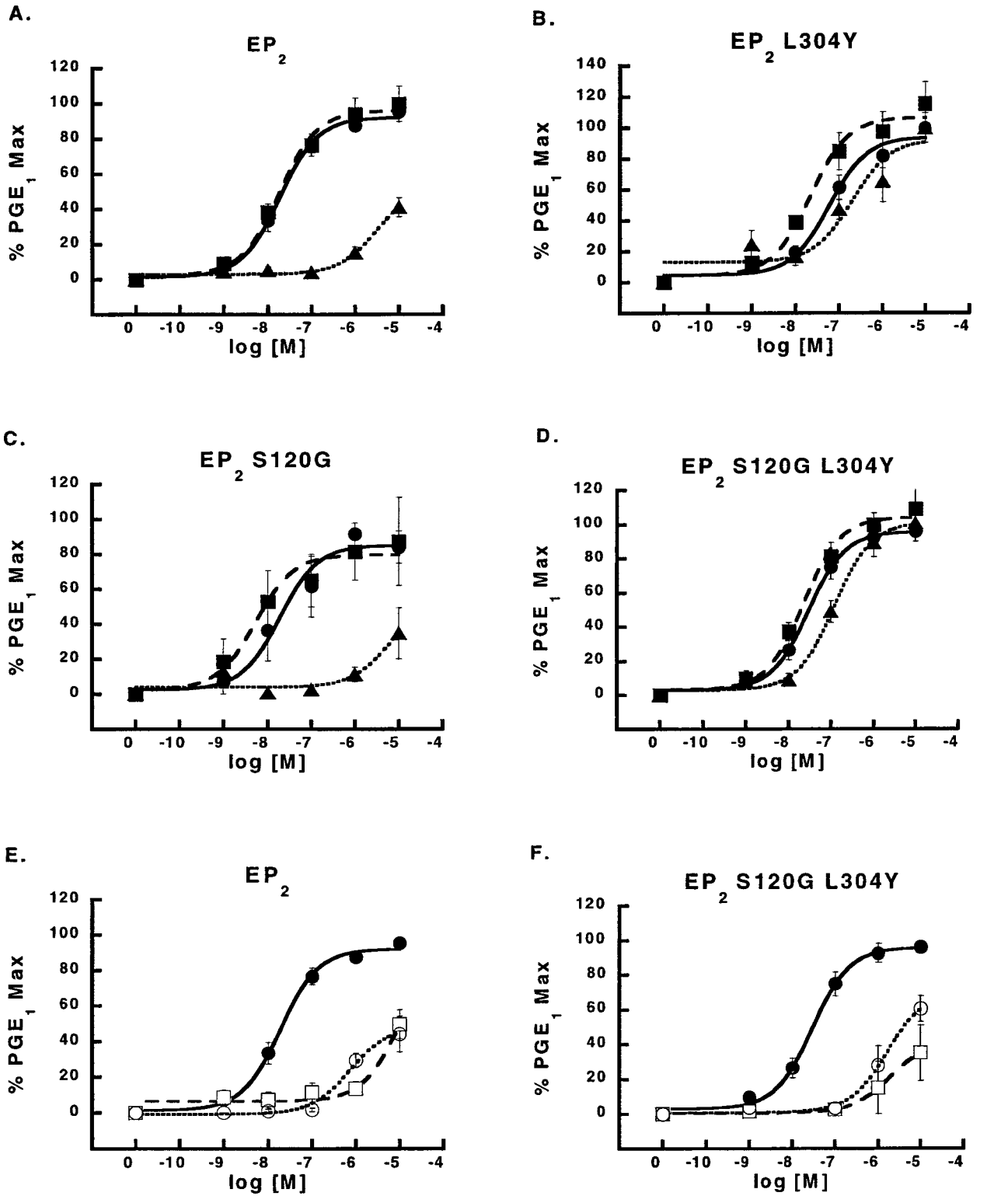


Fig. 2. Activation of wild-type and mutant human EP_2 receptors. Receptors were expressed in CV-1 cells and assayed by the CRE-CAT assay. Drugs used were PGE_1 (●), PGE_2 (■), Iloprost (▲), PGD_2 (□), and $PGF_{2\alpha}$ (○). *Points*, mean ± standard error of the noted number of experiments performed with triplicate determinations. A, The human EP_2 receptor (12 experiments) has EC_{50} values of 34.0 ± 11.0 nM for PGE_1 and 25.5 ± 6.6 nM for PGE_2. The EC_{50} value for iloprost is >10 μM. Basal activity was approximately 10,000 dpm, with a 5.0 ± 2.4-fold increase in the PGE_2 signal. B, Mutant EP_2 L304Y (eight experiments) has EC_{50} values of 99.0 ± 29.5 nM for PGE_1, 33.3 ± 11.2 nM for PGE_2, and 128.9 ± 38.3 nM for iloprost. Basal activity was approximately 15,000 dpm, with a 2.6 ± 0.5-fold increase in PGE_2 signal. C, Mutant EP_2 S120G (four experiments) has EC_{50} values of 47.4 ± 21.1 nM for PGE_1, 33.0 ± 11.7 nM for PGE_2 and >10 μM for iloprost. Basal activity was approximately 17,000 dpm, with a 3.1 ± 0.7-fold increase in the PGE_2 signal. D, Mutant EP_2 S120G L304Y (eight experiments) has EC_{50} values of 36.0 ± 10.0 nM for PGE_1, 37.9 ± 12.3 nM for PGE_2, and 142.1 ± 43.4 nM for iloprost. Basal activity was approximately 10,000 dpm, with a 3.4 ± 0.9-fold increase in the PGE_2 signal. E, Wild type EP_2 receptor (three experiments) shows activity only in response to PGD_2 or $PGF_{2\alpha}$ at concentrations ≥ 1 μM. The response to PGE_1 from A is included for reference. F, Mutant EP_2 S120G L304Y (three experiments) shows activity in response to PGD_2 or $PGF_{2\alpha\ a}$ only at concentrations ≥ 1 μM. The response to PGE_1 from D is included for reference.

does the wild-type EP_2 receptor, which suggests that TM3 is not directly involved in defining ligand specificity.

The double mutant, EP_2 S120G L304Y, responds much as does the single mutant EP_2 L304Y (Fig. 2D). It retains the ability to signal in response to PGE_1 and PGE_2 (EC_{50} values of 36.0 ± 10.0 nM and 37.9 ± 12.3 nM, respectively), and has gained the ability to respond to iloprost (EC_{50} of 142.1 ± 43.4 nM) and carbacyclin (Fig. 3; EC_{50} of 1705.0 ± 734.1 nM). No response to PGD_2 or $PGF_{2\alpha}$ is seen at submicromolar concentrations (Fig. 2F). Interestingly, the overall signal magnitude, as a percent of basal, of the double mutant EP_2 S120G L304Y is consistently superior to that of the single mutant EP_2 L304Y, suggesting that residue 120 in TM3 interacts in some way with residue 304, perhaps by stabilizing the receptor (Fig. 4).

Arginine 302 is a key residue for prostaglandin activity. The conserved arginine residue in TM7 has been proposed to be the counterion for binding of the carboxyl group of prostaglandin compounds. Previous studies have demonstrated that alterations of this residue in EP_3 and TP prostaglandin receptors alters ligand binding and signaling (Funk *et al.*, 1993; Huang and Tai, 1995; Negishi *et al.*, 1995; Audoly and Breyer, 1997; Chang *et al.*, 1997). To confirm the significance of this residue in receptor-ligand interactions for the G_s-coupled branch of the prostaglandin receptor family, this arginine has been substituted with a neutral residue (R302Q) and a negatively charged residue (R302E) to evaluate the change in function of these mutants.

Both mutants, EP_2 R302Q and EP_2 R302E (Fig. 5, A and B), demonstrate a loss of activity compared with the wild-type receptor. Dose-response curves are shifted to the right, with increases in EC_{50} values for both PGE_1 and PGE_2. The EC_{50} values for PGE_1 are 1123.2 ± 346.5 nM for EP_2 R302Q and 694.3 ± 210.8 nM for EP_2 R302E, and for PGE_2 are 238.3 ± 53.8 nM for EP_2 R302Q and 949.8 ± 207.2 nM for EP_2 R302E. Iloprost does not activate either mutant.

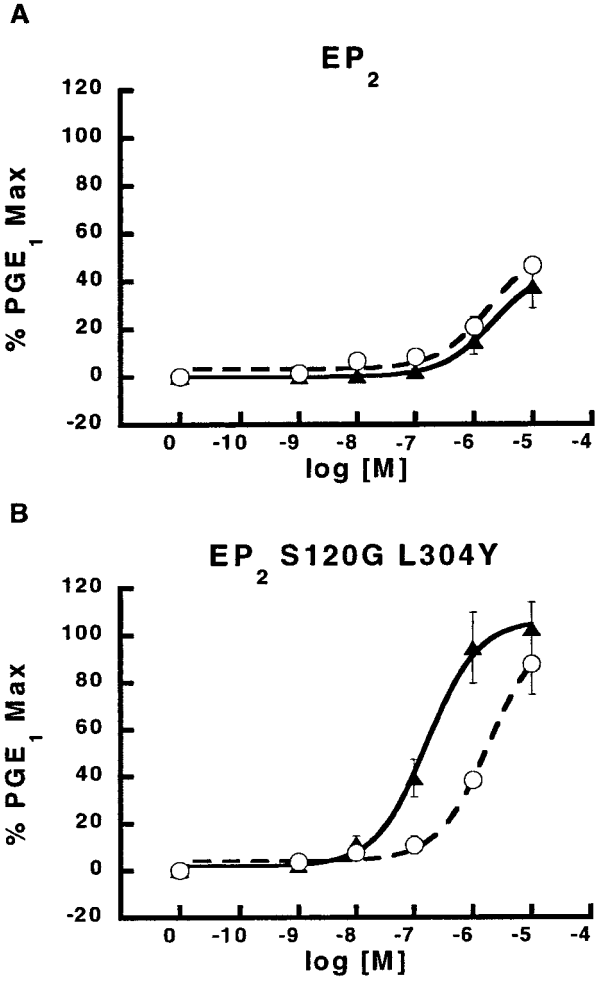


Fig. 3. Activation of EP_2 and EP_2 S120G L304Y mutant receptors by prostacyclin analogs. Receptors were expressed in CV-1 cells and assayed by the CRE-CAT assay. Drugs used were iloprost (▲) and carbacyclin (○). *Points*, mean ± standard error of four experiments performed with triplicate determinations. A, The EP_2 receptor shows only weak stimulation by iloprost and carbacyclin. The EC_{50} values are > 10 μM. B, The EP_2 S120G L304Y receptor has EC_{50} values of 189.8 ± 89.0 nM for iloprost and 1705.0 ± 734.1 nM for carbacyclin.

Discussion

The proposed prostaglandin receptor phylogeny (Regan *et al.* 1994; Toh *et al.* 1995) led us to hypothesize that only a few amino acids determine the selective interactions of the prostaglandins with their receptors. The approximately 100-fold increase in the activity of an IP receptor-selective agonist with a single amino-acid substitution confirms this hypothesis and is consistent with the proposed phylogeny.

The phylogenetic analysis highlighted candidate amino acids for gain of function mutations that could be rapidly screened with our functional assay (Fig. 1B). This approach avoided the difficulties of interpreting loss of functional or binding activity, which can result from defective receptor synthesis and assembly rather than a change in ligand-receptor interactions. Only one recent study that swapped regions of the IP and DP receptors identified a gain of function, although this was not specific for a single type of prostaglandin (Kobayashi *et al.*, 1997). A nonspecific enhancement of activity could result from a general change in the activation kinetics of the receptors.

The role of residue 304 in ligand activation is specific to prostacyclin receptor agonists. The EP_2 L304Y and EP_2 S120G L304Y mutants acquire the ability to respond to the IP ligand iloprost, but not to the DP ligand PGD_2 or the FP ligand $PGF_{2\alpha}$ (Fig. 2F). Thus, the specific gain of function seen in the EP_2 L304Y and EP_2 S120G L304Y mutants is

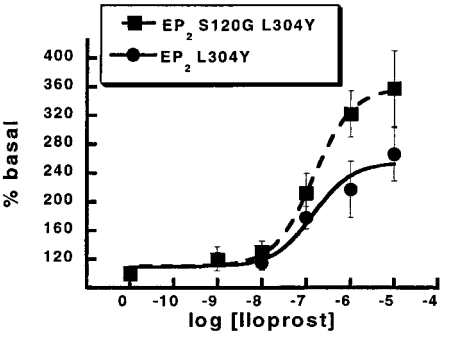


Fig. 4. Comparison of signal magnitude of the EP_2 L304Y and EP_2 S120G L304Y mutant receptors. *Points*, mean ± standard error of four experiments with triplicate determinations, performed on cells transfected in the same experiment with the two mutant cDNAs. The basal activity was approximately 25,000 dpm for EP_2 L304Y and 10,000 dpm for EP_2 S120G L304Y, with the average increase over basal for iloprost of 2.6-fold and 3.6-fold, respectively.

likely caused by an alteration in the vicinity of TM7, at a site that specifically interacts with iloprost.

It seems likely that residue 304 is sensitive to structural alteration of the α side chain. Prostacyclin and its stable analog, iloprost, have a constrained configuration because of an additional ring that is not present in PGE_2. PGE_1, which activates both EP_2 and IP receptors, has a much more flexible α chain and presumably can adopt a configuration that enables interaction with either receptor. The iloprost ω chain, which differs from prostacyclin and PGE_2, does not seem to interact with residue 304 because carbacyclin, another prostacyclin analog that has an ω chain identical to prostacyclin and PGE_2 and a constrained α chain, also gains function with the EP_2 S120G L304Y mutant (Fig. 3). It is interesting to note that the potency of carbacyclin relative to iloprost for the mutant is similar to its relative potency in binding to the IP receptor (Boie *et al.*, 1994). The data are thus consistent with the L304Y substitution's enhancing the ability of the prostacyclin α chain to interact with the EP_2 receptor. One explanation for the result is that the hydroxyl moiety of tyrosine provides a new contact point for prostacyclin and its analogs, compensating for one that prostaglandins with a less constrained α side chain possess.

Other contact points are necessary for the discrimination of prostaglandin ligands. The iloprost potency at the IP receptor is approximately 100-fold greater than its potency at EP_2 L304Y. There must also be amino acids responsible for reducing the potency of PGE_2 at the IP and other non-EP receptors. The region of the binding pocket that enables the selective interactions of PGE_2, as well as PGD_2 and $PGF_{2\alpha}$, may be distinct from TM7 and is likely involved in interactions with the cyclopentane ring. A recent study with chimeric IP/DP receptor constructs suggested a role for TM3 of the DP receptor in cyclopentane ring interactions (Kobayashi *et al.*, 1997). These authors also suggested that TM6 or TM7 is responsible for α chain recognition, which is consistent with our results. In another study, mutagenesis of a conserved hydroxy amino acid in TM6 of the EP_3 receptor seemed to alter the selectivity of that receptor (Negishi *et al.*, 1995). The approach we used here can be applied to other receptor pairs (the EP_2 and DP receptors for instance) to identify significant determinants of cyclopentane ring interactions. A comparison of EP_2 and EP_4 receptors might also determine why PGE_2 is approximately 10-fold less potent at the EP_2 receptor than at other EP receptors.

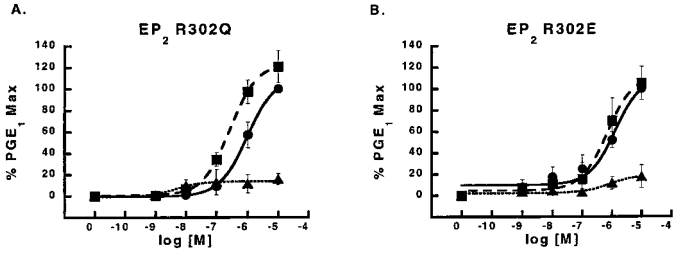


Fig. 5. Activation of EP_2 R302Q and EP_2 R302E mutant receptors. Receptors were expressed in CV-1 cells and assayed by the CRE-CAT assay. Drugs used were PGE_1 (●), PGE_2 (■), and iloprost (▲). *Points*, mean ± standard error of four experiments performed with triplicate determinations. A, The EP_2 R302Q mutant has EC_{50} values of 1123.2 ± 346.5 nM for PGE_1 and 238.3 ± 53.8 nM for PGE_2. Iloprost was not active. The basal activity was approximately 5000 dpm, with a 2.7 ± 0.5-fold increase in the PGE_2 signal. B, The EP_2 R302E mutant has EC_{50} values of 694.3 ± 210.8 nM for PGE_1 and 949.8 ± 297.2 nM for PGE_2. Iloprost was not active. The basal activity was approximately 5000 dpm, with a 2.5 ± 0.4-fold increase in the PGE_2 signal.

Residue 120 in TM3 probably influences ligand activity by interacting with residue 304. The EP_2 S120G mutation alone has wild-type pharmacology, which indicates that this residue is not involved in defining a ligand interaction site (Fig. 2C). However, EP_2 L304Y exhibits a loss of signal magnitude that is rescued by the double mutant, EP_2 S120G L304Y, which indicates that the TM3–TM7 interactions are important for optimal receptor function (Fig. 4). The elevated basal activity of the single mutants may reflect reduced stability of the receptor in the inactive state, which is stabilized by G protein coupling or the double mutant. Further evidence that these two amino acids interact is the fact that the changes at these positions between EP_2 and IP receptors are complementary in side chain length and polarity.

Arg302, which is located in TM7 and corresponds with arginine residues that have been implicated in binding of the prostaglandin carboxylic acid moiety, is predicted on the basis of modeling to be situated at the opposite end of the binding pocket from TM3 and TM6. As expected for an amino acid that is conserved in all prostaglandin receptors, mutagenesis of this residue resulted in a nonselective reduction of agonist activity (Fig. 5). The retention of signal transduction, however minor, with the Arg302 mutants EP_2 R302Q and EP_2 R302E is intriguing. The result is consistent with PG ligands having many receptor contact points, as previously discussed. Mutagenesis of the corresponding residue in other prostaglandin receptors has resulted in a range of responses from total loss of binding or signaling to retention of some signaling. In some studies (Funk *et al.*, 1993; Huang and Tai, 1995) binding alone was evaluated, which may underestimate any residual function present in the TP and EP_3 mutants. In one case, functional studies using the synthetic EP_3 analog sulprostone supplemented binding studies and demonstrated that mutation of the conserved arginine in the EP_3 receptor resulted in complete loss of function (Audoly and Breyer, 1997). However, other studies have demonstrated retention of some EP_3 function when PGE_2 but not sulprostone is the agonist (Negishi *et al.*, 1995; Chang *et al.*, 1997).

There are only a few previous examples of a single amino acid change altering the selectivity of a receptor for endogenous ligands. In the somatostatin receptor subtype 5, substitution of Phe265 in TM6 with tyrosine increased the affinity of the 14-amino-acid form of somatostatin, so that it was comparable with its affinity for somatostatin receptor subtypes 1–4 (Ozenberger and Hadcock, 1995). Mutation of Tyr129 in TM2 of endothelin receptor A to histidine, the corresponding amino acid in endothelin receptor B, enhanced endothelin-3 binding so that its affinity was similar to that of endothelin receptor B (Krystek *et al.*, 1994; Lee *et al.*, 1994). These receptors, as well as the EP_2, should support study of the evolution of ligand-receptor pairs. One would predict that the emergence of the particular amino acid changes that are described and novel endogenous ligands occurred at similar stages of evolution.

In summary, a single residue in TM7 of the EP_2 receptor that is changed in the IP receptor determines the activity of stable prostacyclin analogs. This result is consistent with a model of prostaglandins interacting with their receptors at a

universal contact point (TM7 arginine) and at other residues, probably in a pocket formed by TM7, TM6, and TM3 that enables ligand discrimination. The significant role of a single amino acid in the selectivity of the EP_2 receptor is consistent with the hypothesis that the IP receptor evolved from the EP_2 receptor. In effect, the mutant receptor represents a molecular "missing link" in the evolution of the IP receptor from the EP_2 receptor.

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