Prostanoid receptors belong to the class of heptahelical plasma membrane receptors. For the five prostanoids, eight receptor subtypes have been identified. They display an overall sequence similarity of roughly 30%. Based on sequence comparison, single amino acids in different subtypes of different species have previously been identified by site-directed mutagenesis or in hybrid receptors that appear to be essential for ligand binding or G-protein coupling. Based on this information, a series of mutants of the human FP receptor was generated and characterized in ligand-binding and second-messenger-formation studies. It was found that mutation of His-81 to Ala in transmembrane domain 2 and of Arg-291 to Leu in transmembrane domain 7, which are putative interaction partners for the prostanoid’s carboxyl group, abolished ligand binding. Mutants in which Ser-263 in transmembrane domain 6 or Asp-300 in transmembrane domain 7 had been replaced by Ala or Gln, respectively, no longer discriminated between prostaglandins PGF2α and PGD2. Thus distortion of the topology of transmembrane domains 6 and 7 appears to interfere with the cyclopentane ring selectivity of the receptor. PGF2α-induced inositol formation was strongly reduced in the mutant Asp-300Gln, inferring a role for this residue in agonist-induced G-protein activation.

Key words: G-protein-coupled receptor (GPCR), mutant, structure–function relationship.

INTRODUCTION

Prostanoid receptors belong to the class of heptahelical plasma membrane receptors which couple to heterotrimeric G-proteins [1]. One receptor each has been identified pharmacologically for the prostanoids prostaglandin (PG) D2, PGF2α, prostacyclin (PGI2) and thromboxane A2, named the DP-, FP-, IP- and TP-receptors (-Rs), whereas four receptor subtypes have been characterized pharmacologically for PGE2, named EP1-R–EP4-R [2]. The DP-, EP2-, EP4- and IP-R couple to Gα-proteins, the EP3-R to Gβ and the EP1-R, FP-R and TP-R couple to Gα. In addition, the TP-R appears to couple to G12/13 [3]. cDNAs have been cloned from different species for all prostanoid receptors that had been identified pharmacologically. Additional diversity is generated by alternative splicing, generating C-terminal splice variants, e.g. for the EP3-R, FP-R and TP-R [4].

Despite abundant sequence information, only scattered information is available on structure-function relationships in prostanoid receptors, i.e. identification of the ligand-binding domain or domains involved in signal transduction. The prostanoid receptors display an overall sequence similarity of roughly 30–40%. According to the grade of similarity they can be grouped into phylogenetic families [5]. The affiliation of the family members seems to be determined by the G-protein-coupling specificity rather than by the ligand-binding specificity. Thus the three Gα-coupled receptors form one family that is closely related to the Gβ-coupled EP3-R, while all Gα-coupled receptors form a distinct family. Therefore, sequence alignment has been of limited use to identify amino acids that play a role in ligand-binding specificity. Nevertheless, some information on the function of single amino acids or domains of prostanoid receptors is available: a stretch in the second extracellular loop has been identified that is conserved among all EP receptors and appeared to confer E selectivity [6]. Analysis of the properties of hybrid receptors that were generated by replacing receptor domains of the IP-R with the homologous domain of the closely related DP-R indicate that transmembrane domain 1 and 2 might be involved in recognizing the ring substituents, while transmembrane domains 6 and 7 might recognize the conformation of the α and ω side chains [7]. Studies with EP3-R and TP-R from various species indicate the significance of an ionic interaction of an Arg in transmembrane domain 7 with the prostanoid’s carboxyl group [8,9]. Polar amino acids in transmembrane domains 5 and 6 have also been inferred in ligand binding in the EP3-R [9]. Single amino acids in transmembrane domain 7 and the second intracellular loop have been inferred in ligand-dependent signal transduction in the EP3-R [10,11] and TP-R [10].

It was the purpose of the current study to analyse the function in ligand binding and signal transduction of single amino acids of the human FP-R that appeared to be crucial in ligand binding or signal transduction in homologous positions in other prostanoid receptors.

EXPERIMENTAL

Materials

All materials were of analytical grade and from commercial sources indicated throughout the text. Oligonucleotides were custom-synthesized by MWG Biotech (Ebersberg, Germany).

Generation of a cDNA for a FLAG-tagged human FP-R

Total RNA was isolated from human placenta using the SV Total RNA Isolation System (Promega, Madison, WI, U.S.A.).
Total RNA was used for oligo(dT)-primed cDNA synthesis using Superscript II (Gibco-BRL, Eggenstein, Germany) according to the standard protocol provided with the kit. A cDNA coding for a human FP-R was amplified from this cDNA with primers hFP-f (5'-gcggcgtgccggATGGTGCAGAGCAGGACGACAGCAGACAGTCCATGAACTTCAAAAGAG-3') and hFP-r (5'-gcggcgtgcagtaggtgcttggtgatttctgtgc-3'), containing additional restriction sites for forced cloning (underlined) using Power script polymerase (PAN Biotech, Aidenbach, Germany), according to the following program: 3 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C, followed by 10 min at 72 °C. The PCR product was gel-purified and cloned into pUC57/T. The plasmid served as the template for a second PCR to introduce a sequence for an N-terminal FLAG tag (N-Asp-Lys-Tyr-Asp-Asp-Asp-Lys) recognized by the monoclonal antibody FLAG-M2 (Sigma, Deisenhofen, Germany), into the cDNA of the human FP-R (hFP-R) using FLAG-hFP-f as the forward primer (5'-gcggcgtgccggATGGTGCAGAGCAGGACGACAGCAGACAGTCCATGAACTTCAAAAGAG-3') and hFP-r as the reverse primer. The resulting PCR product was modified by restriction digestion of the primer-encoded restriction sites and cloned into the Xhol/XbaI site of the eukaryotic expression vector pSVL.

PCR-based site-directed mutagenesis

The FLAG-hFP-R cDNA served as a template for PCR-based site-directed mutagenesis. Mutations were generated in 5' and 3' receptor cDNA fragments using the following primer combinations, where mutated nucleotides are shown in bold and the first primer pair in each case was used to generate the mutated 5'-cDNA fragment and the second primer pair to generate the mutated 3'-cDNA fragment. FLAG-hFP-H81A-R: FLAG-hFP-f/FLAG-hFP-r. FLAG-hFP-H81A-R: FLAG-hFP-f/FLAG-hFP-r. FLAG-hFP-XbaI-R: FLAG-hFP-f/FLAG-hFP-r. FLAG-hFP-NcoI-R: FLAG-hFP-f/FLAG-hFP-r. FLAG-hFP-ATG-R: FLAG-hFP-f/FLAG-hFP-r. FLAG-hFP-S182P-R: FLAG-hFP-f/FLAG-hFP-r. FLAG-hFP-S182P-R: FLAG-hFP-f/FLAG-hFP-r. FLAG-hFP-S206A-R: FLAG-hFP-f/FLAG-hFP-r. FLAG-hFP-S206A-R: FLAG-hFP-f/FLAG-hFP-r. The 5' and 3' receptor constructs cloned into pSVL and transiently transfected after 24 h using the DEAE-dextran method with wild-type pSVL/FLAG-rFP-R or the mutant receptor constructs cloned into pSVL (5 μg/plate). Assays were performed 72 h after transfection.

Determination of receptor cell-surface expression by immunocytochemistry and cyto-ELISA

Subcellular distribution of the receptor proteins was determined by confocal laser microscopy essentially as described previously [11]. FLAG-tagged receptor proteins were detected by mouse anti-FLAG-M2 antibody and a Cy3-labelled goat anti-mouse antibody. Plasma membrane proteins were selectively labelled in intact cells by biotinylation with biotin hydrazide prior to fixation and subsequent visualization with streptavidin–FITC.

The cyto-ELISA was performed 24 h after transfection on 24-well plates. All steps were performed at 4 °C. Cells were incubated for 1 h with a blocking buffer containing 3% BSA and 20% bovine serum in PBS, washed once with PBS and then incubated for 2 h with 250 μl of PBS/well containing 1% BSA, 6.6% bovine serum and 2.5 μg/ml mouse anti-FLAG-M2 antibody. Unbound FLAG-M2 antibody was removed by six washes with PBS. Cell-bound FLAG-M2 antibody was then recovered by incubating cells for 30 min in a stripping buffer containing 100 mM sodium citrate and 50 mM NaCl, pH 3.0. Cells were scraped into the stripping buffer. The suspension was neutralized with 1 M Tris and centrifuged at 15000 × g for 5 min. The monoclonal FLAG-M2 antibody in the supernatant was quantified in a sandwich ELISA using a rabbit anti-mouse IgG antibody as a capture antibody, which was coated on to the ELISA plates, and a biotinylated goat anti-mouse IgG antibody followed by streptavidin–peroxidase and ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)] for detection. A standard curve was generated with 0–1 ng of FLAG-M2 antibody/well.

Membrane isolation and PGF$_2$α-binding assay

Membrane preparation and binding assays were performed essentially as described previously [12]. For membrane preparations, transfected cells were scraped into a homogenization buffer containing 50 mM Tris/HCl, pH 7.5, 5 mM EDTA and 0.2 mM Pefabloc SC (Biomol, Hamburg, Germany), 10 μg/ml leupeptin and 10 μg/ml soya bean trypsin inhibitor as protease inhibitors. After homogenization by vigorous pipetting and vortex mixing a crude membrane fraction was prepared by centrifugation of the homogenate at 100000 g for 30 min. The resulting pellet was suspended in binding buffer containing 10 mM Mes/NaOH, pH 6.2, 10 mM MgCl$_2$ and 1 mM EDTA and stored at −70 °C. Protein was determined according to Bradford [13]. For saturation binding assays, membranes (20–50 μg of protein) were incubated with 5 nM [3H]PGF$_2$α (6.7 TBq/mmol) and increasing concentrations of unlabelled PGF$_2$α in 100 μl of binding buffer for 1 h at 20 °C. Non-specific binding was determined in the presence of 10 μM PGF$_2$α. Bound and unbound ligand were separated by rapid vacuum filtration through GF 52 filters (Schleicher & Schüll, Dassel, Germany).
Site-directed mutagenesis of the human FP prostanoid receptor

Figure 1 Immunocytochemical localization of wild-type and mutant hFP-R proteins

COS-7 cells were cultured on coverslips and transfected in the eukaryotic expression vector pSVL with the cDNAs of the receptors indicated. After transfection (24 h) the intact cells were biotinylated for later detection of the plasma membranes with streptavidin–fluorescein. After fixation receptor proteins were detected with the monoclonal FLAG-M2 antibody and a Cy3-labelled secondary antibody. The labelled plasma membrane appears green, the receptor protein red. Co-localization in the overlay is indicated by yellow. Receptor proteins appear to be located in the plasma membranes of cells transfected with wild-type receptor and all mutant receptors except for the mutant hFP-R291L. This latter mutant appears to be located predominantly in an intracellular compartment, possibly the endoplasmic reticulum or the Golgi compartment. The mutant D300N appears to be located in a vesicular sub-plasma membrane compartment in addition to the plasma membrane.

Filters were washed four times with 4 ml of ice-cold binding buffer. Radioactivity retained on the filter was counted in 5 ml of Hydroluma (Baker, Grob, Gerau, Germany). Binding constants were calculated by non-linear regression analysis (LIGAND) [14]. The affinities for PGE$_2$ and PGD$_2$ were determined by competition binding assays with 5 nM $^3$H]PGF$_{2\alpha}$ and increasing concentrations of unlabelled PGE$_2$ and PGD$_2$. Binding constants were calculated by non-linear regression analysis (LIGAND) on the basis of the $K_d$ determined for PGF$_{2\alpha}$ by saturation binding assays.

Determination of PGF$_{2\alpha}$-stimulated inositol phosphate formation

COS-7 cells were seeded in a density of 100 000 cells/3.5 cm-diameter plates, cultured for 48 h, transfected as described above; 24 h post-transfection, medium was replaced by inositol-free DMEM containing 10 % dialysed fetal bovine serum and 1 $\mu$Ci/ml $^3$H]myo-inositol. After 24 h free inositol was removed by extensive washing. Cells were then incubated with Krebs–Henseleit buffer containing 10 mM LiCl to inhibit degradation of inositol phosphates. PGF$_{2\alpha}$ was added to the cells at the concentration indicated. After 15 min the reaction was stopped by removing the buffer and freezing cells in liquid nitrogen. Cells were scraped into 750 $\mu$l of 10 mM formic acid. The homogenate was neutralized with 3 ml of 10 M ammonia and loaded on to 1.5 ml Dowex formate columns. Columns were washed with 4 ml of 0.04 M ammonium formate/0.04 M formic acid, pH 5.0. Inositol phosphates were eluted with 4 ml of 2 M ammonium formate/2 M formic acid, pH 5.0. Radioactivity in aliquots of the fractions was determined using Hydroluma scintillant.

RESULTS

Expression of wild-type and mutated receptor proteins

The codons for single amino acids that had been implicated in ligand binding or signal transduction were replaced by site-directed mutagenesis. The rationale for the substitutions was either to replace hydrophilic Ser residues in the hydrophobic transmembrane domains with the most closely related hydrophobic amino acid, i.e. Ala in mutants S206A, S258A and S263A, or to replace an amino acid that is specific for the FP-R in an otherwise highly conserved stretch, i.e. Ser-182, with Pro, which is found in the corresponding position in all PGE$_2$ receptors, or to introduce mutations that had previously been shown to cause altered function when introduced into the corresponding position of other prostanoid receptors, i.e. H81A, T138A, R291L and D300N. The resulting cDNAs were cloned into the expression vector pSVL and expressed transiently in COS-7 cells. Cell-surface expression of the wild-type and mutant receptor proteins was determined by confocal laser microscopical analysis of immunocytochemistry (Figure 1) and cyto-ELISA (Figure 2). Both methods revealed that wild-type receptor and all mutant receptors except for the mutant hFP-R291L were synthesized,
Cells expressing either wild-type or mutant receptors were incubated with a monoclonal antibody against the FLAG epitope. Unbound antibody was removed by extensive washing. Bound antibody was then eluted in acid buffer and quantified in a sandwich ELISA procedure as detailed in the Experimental section. The receptor cell-surface expression was set equal to the amount of antibody that was eluted from the cell surface. Values are means ± S.E.M. from three independent experiments. *P < 0.05, significantly different from untransfected control.

**Table 1** $K_d$ values of wild-type and mutant hFP-Rs

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Position of mutation</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>–</td>
<td>6.4 ± 1.7 (4)</td>
</tr>
<tr>
<td>H81A</td>
<td>TM2</td>
<td>No binding</td>
</tr>
<tr>
<td>T138A</td>
<td>In2</td>
<td>8.4 ± 1.3 (4)</td>
</tr>
<tr>
<td>S182P</td>
<td>Ex2</td>
<td>5.9 ± 1.3 (3)</td>
</tr>
<tr>
<td>S206A</td>
<td>TM5</td>
<td>12.4 ± 4.3 (4)</td>
</tr>
<tr>
<td>S258A</td>
<td>TM6</td>
<td>7.2 ± 1.2 (4)</td>
</tr>
<tr>
<td>S263A</td>
<td>TM6</td>
<td>30.6 ± 6.5 (5)</td>
</tr>
<tr>
<td>R291L</td>
<td>TM7</td>
<td>No binding</td>
</tr>
<tr>
<td>D300N</td>
<td>TM7</td>
<td>10.0 ± 2.3 (3)</td>
</tr>
</tbody>
</table>

Figure 2  Quantification of receptor cell-surface expression by cyto-ELISA

Figure 3  PGF$_{2\alpha}$-stimulated inositol formation in cells expressing wild-type or mutant FP-R

Transiently transfected cells were labelled with [³H]myo-inositol for 24 h and then stimulated with the PGF$_{2\alpha}$ concentration indicated. Cells were extracted with 10 mM formic acid. Radioactively labelled inositol phosphates (InsP$_x$) were separated from other labelled compounds by ion-exchange chromatography on DOWEX formate and quantified by liquid-scintillation counting. To allow pooling of data from independent transfection experiments with various labelling efficiencies, inositol phosphate formation is expressed as a percentage of the inositol formation that was observed in the same experiment. Values are means ± S.E.M. from six (wild-type) or three (mutant receptors and untransfected control cells) independent experiments.

**Figure 2** Quantification of receptor cell-surface expression by cyto-ELISA

**Figure 3** PGF$_{2\alpha}$-stimulated inositol formation in cells expressing wild-type or mutant FP-R

As has been reported previously [14a], cells expressing the hFP-R showed a PGF$_{2\alpha}$-dependent increase of inositol phosphates that was absent from non-transfected cells (Figure 3), indicating that the receptor coupled to G$_i$. At receptor-saturating PGF$_{2\alpha}$ concentrations all receptor mutants that bound PGF$_{2\alpha}$, with the exception of hFP-D300N-R, also elicited agonist-dependent inositol formation that was comparable with the wild-type receptor (results not shown). Wild-type receptor and all mutant receptors, except hFP-S263A-R and hFP-D300N-R, bound PGF$_{2\alpha}$ with an approx. 3–4-fold lower affinity than PGF$_{2\alpha}$. PGF$_{2\alpha}$ was bound with an approx. 60-fold lower affinity. Thus wild-type receptor and all mutant receptors, except hFP-S263A-R and hFP-D300N-R, bound PGF$_{2\alpha}$, $>$ PGF$_{2\alpha}$, $>$ PGF$_{2\alpha}$. By contrast, the affinity towards PGF$_{2\alpha}$ and PGD$_2$ did not differ significantly for the mutants hFP-S263A-R and hFP-D300N-R, indicating a loss of selectivity between PGF$_{2\alpha}$ and PGD$_2$, in these mutants.

**Signal transduction properties of wild-type and mutated receptor proteins**

As has been reported previously [14a], cells expressing the hFP-R showed a PGF$_{2\alpha}$-dependent increase of inositol phosphates that was absent from non-transfected cells (Figure 3), indicating that the receptor coupled to G$_i$. At receptor-saturating PGF$_{2\alpha}$ concentrations all receptor mutants that bound PGF$_{2\alpha}$, with the exception of hFP-D300N-R, also elicited agonist-dependent inositol formation that was comparable with the wild-type receptor (results not shown). Wild-type receptor and the mutants hFP-T138A-R and hFP-D300N-R, which were suspected of inositol formation at a receptor-saturating PGF$_{2\alpha}$ concentration (10 µM) in wild-type-expressing cells in the same experiment. Values are means ± S.E.M. from six (wild-type) or three (mutant receptors and untransfected control cells) independent experiments.
the mutant hFP-T138A-R did not differ significantly from wild-type receptor. Maximal agonist-induced inositol formation and EC50 value were almost identical with that of the wild-type receptor. However, maximal agonist-induced inositol formation was strongly reduced in the mutant hFP-D300N-R, which also displayed a slight right shift in the dose–response curve (Figure 3).

**DISCUSSION**

Sequence alignment of a large number of prostanoid receptor sequences revealed regions that are highly conserved among all prostanoid receptor subtypes and others that are unique to a certain receptor subtype but which are conserved in the same receptor subtype between different species. Thereby, sequence alignment has provided some indication of which receptor domains might potentially be essential for ligand-binding specificity or G-protein coupling. Consequently, single amino acids were substituted in these domains by site-directed mutagenesis. So far, most site-directed mutagenesis studies have focused on one particular structural aspect in one specific receptor subtype. Therefore, structure–function information is scattered. It is, however, not safe to translate findings from one receptor subtype to another, even if the mutation was introduced into a region that is conserved among the different receptor subtypes. Therefore, in the current study mutations were introduced into the human FP-R that had previously been shown to alter receptor function when introduced into homologous positions in other prostanoid receptor subtypes.

**FLAG-tagged wild-type hFP-R**

The cDNA of the hFP-R was extended at the 5′-end in order to incorporate an N-terminal FLAG tag for immunological detection of the receptor protein. As expected, the N-terminal tag did not modify the receptor’s properties. The Ka value of the tagged receptor was close to the Ka values for the hFP-R that have been reported in the literature [15]. It had previously been shown that in transiently transfected cells the rat EP3-R was retained in the endoplasmic reticulum [11]. Therefore, intracellular retention of the FLAG-hFP-R was excluded. It was confirmed by laser scanning immunocytochemistry that the receptor was correctly inserted into the plasma membrane in transiently transfected COS-7 cells (Figure 1).

**Ligand binding**

**hFP-H81A-R**

Molecular modelling of the rat FP-R had led to the hypothesis that His-81 in the second transmembrane domain is in close proximity to the highly conserved Arg-291 in the seventh transmembrane domain, which was assumed to interact with the prostanoid’s carboxyl group (see below). Replacement of the His-81 with Ala in the rat receptor led to a complete loss of binding [12]. This finding was confirmed with hFP-R (Table 1), indicating that His-81 is essential for high-affinity binding. As discussed previously [14] the loss of binding could possibly be attributed to a distortion of the site harbouring the prostanoid’s carboxyl group. The hypothesis of Kobayashi et al. [7], that His-81 in the second transmembrane domain might be relevant for the recognition of the substitution pattern at the cyclopentane ring, as was inferred by their finding with IP-/DP-R hybrids [7,16], appears to be less likely. Firstly, substitution of this FP-typical histidine in the rat FP-R with the EP3-R-typical glutamine led to a reduction in the affinity for PGF2α, with no evidence for a shift in ligand-binding specificity [14]. Second, molecular modelling precludes a role for this residue of the second transmembrane domain in cyclopentane-ring recognition if the assumption is made that the carboxyl group of the prostanoid interacts with the arginine in the seventh transmembrane domain, because these residues in transmembrane domains 2 and 7 appear to be too close to allow the full length of the α-chain and the ring of prostaglandin F2α in between them.

**hFP-S182P-R**

The FP-R is the only prostanoid receptor that has a serine in an otherwise highly conserved region of the second extracellular loop. All other prostanoid receptors have a proline in the homologous position. It has previously been shown that substitution of this prolyl residue in the rabbit EP3-R by the FP-typical serine decreased the receptor’s affinity for PGE2 and increased the affinity for PGF2α [6]. The authors discussed that the second extracellular loop might have a gate function which restricts the access to the ligand-binding pocket formed by the transmembrane domains. It was therefore assumed that substitution of the FP-typical serine in this position of the hFP-R by the proline found in all other prostanoid receptors would change the ligand-binding specificity of the hFP-R. However, the hFP-S182P-R had identical binding properties to the wild-type receptor (Table 1). Neither was the affinity for PGF2α decreased nor was the affinity for the other prostanoids tested increased.

**hFP-S206A-R**

Modification of Ser-201 in the human TP-R, which by sequence alignment appears to be located at a homologous position to Ser-206 in the hFP-R, resulted in a 5-fold reduction in affinity for the agonist I-BOP ((1S[1-α,2-β(5Z),3-α(1E,3R*,4-α)]-7-[3-hydroxy-4-(4′-iodophenox)-1-butanyl]-7-oxabicyclo-[2.2.1]heptan-2-yl)-5-heptenoic acid), whereas affinity for the structurally related agonist SQ29548, which lacks the α-chain hydroxy group, remained unaffected [17]. The authors proposed that this serine might form a hydrogen bond with the 15-hydroxy group of I-BOP. It was therefore assumed that elimination of the hydroxyl group in the analogous position of the hFP-R might also result in a reduction of ligand-binding affinity for all natural prostanoids carrying a 15-hydroxy group. The hFP-S206A-R indeed had an approx. 2-fold lower affinity for PGF2α, PGD2 and PGE2 than the wild-type receptor; however, this reduction in affinity did not reach statistical significance (Table 1).

**hFP-S258A-R and hFP-S263A-R**

Both serines are located in transmembrane domain 6. Their positions correspond to Ser-268 and Ser-273, respectively, in the mouse EP3α-R. It has previously been shown that Ser-268 is essential for ligand binding in the mouse EP3α-R [18], where substitution of this residue by alanine led to a complete loss of binding while substitution with threonine did not affect the binding of EP3-R agonists but abolished the discrimination between EP-subtype-specific ligands. Substitution of the corresponding serine in the human thromboxane receptor by alanine had less pronounced effects on the affinity [17]. The Ser corresponding to the hFP-R Ser-263 appeared not to contribute to ligand binding in the mouse EP3α-R [18]. By contrast, replacement of S258A did not affect ligand-binding or signal transduction properties of the hFP-R. However, the S263A substitution resulted in an approx. 5-fold reduction of the
receptor’s affinity for PGF<sub>2α</sub> and abolished the discrimination between the F and D rings (Table 1). Taken together, these results indicate that the sixth transmembrane domain contributes to the ligand-binding pocket of prostanoid receptors and possibly contributes to the discrimination between the different cyclopentane rings.

**hFP-R291L-R**

This arginine in the seventh transmembrane domain is conserved among all prostanoid receptors [1]. It has been assumed to interact with the prostanoid’s carboxyl group by analogy with the homologous lysyl residue, which is responsible for the tight binding of retinal to opsin [19]. Substitution of this residue with different amino acids in several prostanoid receptors interfaced to a greater or lesser extent with prostanoid binding [8,20–22]. Thus replacement of this arginine in the mouse EP3-R by glutamine resulted in a receptor with almost unaltered binding properties, while substitution with asparagine or leucine resulted in receptors with 5- and 40-fold lower affinity, respectively [8]. Replacement of the homologous residue in the rabbit EP3-R by alanine or aspartic acid caused a complete loss of binding [9]. Similarly, substitution of the analogous arginine by glutamine in the human TP-R abolished binding [21].

The importance of the carboxyl group of PGF<sub>2α</sub> for high-affinity binding has been well documented. The affinity of the FP-R for PGF<sub>2α</sub> derivatives lacking the carboxyl group was lower by several orders of magnitude [23]. None of the previous studies confirmed the correct expression of the receptor protein in the plasma membrane. Here we show that, in line with all the previous findings, substitution with leucine of the conserved Arg in transmembrane domain 7 of the hFP-R results in a complete loss of PGF<sub>2α</sub> binding (Table 1). However, as shown by confocal laser scanning microscopical immunocytochemistry, the mutant receptor protein was completely retained within the cell, probably in the endoplasmic reticulum (Figures 1 and 2). It was not transported to the plasma membrane. Therefore, the lack of binding might not be due to the loss of a charged interaction partner for the prostanoid’s carboxyl group, but could be attributed to a general misfolding of the receptor protein that prevented correct processing and insertion into the plasma membrane. The conclusions of the previous studies [8,20–22] should be reconsidered in view of this new finding.

**hFP-D300N-R**

This receptor mutant had a similar affinity for PGF<sub>2α</sub> to the wild-type receptor, but, unexpectedly, no longer discriminated between the F and D configurations of the cyclopentane ring (Table 1). If the assumption is made that Arg-291 in the seventh transmembrane domain interacts directly with the carboxyl group of the prostanoid, it is probably impossible for Asp-300 to contact a hydroxyl/oxo group of the cyclopentane ring. Therefore, the loss of discrimination between F and D ring in this mutant has most likely to be attributed to a more global reorientation of the ligand-binding-pocket-forming helices towards each other. A similar observation was made with the EP2-R. A Leu-to-Tyr substitution in transmembrane domain 7 conferred increased responsiveness of the receptor to the IP-R agonist iloprost [5].

**Signal transduction**

**hFP-T138A-R**

The second intracellular loop has previously been shown to be essential for G-protein coupling in the human TP-R [10]. All G<sub>q</sub>-coupled prostanoid receptors share threonine in the position homologous to Thr-138. It was therefore assumed that this threonine might also be essential for coupling to G<sub>q</sub> in hFP-R. In contrast with expectations, the substitution of threonine by alanine in the mutant receptor protein did not modify signal transduction properties (Figure 3). Maximal agonist-induced inositol phosphate formation and EC<sub>50</sub> in cells transfected with hFP-T138A-R were identical with those cells transfected in wild-type receptor.

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