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Identification of a Ser/Thr cluster in the C-terminal domain of the human prostaglandin receptor EP4 that is essential for agonist-induced β -arrestin1 recruitment but differs from the apparent principal phosphorylation site

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hEP4-R (human prostaglandin E₂ receptor, subtype EP4) is a G_s-linked heterotrimeric GPCR (G-protein-coupled receptor). It undergoes agonist-induced desensitization and internalization that depend on the presence of its C-terminal domain. Desensitization and internalization of GPCRs are often linked to agonist-induced β -arrestin complex formation, which is stabilized by phosphorylation. Subsequently β -arrestin uncouples the receptor from its G-protein and links it to the endocytotic machinery. The C-terminal domain of hEP4-R contains 38 Ser/Thr residues that represent potential phosphorylation sites. The present study aimed to analyse the relevance of these Ser/Thr residues for agonist-induced phosphorylation, interaction with β -arrestin and internalization. In response to agonist treatment, hEP4-R was phosphorylated. By analysis of proteolytic phosphopeptides of the wild-type receptor and mutants in which groups of Ser/Thr residues had been replaced by Ala, the principal

phosphorylation site was mapped to a Ser/Thr-containing region comprising residues 370–382, the presence of which was necessary and sufficient to obtain full agonist-induced phosphorylation. A cluster of Ser/Thr residues (Ser-389–Ser-390–Thr-391–Ser-392) distal to this site, but not the principal phosphorylation site, was essential to allow agonist-induced recruitment of β -arrestin1. However, phosphorylation greatly enhanced the stability of the β -arrestin1–receptor complexes. For maximal agonist-induced internalization, phosphorylation of the principal phosphorylation site was not required, but both β -arrestin1 recruitment and the presence of Ser/Thr residues in the distal half of the C-terminal domain were necessary.

Key words: desensitization, G-protein-coupled receptor kinase, internalization, phosphopeptide sequencing, prostaglandin receptor.

INTRODUCTION

PGE₂ (prostaglandin E₂) regulates a variety of physiological and pathophysiological processes ([1,2] and references cited therein). It binds to specific PGE₂ receptors, EP-Rs, which belong to the class of GPCRs (G-protein-coupled receptors) with seven transmembrane domains. Four subtypes of EP-R have been identified that differ in their affinity for synthetic ligands and in their G-protein coupling specificity: EP1-Rs are linked to G_q and to an as yet unidentified G-protein, and mediate an increase in cytosolic Ca²⁺ concentration, most probably by activation of Ca²⁺ channels; EP2-Rs and EP4-Rs couple to G_s and increase intracellular cAMP; and EP3-Rs couple to G_i and decrease hormone-stimulated cAMP formation [3]. These receptors display an overall sequence identity of approx. 40%, with the putative transmembrane domains being most highly conserved [3].

The EP4-R is widely distributed throughout the body, and its mRNA can be detected in almost every tissue. The EP4-R has been shown to be involved in the induction of bone formation [4] and in intestinal mucin secretion during inflammation [5], and it is the primary receptor conferring PGE₂-dependent renal vasodilatation [6]. The majority of the offspring of gene knockout mice lacking the EP4-R die soon after birth as a consequence

of impaired closure of the ductus arteriosus [7], inferring a central role for this receptor in the development of the vascular system. The EP4-R, in contrast with the second G_s-linked EP-R, the EP2-R, undergoes rapid agonist-induced desensitization [8]. It has a long serine- and threonine-rich C-terminal domain. Agonist-induced desensitization, but not G-protein coupling, was lost following truncation of the C-terminal domain of the hEP4-R (human EP4-R) [9]. Conversely, the C-terminal domain of the hEP4-R conferred agonist-induced desensitization in a receptor hybrid in which it replaced the analogous region of the non-desensitizable EP3 β -R [10]. Apparently, the C-terminal domain of the hEP4-R was necessary and sufficient to confer agonist-induced receptor desensitization.

Based on a model developed from studies of the β_2 -adrenergic receptor, desensitization of GPCRs is initiated by receptor phosphorylation, which facilitates binding of the adaptor protein β -arrestin to the ligand-activated receptor. Receptor phosphorylation is not an absolute prerequisite for interaction with β -arrestin, but it markedly increases the affinity of β -arrestin for the agonist-occupied receptor [11]. β -Arrestin binding to the receptor physically prevents further activation of G-proteins by the ligand-occupied receptor, a phenomenon called uncoupling [12]. Receptor phosphorylation can be catalysed either by second

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; (h)EP4-R, (human) prostaglandin E₂ receptor, subtype EP4; FCS, fetal calf serum; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; HEK, human embryonic kidney; IBMX, isobutylmethylxanthine; PGE₂, prostaglandin E₂.

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Table 1 Sequences of oligonucleotides employed for site-directed mutagenesis

Sequences are given in the 5' to 3' direction. 5'-Overlapping primer sequences are shown in lower case. Recognition sites for restriction enzymes are underlined. Translated sequences are shown in upper case. The sequence coding for the FLAG epitope is shown in bold, italic lower case. It is preceded by a Kozak sequence (italic) inserted to enhance translation efficiency. Mutations are shown in bold. Sequences that were re-introduced into the cDNA of hEP4-ST335-484A-R are shown in bold, italic upper case. 'Positions' refers to the positions in the published sequence (GenBank accession number L25124).

Primer	Sequence	Position
FLAG-hEP4-f	5'-gcgccgaagcctccaccatg gactacaaggacgacgacgacaag TCCACTCCCGGGGTCAATTGCTCC	589-612
hEP4-SnaBI-r	5'-GAGCACTGTCTT ACGT AGG AGGATATATATCC	1596-1565
hEP4-SnaBI-f	5'-GGATATATATCCCT ACT AAGACAGTGCTC	1565-1596
hEP4-Xba-r	5'-gcgccgctctagaCTACTATATAC ATTTTCTGATAA	2051-2032
hEP4-SnaBI/KpnI-f	5'-cgccggggtaccctcTCC TACGTAAGC AGTGC CGCT AAAGCAATAG	1577-1609
hEP4-3-r	5'-tcc CCCGGC CGATGAAGG CGCGAG CGTGGCCTGC ATAGCAGCAGCTGCC CTTTGACT	1739-1681
hEP4-6-r	5'-TGTG CAGCGTCTGCGGC CTCTGCTATTTCGAAAG CCCTCAGTGC GGCG CGTCTCTCTGG	1916-1857
hEP4-6-f	5'-GAC GCCGCC CACTGAGG GGCTTTGCGAATAGCAGAG CG CCGACAGCCTGC CACAGGGTCAG	1864-1923
hEP4-2-f	5'-ggc GGGGCCCGCAGGGAGCGCGCCGGACAGCACTGC GCAGAC CGCTCA AAAGG	1639-1689
hEP4-5-r	5'-CTGCCT CCAAGGCC ATTTC AGCGAGGTCTGGCAGTGC GAGGTCT	1820-1776
hEP4-4-f	5'-tcc CCCGGGAGCTGAACGAGATCGCCGCTGC AGCT CAAGCCCTCCTGCCAG	1730-1777
hEP4-7-f	5'- ACGCAGAGGCTGTCTTACTGGTGGATGAGGCTGGTGGGGCCGGCAGG	1925-1971
hEP4-7-r	5'- CCGGCCACCAGCCTCATCCACCAGTAAGACAGCCTCTGCGTCTGA	1967-1920
hEP4-8-r	5'-gctctagaTTATATACATTTTCT GCTAAGTTCAGTGC TT CAGCGGGAAATGCG ACTTGCAGG GGCGCC CCCT	2052-1987
hEP4-9-f	5'-GCTGAAGGAGAT CGCCGCTGC AGCTCAGACCCTCCTGCCAG	1737-1777
hEP4-9-r	5'-CTGGCAGGAGGCTGAG CTGCAGCGGC GATCTCCTTCAG	1777-1738
hEP4-10-f	5'-GCTGAAGGAGAT AGCAGTAC ACTCAGGCCCTCCTGCCAG	1737-1777
hEP4-10-r	5'-CTGGCAGGAGGCGCTGAG ATGACTGCT GATCTCCTTCAG	1777-1738

messenger-activated kinases such as protein kinase A or protein kinase C, or by GRKs (GPCR kinases) [13,14].

In addition to uncoupling, ligand-induced receptor endocytosis may contribute to desensitization; this process is known as receptor internalization or sequestration. Receptor sequestration is believed to involve clathrin-coated or caveolin-rich vesicles [15,16] and to result from specific interactions of the agonist-occupied receptor with components of the endocytotic machinery. The molecular mechanisms that trigger internalization of agonist-occupied receptors vary among different receptors ([17] references cited therein). For many GPCRs, receptor sequestration depends on phosphorylation. In these cases, binding of β -arrestin, which also has a high affinity for proteins of the endocytotic machinery, such as clathrin and the adaptor protein AP-2, targets the agonist-activated and phosphorylated receptor to clathrin-coated pits [18]. However, uncoupling and sequestration are not always linked. Mutation of phosphorylation sites in the N-formyl peptide receptor [19] or suppression of m2-muscarinic acetylcholine receptor phosphorylation by dominant-negative GRK2^{K220R} suppressed uncoupling but left sequestration unaffected [20], whereas mutation of defined receptor phosphorylation sites of the lutropin/chorionic gonadotropin receptor retarded receptor internalization but had no effect on uncoupling from the G-protein [21].

In a previous study, it was shown that the C-terminal domain of the hEP4-R was phosphorylated by agonist stimulation both in the wild-type hEP4-R and in a G_i-coupled EP3-R/hEP4-R hybrid. Agonist-induced receptor phosphorylation was augmented by GRK overexpression [22], indicating that GRKs most probably phosphorylate the C-terminal domain of the receptor in response to the agonist. Unlike protein kinase A and protein kinase C, GRKs lack a common consensus motif for receptor phosphorylation, and it was, therefore, impossible to predict which of the 38 serine/threonine residues in the hEP4-R C-terminal domain may serve as substrate(s) for GRK-dependent phosphorylation.

The present work aimed at identifying those Ser/Thr residues in the C-terminal tail of the hEP4-R that undergo agonist-induced phosphorylation and/or confer agonist-induced receptor- β -arrestin interaction and internalization. For this purpose,

potential phosphorylation sites were gradually eliminated by site-directed mutagenesis, and phosphorylated residues were identified by radio Edman degradation of the agonist-exposed wild-type receptor. The interaction of the receptor with GFP (green fluorescent protein)-labelled β -arrestin1 was studied by *in vivo* confocal laser microscopy, and receptor internalization was monitored by measuring the sequestration of radioactively labelled ligand from the cell surface.

It was found that agonist-induced phosphorylation, interaction with β -arrestin1 and internalization of the hEP4-R depended on the presence of different clusters of Ser/Thr residues in the C-terminal receptor domain. Maximal receptor phosphorylation was not required for internalization, but was required for formation of stable hEP4-R- β -arrestin1 complexes. Maximal internalization depended on agonist-induced hEP4-R- β -arrestin1 interactions and the additional presence of Ser/Thr residues in the distal half of the C-terminal domain that were required neither for arrestin recruitment nor for maximal phosphorylation.

EXPERIMENTAL

Materials

All materials were of analytical grade and from commercial sources. [³H]PGE₂, [³²P]P_i, Sepharose CL-4B and Protein G-Sepharose Fast Flow were obtained from Amersham/Pharmacia (Freiburg, Germany), and unlabelled PGE₂ and IBMX (isobutylmethylxanthine) were purchased from Calbiochem-Novabiochem (Bad Soden, Germany). Cell culture media were obtained from Invitrogen (Karlsruhe, Germany). Primers (Table 1) were synthesized by NAPS (Göttingen, Germany) and MWG (Ebersberg, Germany). Anti-FLAG monoclonal antibody M2 was from Sigma (Heidelberg, Germany). The sources of other materials are given in the text.

Plasmid construction

Cloning of the cDNA for a FLAG-epitope-tagged hEP4-R cDNA was carried out as described previously [22]. cDNAs

for mutant receptors containing Ser/Thr → Ala mutations in the C-terminal domain were generated by PCR-based site-directed mutagenesis using proof-reading Powerscript-Polymerase (PAN-Systems, Aichenbach, Germany) and the following programme: 3 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C, and a final step of 10 min at 72 °C. For a detailed protocol, see the Appendix. The plasmid encoding β -arrestin1-enhanced GFP has been described recently [23].

Cell culture and transfection

HEK293 (human embryonal kidney 293) cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) FCS (fetal calf serum), penicillin (100 units/ml) and streptomycin (100 μ g/ml). For generation of receptor-expressing cell lines, HEK293 cells were seeded at a density of 1.5×10^6 cells/10 cm diameter plate. After 24 h, cells were transfected with expression vectors for wild-type or mutant hEP4-R using a modified calcium phosphate method including 5% (v/v) bovine serum and 2.5 μ g/ml 25-hydroxycholesterol (Sigma) in the transfection medium. Transfectants stably expressing either wild-type hEP4-R or receptor mutants were isolated in DMEM containing 10% (v/v) FCS, penicillin (100 units/ml), streptomycin (100 μ g/ml) and 0.5 mg/ml G-418 as a substrate of the selection marker aminoglycoside phosphotransferase (NEO). Clonal cell lines were isolated by single-cell cloning and tested for expression by PGE₂ binding. For expression of GFP-tagged β -arrestin1, cell lines expressing wild-type or mutant hEP4-R were seeded on to poly-L-lysine-coated glass cover slips at a density of 2×10^5 cells/3.5 cm diameter plate. After 24 h, cells were transfected with Lipofectamine (Invitrogen) with an expression vector for GFP-tagged β -arrestin1. Assays were performed 24 h after transfection.

Membrane isolation and PGE₂ binding assay

Transfected cells were scraped into a hypotonic homogenization buffer containing 50 mM Tris/HCl, pH 7.5, 5 mM EDTA and 0.2 mM Pefabloc SC (Biomol, Hamburg, Germany), with 10 μ g/ml leupeptin and 10 μ g/ml soybean trypsin inhibitor as protease inhibitors. Cells were swollen for 10 min on ice and then disrupted by vigorous vortexing. A crude membrane fraction was prepared by centrifugation of the homogenate at 35 000 g for 20 min. The resulting pellet was suspended in binding buffer containing 25 mM Mes/NaOH, pH 6.2, 10 mM MgCl₂ and 1 mM EDTA. Membranes were stored at -70 °C. PGE₂ binding was assayed by incubating membranes (20–50 μ g of protein) with 0.5–5 nM [³H]PGE₂ and various concentrations of unlabelled PGE₂ in 100 μ l of binding buffer for 1 h at 20 °C. Non-specific binding was determined in the presence of 25 μ M PGE₂. Bound and unbound ligand were separated by rapid vacuum filtration through GF 52 filters (Schleicher & Schüll, Dassel, Germany). Filters were washed five times with 4 ml of ice-cold binding buffer. Radioactivity retained on the filter was counted in 5 ml of Hydroluma (Baker, Deventer, The Netherlands). Binding constants were calculated by non-linear regression analysis (LIGAND [24]).

Cell surface ligand binding

Cells in 24-well plates (1×10^5 cells/well) were washed once with a Hepes-buffered salt solution (15 mM Hepes, 4.7 mM KCl, 1.2 mM KH₂PO₄, 11 mM glucose and 2.2 mM CaCl₂) and then incubated for 2 h at 4 °C in 200 μ l of buffer containing 5 nM [³H]PGE₂, in the presence or absence of 10 μ M unlabelled PGE₂ to determine non-specific binding in the same buffer. Plates were

washed three times with ice-cold Hepes-buffered salt solution, and cell-associated radioactivity was released by lysing cells in 400 μ l of 0.3 M NaOH/1% (w/v) SDS. The radioactivity in the cell lysates was counted in 5 ml of Hydroluma.

cAMP determination

Receptor-expressing cells were harvested in homogenization buffer and crude membranes were prepared as described above. Membranes were suspended at a protein concentration of 1 μ g/ml in 60 mM Tris/HCl, pH 7.5, containing protease inhibitors as above. cAMP formation was determined by incubating 30 μ l of this membrane suspension with 50 μ l of assay mixture (4 mM ATP, 20 mM MgCl₂, 2 mM dithiothreitol, 20 μ M GTP, 2 mM IBMX, 4 mM EDTA, 10 mM phosphocreatine and 20 units/ml creatine kinase, pH 7.5) and PGE₂ at the concentrations indicated in a total volume of 100 μ l for 5 min at 37 °C. The reaction was terminated by heating the mixture to 95 °C for 10 min. cAMP in the supernatant was determined by RIA (Amersham Pharmacia) according to the manufacturer's instructions.

Intact cell phosphorylation and immunoprecipitation

Receptor-expressing HEK293 cells in six-well plates were washed two times with phosphate-free DMEM and prelabelled for 60 min with 150 μ Ci/ml [³²P]P_i in 500 μ l of the same medium containing 10% (v/v) FCS that had been dialysed extensively against 10 mM Hepes buffer, pH 7.5, containing 150 mM NaCl. After cell labelling, PGE₂ was added for 10 min at 37 °C in a volume of 500 μ l of phosphate-free DMEM containing 10% (v/v) dialysed FCS. After stimulation, the medium was removed and the cells were washed once with ice-cold 10 mM Hepes buffer, pH 7.5, containing 150 mM NaCl. Cells were scraped into 800 μ l of lysis buffer/well [1% (v/v) Triton X-100, 0.05% (w/v) SDS, 50 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 0.2 mM Pefabloc SC, 10 μ g/ml leupeptin and 10 μ g/ml soybean trypsin inhibitor], transferred to Microfuge tubes and centrifuged for 30 min at 35 000 g to remove insoluble material. The supernatant was precleared for 60 min at 4 °C with 100 μ l of 10% (v/v) Sepharose 4B in lysis buffer containing 0.1% (w/v) BSA. The precleared supernatant was incubated for 2 h at 4 °C with an immune complex of 15 μ g of anti-FLAG monoclonal antibody M2 and 100 μ l of 10% (v/v) Protein G-Sepharose FF in lysis buffer containing 0.2% (w/v) BSA, which was preformed by incubation for 60 min at 4 °C. Immune complexes were collected by centrifugation and washed five times with ice-cold lysis buffer. Bound receptor proteins were displaced from the immune complexes by incubation for 60 min at 37 °C and 10 min at 60 °C under vigorous shaking with Laemmli sample buffer containing 5% (v/v) β -mercaptoethanol. The amount of receptor proteins loaded on to SDS/PAGE was equalized by parallel determination of cell surface ligand binding by receptor-expressing cells as described above. Gels were stained with Coomassie Blue and dried for PhosphorImager analysis. To quantify receptor phosphorylation, the intensity of PhosphorImager bands was calculated using Quantity One (Bio-Rad, Madison, WI, U.S.A.).

Mapping of phosphorylation sites

Receptor-expressing HEK293 cells in a 10 cm diameter plate were labelled with 2 mCi of [³²P]P_i for 5 h, stimulated with 1 μ M PGE₂ for 10 min, lysed in 2.4 ml of lysis buffer and immunoprecipitated as described above.

Generation of tryptic peptides

Immunoprecipitated proteins were separated by SDS/PAGE and blotted on to a PVDF membrane. Phosphorylated receptors were detected by phosphorimaging and the corresponding bands were cut out. The membrane pieces were blocked for 1 h at room temperature with cleavage buffer [50 mM (NH₄)HCO₃] containing 0.1% (v/v) Tween 20 and washed five times with cleavage buffer. Receptors were cleaved with 1 µg of modified sequencing-grade trypsin (Promega) in 200 µl of cleavage buffer for 12 h at 37 °C. Peptides released into the supernatant were vacuum dried and oxidized with 50 µl of performic acid for 1 h on ice. Reactions were diluted with 500 µl of water, frozen and vacuum dried. Another digestion with 1 µg of trypsin was performed in 50 µl of cleavage buffer for 12 h at 37 °C.

Generation of CNBr/endoproteinase GluC peptides

Immunoprecipitated proteins on Protein G–Sepharose FF beads were cleaved for 16 h with 250 µl of 100 mM CNBr in 70% (v/v) formic acid. The reaction was diluted with 500 µl of water and centrifuged (15 000 g for 5 min). The supernatant containing the released peptides was frozen and vacuum dried. The dried peptides were dissolved in 500 µl of water and freeze-dried three times to remove residual formic acid. Peptides were redissolved in 100 µl of 25 mM (NH₄)HCO₃ and cleaved with 2 µg of sequencing-grade endoproteinase GluC (Roche, Mannheim, Germany) for 16 h at 25 °C.

Two-dimensional mapping of phosphopeptides

Samples were vacuum-dried, dissolved in 10 µl of electrophoresis buffer (formic acid/acetic acid/water, 46:156:1790, by vol., pH 1.9), and phosphopeptides were separated by electrophoresis on cellulose thin layer plates (Merck, Darmstadt, Germany) in the first dimension (1000 V, 45 min, electrophoresis buffer) followed by chromatography in the second dimension (isobutyric acid/butan-1-ol/pyridine/acetic acid/water (1250:38:96:58:558, by vol.) for 16 h at room temperature. Plates were dried, phosphopeptides were visualized by phosphorimaging and the corresponding spots were scraped off the plates. Peptides were extracted with 2 × 250 µl of 20% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid in a sonicated water bath and vacuum-dried. Peptides (40–500 c.p.m.) were sequenced on a solid-phase sequencer (ABI 477); 10–20 sequencing cycles were performed. The eluates of each cycle were spotted on to membranes and their ³²P content was quantified by phosphorimaging.

Molecular mass determination of CNBr-derived peptides

CNBr-derived peptides from PGE₂-stimulated hEP4-Rs were generated as described above. Peptides were separated by SDS/PAGE on 16.5% Tris/Tricine peptide gels according to Schagger and von Jagow [25]. Gels were dried and analysed using a PhosphorImager after 2 days. Molecular masses of ³²P-labelled peptides were calculated by comparison with ¹⁴C-labelled peptide molecular mass standards (Amersham Pharmacia).

Monitoring of agonist-induced β-arrestin1–GFP translocation in living cells by confocal laser scanning microscopy

Confocal microscopy was performed using a Zeiss laser scanning microscope (LSM 510). hEP4-R wild-type or mutant receptor-expressing cells were grown on glass cover slips for 24 h and then transiently transfected with a β-arrestin1–GFP expression vector using Lipofectamine (Invitrogen). After 24 h, cells were transferred to a tempered (37 °C) microscope stage filled with 1 ml

of Krebs–Hepes buffer, pH 7.4, and treated with 1 µM PGE₂ for 0–30 min. Images were collected sequentially using single-line excitation (488 nm).

Internalization assay

Cells were seeded in 24-well plates at a density of 1 × 10⁵ cells/well. Assays were performed 48 h later. Cell surface ligand binding was performed for 2 h at 4 °C or for 30 min at 37 °C as described above. Plates were placed on ice and washed three times with ice-cold Hepes-buffered salt solution. Surface-bound ligand was eluted for 2 × 5 min with 200 µl of ice-cold 50 mM glycine/HCl, pH 3.0, containing 150 mM NaCl. The residual cell-associated radioactivity was released by lysing cells in 400 µl of 0.3 M NaOH/1% (w/v) SDS. The radioactivity in the pH 3.0 eluate and in the cell lysate was counted in 5 ml of Hydroluma.

RESULTS

According to the paradigm of the β₂-adrenergic receptor, agonist-dependent phosphorylation and β-arrestin recruitment are related [11]. Therefore, experiments to map the site of phosphorylation of the hEP4-R were carried out first. The C-terminal domain of the hEP4-R contains 38 Ser or Thr residues that are potential sites for agonist-induced receptor phosphorylation. In order to confine the range within the C-terminal domain, which contains the relevant phosphorylation sites, mutants were generated that lacked all or some of the potential phosphorylation sites (Figure 1). cDNAs of wild-type and mutant receptors were sequenced to exclude the possibility that mutations other than the ones intended were introduced by PCR artifacts. The cDNAs were cloned into the expression vector pcDNA3 for transfection of HEK293 cells. Clonal cell lines expressing wild-type or mutant receptors were generated.

Characterization of the ligand binding and signal transduction properties of wild-type hEP4-R and receptor mutants

The binding properties of the receptor proteins were characterized by saturation binding assays in membranes of the cells. The *K*_d value for PGE₂ binding was approx. 2 nM, and did not differ significantly between the wild-type hEP4-R and the mutants (Table 2). The cell clones selected expressed the respective receptor proteins to similar levels. The maximal binding capacity was approx. 2 pmol/mg of membrane protein, and there were no statistically significant differences between cell lines expressing the wild-type receptor or the receptor mutants. Wild-type and mutant receptors both coupled to G_s. Control of coupling, which was shown previously to be dependent on the presence of an intact C-terminal domain in the EP3-R [26], was not affected by the mutants. The EC₅₀ for PGE₂-stimulated cAMP formation in membranes of the cell lines varied between 0.5 and 8 nM (Table 2). However, the differences in EC₅₀ values between mutant and wild-type receptors did not attain statistical significance. EC₅₀ values were thus all in the range of the *K*_d values.

Localization of the main phosphorylation sites in the C-terminal domain of the hEP4-R by characterization of receptor mutants

Stimulation of cells expressing the wild-type hEP4-R with 1 µM PGE₂ for 10 min resulted in phosphorylation of the receptor protein (Figure 2). The hEP4-R protein was immunoprecipitated with the anti-FLAG M2 antibody, and the proteins in the precipitate were separated by SDS/PAGE. The phosphorylated receptor appeared as a single broad band of approx. 80 kDa by

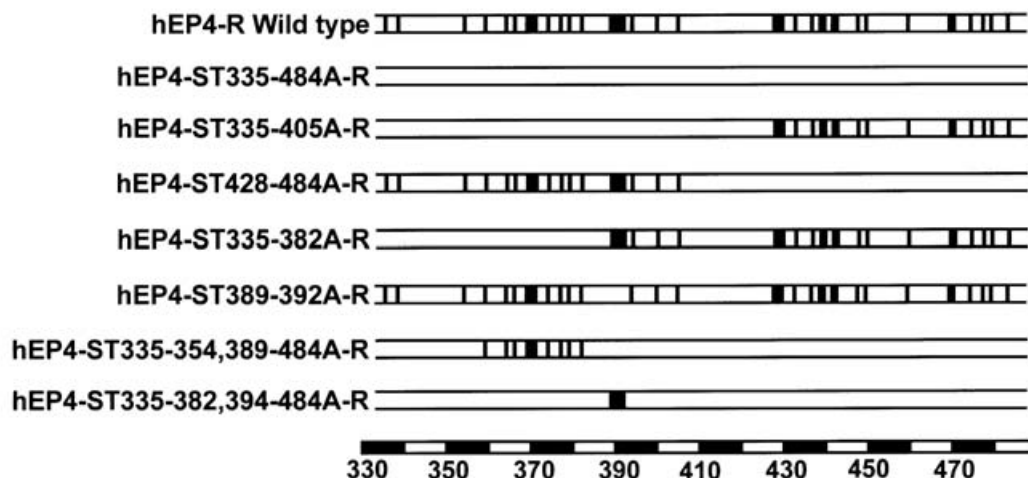


Figure 1 Potential phosphorylation sites in the hEP4-R C-terminal domain, and Ser/Thr → Ala substitution mutants

Potential phosphorylation sites are represented by black bars.

Table 2 Binding and signalling characteristics of wild-type and mutant hEP4-Rs

Membranes from HEK293 cells stably expressing wild-type or mutant hEP4-Rs were prepared and examined for [³H]PGE₂ binding and PGE₂-induced cAMP formation. *K_d* and *B_{max}* values were determined by non-linear regression analysis of saturation binding assays. cAMP formation was stimulated by 10⁻¹¹–10⁻⁵ M PGE₂. EC₅₀ values were determined by non-linear regression analysis. Details are described in the Experimental section. Values are means ± S.E.M. from the numbers of independent experiments indicated in parentheses.

Receptor	<i>K_d</i> (nM)	<i>B_{max}</i> (pmol/mg)	EC ₅₀ (nM)
hEP4-R wild type	3.0 ± 1.3 (4)	1.0 ± 0.4 (4)	2.2 ± 0.6 (3)
hEP4-ST335–484A-R	3.2 ± 1.0 (4)	1.2 ± 0.3 (4)	1.7 ± 0.9 (3)
hEP4-ST335–405A-R	3.9 ± 1.9 (3)	1.4 ± 0.4 (3)	1.1 ± 0.1 (3)
hEP4-ST428–484A-R	1.6 ± 0.3 (3)	2.0 ± 0.5 (3)	0.6 ± 0.5 (3)
hEP4-ST335–382A-R	2.3 ± 0.9 (3)	2.0 ± 0.1 (3)	2.7 ± 1.6 (3)
hEP4-ST389–392A-R	2.9 ± 0.9 (3)	1.4 ± 0.4 (3)	8.3 ± 2.6 (3)
hEP4-ST335–354,389–484A-R	4.0 ± 3.3 (3)	1.8 ± 0.7 (3)	2.7 ± 3.0 (7)
hEP4-ST335–382,394–484A-R	1.7 ± 0.3 (3)	2.0 ± 0.5 (3)	4.7 ± 1.2 (3)

phosphorimaging (Figure 2). In accordance with previous observations [22], this phosphorylation was neither mimicked by receptor-independent activation of protein kinase A or protein kinase C nor inhibited by staurosporine (results not shown), and thus was most probably mediated by GRKs. It was shown previously that the C-terminal domain of the hEP4-R was necessary and sufficient to confer agonist-induced receptor phosphorylation [22]. In line with these results, the mutant hEP4-ST335–484A-R, in which all 38 potential phosphorylation sites were replaced by Ala, was no longer phosphorylated upon agonist exposure (Figure 2). Similarly, substitution with Ala of all potential phosphorylation sites in the proximal part of the C-terminal domain of the hEP4-R (hEP4-ST335–405A-R) resulted in a decrease in agonist-induced receptor phosphorylation to approx. 20% of the level observed with the wild-type receptor (Figure 2). In contrast, a receptor mutant lacking all potential phosphorylation sites in the distal part of the C-terminal domain, but retaining the phosphorylation sites in the proximal part (hEP4-ST428–484A-R), was phosphorylated to a similar extent as the wild-type receptor upon stimulation with PGE₂ (Figure 2).

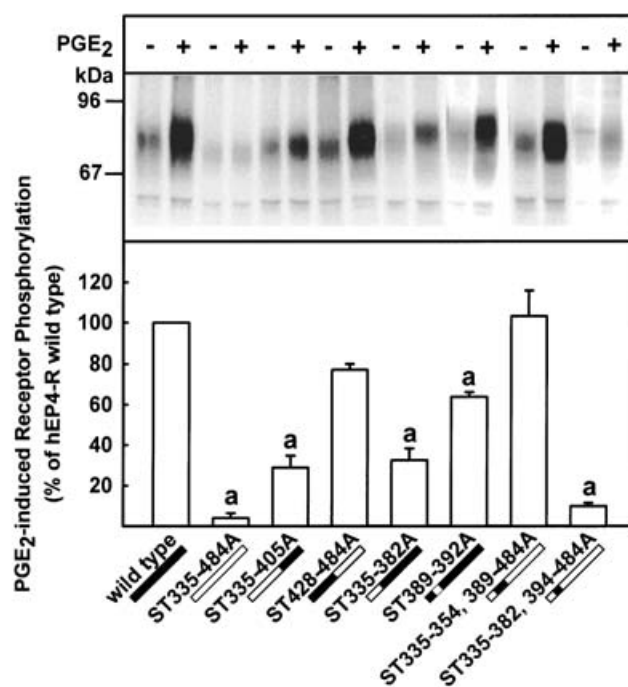


Figure 2 Effects of Ser/Thr → Ala mutagenesis on PGE₂-induced hEP4-R phosphorylation

[³²P]P_i-labelled HEK293 cells stably expressing FLAG-tagged wild-type or mutant hEP4-Rs were stimulated for 10 min at 37 °C with 1 μM PGE₂. Receptors were immunoprecipitated with anti-FLAG M2 antibody and normalized for cell surface receptor expression. Receptor proteins were resolved under reducing conditions by SDS/PAGE and phosphorylation was determined by PhosphorImager analysis after 2 days. Agonist-induced receptor phosphorylation was calculated by subtracting the values of basal phosphorylation in the absence of stimulus for each receptor variant, and was normalized to that obtained in cells expressing wild-type hEP4-R. The upper panel shows a representative PhosphorImager analysis, and the lower panel shows the quantitative analysis from pooled data from at least three independent experiments. Data are means ± S.E.M.; **P* < 0.05 compared with cells expressing wild-type hEP4-R.

Thus agonist-dependent phosphorylation appeared to depend predominantly on the presence of Ser/Thr residues in the proximal part of the C-terminal domain. Therefore this region was studied

in more detail. The proximal part of the C-terminal domain contains two clusters of Ser/Thr residues. The first cluster is located around amino acid 370, and the second cluster around amino acid 390. Replacement by Ala of all Ser/Thr residues up to Ser-382, including the first cluster (hEP4-ST335–382A-R), reduced agonist-induced phosphorylation by approx. 70%, whereas replacement of Ser/Thr residues in the second cluster (hEP4-ST389–392A-R) led to only a 40% decrease in agonist-induced phosphorylation (Figure 2). This indicated that the Ser and Thr residues around the first cluster might be more relevant for agonist-induced receptor phosphorylation than those around the second cluster. This hypothesis was strengthened by the analysis of two additional mutants. A mutant containing only Ser/Thr residues in the region 359–382, including the first cluster (hEP4-ST335–354,389–484A-R), was phosphorylated to a comparable extent as the wild-type receptor, whereas a mutant containing only the second cluster (hEP4-ST335–382,394–484A-R) showed almost no agonist-induced phosphorylation (Figure 2).

In conclusion, analysis of mutant receptors indicated that PGE₂-induced hEP4-R phosphorylation depended predominantly on the presence of Ser/Thr residues within the region 359–382. The possibility cannot be excluded that Ser/Thr residues in the region 389–392 (Ser-389–Ser-390–Thr-391–Ser-392) and/or Ser/Thr residues in the distal part of the C-terminal domain might also be subject to agonist-induced phosphorylation; however, it appeared that they contributed to total agonist-induced receptor phosphorylation to a much lesser extent.

Identification by radio Edman degradation of phosphorylation sites in the wild-type receptor and the mutant receptor hEP4-ST335–354,389–484A-R

In order to identify those amino acids in the C-terminal domain of the hEP4-R that are phosphorylated upon agonist stimulation, proteolytic fragments of the C-terminal domain of the agonist-stimulated ³²P-labelled wild-type receptor and receptor mutant hEP4-ST335–354,389–484A-R were isolated by two-dimensional separation and then subjected to radio Edman degradation. A tryptic digest of the wild-type receptor yielded four ³²P-labelled peptides that could be separated by two-dimensional chromatography (Figure 3A). Peptide 1 contained most (60%) of the radioactivity. The residual radioactivity was distributed equally among the other three peptides. Tryptic cleavage of the mutant hEP4-ST335–354,389–484A-R, which contained only the potential Ser/Thr phosphorylation sites within the region encompassing residues 359–382, yielded the same four phosphopeptides as the wild-type receptor and one additional phosphopeptide (peptide 5) that contained a minor fraction of the radioactivity (Figure 3A). Notably, there was no phosphorylated tryptic fragment that appeared exclusively in the digest of the wild-type receptor. Therefore, upon agonist stimulation, the vast majority of the ³²P radioactivity in the wild-type receptor must have been incorporated into Ser/Thr residues within region 359–382 that are also present in the mutant receptor. There are three potential tryptic fragments in this region. In order to assign these tryptic fragments to the phosphopeptides, the phosphopeptides were extracted and sequenced (Figure 3B). Radioactivity eluted in the first sequencing cycle of peptide 1 (Figure 3B), in the second sequencing cycle of peptide 2, in the fourth sequencing cycle of peptide 3, in the second and the sixth sequencing cycles of peptide 4, and in the second and third sequencing cycles of peptide 5. Peptide 4 was only successfully sequenced from the mutant hEP4-ST335–354,389–484A-R. Peptide 1, which contained most of the radioactivity, could not be assigned to one particular tryptic

fragment, since a serine or threonine residue was located at position 1 in all of the three tryptic fragments containing potential phosphorylation sites between Ser-359 and Ser-382. Only one tryptic fragment in the relevant part of the C-terminal domain contains a Ser/Thr residue at position 2. Therefore peptide 2 was assigned to the tryptic fragment Thr-369–Arg-378 phosphorylated at Ser-370. Peptide 3 was assigned to the tryptic fragment Ser-379–Arg-383 phosphorylated at Ser-382. Peptide 4 represented tryptic fragment Thr-369–Arg-378 with a double phosphorylation at Ser-370 and Ser-374, and peptide 5 was assigned to the same tryptic fragment with phosphorylation at Ser-370 and Ser-371 (Figure 3B).

Radio Edman degradation of tryptic fragments of the C-terminal domain did not allow us to assign the main phosphopeptide to one particular tryptic fragment. Therefore the agonist-exposed, ³²P-labelled wild-type receptor was cleaved with CNBr (cleavage after methionine), and the resulting phosphopeptides were separated by SDS/PAGE. CNBr cleavage of the hEP4-R C-terminal domain yielded two hypothetical fragments including Ser/Thr residues between Ser-359 and Ser-382 (fragment 1, Val-271–Met-373; fragment 2, Ser-374–Met-421), with calculated molecular masses of 11.6 and 5.1 kDa respectively (Figure 4A). Two major ³²P-labelled peptides were separated by SDS/PAGE of the CNBr fragments of the wild-type receptor. Peptide 1, which contained most (60%) of the radioactivity, had an apparent molecular mass of 6.3 kDa, whereas peptide 2, which contained 20% of the total radioactivity, had a molecular mass of approx. 11.7 kDa. Phosphorylation of the hEP4-R was shown to lead to retardation in the electrophoretic mobility of the receptor protein [27]. Therefore peptide 1 most probably represented fragment 2 (Ser-374–Met-421), while peptide 2 represented fragment 1 (Val-271–Met-373). Under this assumption, the major phosphopeptide 1 of the tryptic map would represent fragment Ser-379–Arg-383, phosphorylated at Ser-379 (Figure 3B). In an attempt to further support this hypothesis, the agonist-exposed, ³²P-labelled wild-type receptor was cleaved with CNBr and subsequently with endoproteinase GluC (cleavage after glutamic acid). The resulting phosphopeptides were separated as described above. The majority of the radioactivity was concentrated in one peptide (Figure 4B), which was purified and subjected to radio Edman degradation. The radioactivity eluted in sequencing cycles 1, 4, 6 and 9 (Figure 4B). There is only one potential CNBr/GluC fragment that contains Ser/Thr residues in these positions, i.e. fragment Ser-374–Glu-384. The phosphorylated amino acids then correspond to Ser-374, Ser-377, Ser-379 and Ser-382. With the exception of Ser-377, all of these phosphorylated residues had also been detected in the tryptic fragments (Figure 3B).

Although the analysis of the phosphopeptides after tryptic cleavage and CNBr/GluC cleavage did not yield 100% congruent results, and do not allow us to identify single amino acids as major substrates for agonist-induced phosphorylation, the data from the phosphopeptide analysis together with the analysis of the phosphorylation behaviour of the mutant receptors strongly support the hypothesis that the main site of agonist-induced receptor phosphorylation in the hEP4-R C-terminal domain is located proximal of Ser-382, presumably between Ser-370 and Ser-382, whereas Ser/Thr residues distal of Ser-382 were not relevant for maximal agonist-induced receptor phosphorylation.

Agonist-induced subcellular redistribution of β -arrestin1-GFP in cells expressing wild-type and mutant hEP4-Rs

Agonist-induced receptor phosphorylation and receptor- β -arrestin interactions are related for most GPCRs [28]. In order

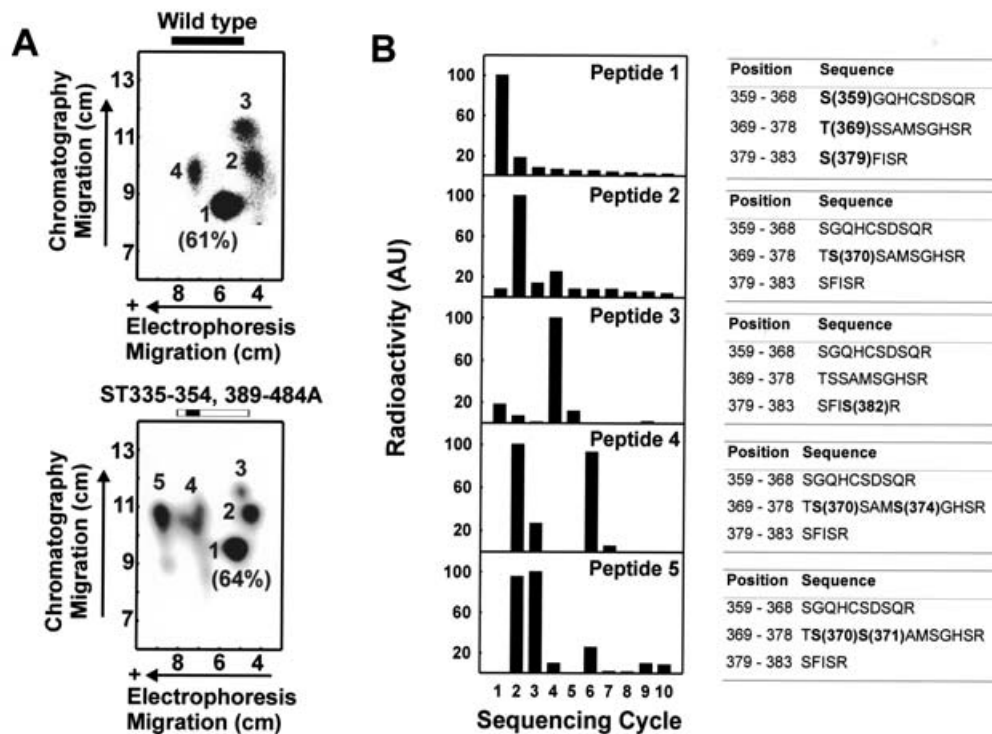


Figure 3 Analysis of tryptic phosphopeptides generated from wild-type hEP4-R and mutant hEP4-ST335-354,389-484A-R

(A) $^{32}\text{P}_i$ -labelled HEK293 cells stably expressing FLAG-tagged wild-type hEP4-R or mutant hEP4-ST335-354,389-484A-R were stimulated for 10 min at 37 °C with 1 μM PGE₂. Following immunoprecipitation and SDS/10%-PAGE, isolated proteins were transferred on to PVDF membranes and analysed using a PhosphorImager. The ^{32}P -labelled receptors were digested *in situ* with trypsin and the resulting peptides were separated on TLC plates by high-voltage electrophoresis and subsequent chromatography. + indicates the polarity during electrophoresis. Thereafter, phosphopeptides were localized by PhosphorImager analysis and the relative contributions of each peptide to total radioactivity were quantified. (B) Phosphopeptides were eluted and subjected to 10 cycles of Edman degradation. Cleaved phenylthiohydantoin-amino acids were collected and analysed using a PhosphorImager to locate the positions of the phosphorylation sites. The content of ^{32}P radioactivity of each sequencing cycle was quantified and expressed in arbitrary units (AU). Phosphorylation sites were identified by scanning potential tryptic fragments, which include Ser/Thr residues between Ser-359 and Ser-382, for Ser/Thr residues at the respective positions determined by phosphopeptide sequencing ($n = 1$). Identified phosphorylation sites are highlighted in bold.

to analyse the interdependence of the phosphorylation and β -arrestin interaction of the hEP4-R, cells expressing either wild-type or mutant receptors were transfected transiently with a β -arrestin1-GFP expression vector. The PGE₂-induced subcellular redistribution of β -arrestin1-GFP was monitored for 30 min in living cells by laser scanning microscopy.

In the absence of agonist, β -arrestin1-GFP was distributed uniformly in the cytoplasm of cells expressing wild-type or mutant hEP4-Rs (Figure 5). Only a small amount of β -arrestin1-GFP was located in the nucleus. The intracellular distribution of β -arrestin1 was thus similar to its distribution reported previously for HEK293 cells [29]. In cells expressing wild-type hEP4-R, β -arrestin1-GFP was redistributed from the cytoplasm to the plasma membrane within 2 min of PGE₂ addition (Figure 5). After longer agonist exposure, β -arrestin1-GFP was redistributed from the plasma membrane to endocytotic vesicles. These vesicles were first detected after 5 min, grew in size and number to a maximum at 10 min, and were still observed after 30 min of agonist treatment. In contrast, in cells expressing the mutant receptor hEP4-ST335-484A-R that lacks all Ser/Thr residues in the C-terminal domain, PGE₂ induced neither acute recruitment of β -arrestin1-GFP to the plasma membrane nor its redistribution into intracellular vesicles upon prolonged agonist exposure. Similarly, substitution of all Ser/Thr residues in the proximal half of the C-terminal domain (mutant hEP4-ST335-405A-R) abolished PGE₂-induced β -arrestin1-GFP translocation (Figure 5). In cells expressing the mutant hEP4-ST428-484A-R, which retains the Ser/Thr residues

in the proximal half but lacks all of them in the distal half of the C-terminal domain, β -arrestin1-GFP was recruited to the plasma membrane within 2 min of stimulation by PGE₂. In contrast with cells expressing wild-type hEP4-R, however, the majority of the β -arrestin1-GFP remained at or close to the plasma membrane even after prolonged agonist treatment, and only a very small amount of β -arrestin1-GFP trafficked into endocytotic vesicles (Figure 5), which were smaller in number and size than in cells expressing wild-type receptor. These results indicate that Ser/Thr residues in the proximal part of the hEP4-R C-terminal domain are essential for the receptor- β -arrestin1 interaction. In addition, Ser/Thr residues in the distal part appear to be necessary for efficient direction of hEP4-R- β -arrestin1 complexes into endocytotic vesicles.

The proximal part of the C-terminal domain also contains the main site of agonist-induced phosphorylation of the hEP4-R, which appears to be located between Ser-370 and Ser-382. To analyse if these phosphorylation sites are identical with the Ser/Thr residues required for the recruitment of β -arrestin1-GFP to the plasma membrane, β -arrestin1-GFP translocation was studied in cells expressing receptor mutants hEP4-ST335-354,389-484A-R (retaining the putative main site of phosphorylation) and hEP4-ST335-382A-R (lacking the putative main site of phosphorylation, but retaining all Ser/Thr residues distal of this site). Surprisingly, no β -arrestin1-GFP translocation was observed in cells expressing hEP4-ST335-354,389-484A-R (Figure 5), although the receptor was phosphorylated to a similar extent as

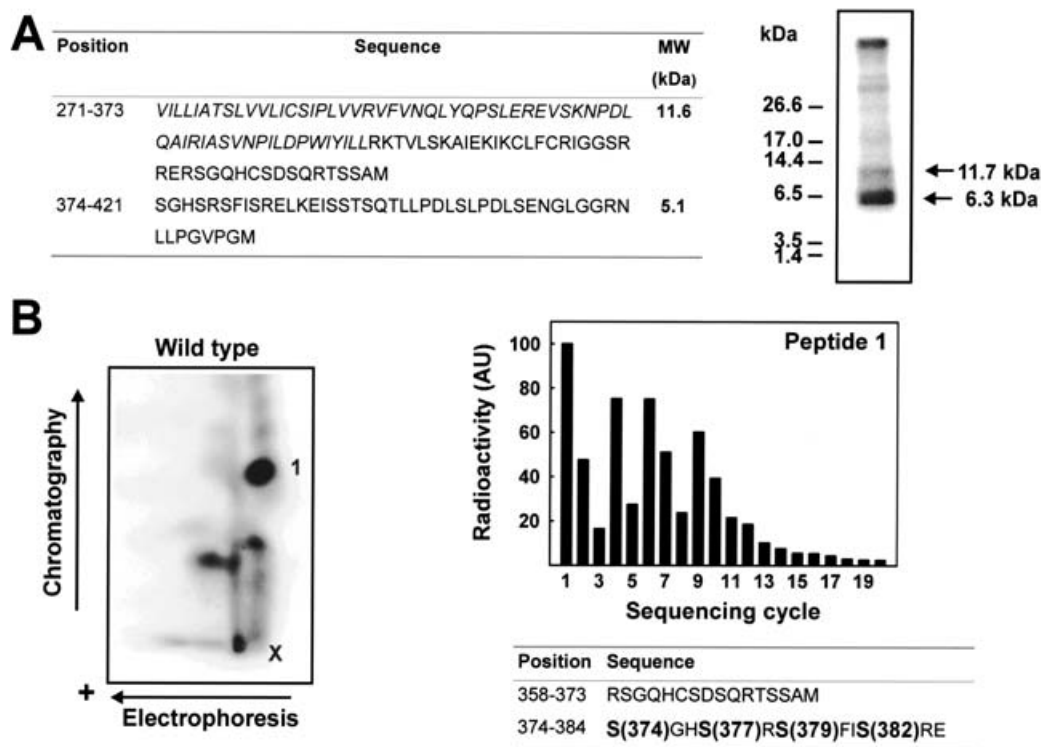


Figure 4 Analysis of CNBr and CNBr/endoproteinase GluC phosphopeptides generated from wild-type hEP4-R

(A) [32 P]P_i-labelled HEK293 cells stably expressing FLAG-tagged wild-type hEP4-R were stimulated for 10 min at 37 °C with 1 μ M PGE₂. Following immunoprecipitation, receptors were digested *in situ* with CNBr on immune complexes. Phosphopeptides were resolved by SDS/PAGE on 16.5% Tris/Tricine gels and analysed using a PhosphorImager. Molecular masses of the phosphopeptides were estimated relative to 14 C-labelled low-molecular-mass standard peptides ($n = 2$). The potential molecular masses of hypothetical CNBr fragments including potential phosphorylation sites between Ser-359 and Ser-382 were calculated using GenePro5. (B) Phosphopeptides generated by CNBr cleavage of wild-type hEP4-R were subsequently digested with endoproteinase GluC. The resulting peptides were separated and analysed by PhosphorImager and phosphopeptide sequencing as described above for tryptic peptides. The main CNBr/endoproteinase GluC phosphopeptide was subjected to 20 cycles of Edman degradation. Phosphorylation sites were identified by scanning of potential CNBr/endoproteinase GluC fragments, which include phosphorylation sites between Ser-359 and Ser-382, for Ser/Thr residues at the respective positions determined by phosphopeptide sequencing ($n = 1$). Identified phosphorylation sites are highlighted in bold.

wild-type hEP4-R (Figure 2). In contrast, in cells expressing hEP4-ST335–382A-R, β -arrestin1–GFP was recruited to the plasma membrane within 2 min of stimulation by PGE₂ (Figure 5), but no translocation of β -arrestin1–GFP into endocytotic vesicles was observed after prolonged agonist treatment. β -Arrestin1–GFP remained at or close to the plasma membrane until 10 min of agonist exposure, and then returned gradually into the cytoplasm, where most of it was evenly distributed after 30 min of agonist exposure. These results led to the hypothesis that the presence of the Ser/Thr cluster comprising residues 389–392 might be mandatory for the interaction of the hEP4-R with β -arrestin1. To prove this concept, PGE₂-induced β -arrestin1–GFP translocation was monitored in cells exclusively lacking Ser/Thr residues 389–392 (hEP4-ST389–392A-R) or containing this cluster as the only Ser/Thr residues in the C-terminal domain (hEP4-ST335–382,394–484A-R). Substitution of the Ser/Thr cluster 389–392 by Ala completely abolished translocation of β -arrestin1–GFP to the plasma membrane in hEP4-ST389–392A-R-expressing cells (Figure 5), and β -arrestin1–GFP did not redistribute into intracellular vesicles after prolonged agonist exposure. In contrast, in cells expressing hEP4-ST335–382,394–484A-R, a small but significant amount of β -arrestin1–GFP was recruited to the plasma membrane within 2 min of agonist stimulation. Plasma membrane localization of β -arrestin1–GFP in these cells was very transient, and it was redistributed from the plasma membrane back to the cytoplasm within 10 min of agonist treatment (Figure 5).

Agonist-induced internalization of wild-type and mutant hEP4-Rs

Agonist-induced receptor internalization was tested by measuring internalization of the ligand-occupied receptor. This was monitored by the decrease in cell surface-accessible radioactively labelled ligand at 37 °C, a temperature that permits internalization, in comparison with that at 4 °C, a temperature that does not permit internalization (Figure 6). At a concentration of 5 nM [3 H]PGE₂ 45% of the PGE₂ binding sites were internalized into intracellular compartments (Figure 6). This receptor internalization was abolished in cells expressing the hEP4-ST335–484A-R mutant, which lacks all Ser/Thr residues in the C-terminal domain. Thus Ser/Thr residues in the hEP4-R C-terminus are apparently essential determinants of agonist-induced receptor internalization. Substitution by Ala of Ser/Thr residues either in the proximal part (hEP4-ST335–405A-R) or in the distal part (hEP4-ST428–484A-R) of the C-terminal domain reduced PGE₂-induced receptor internalization to 20% of total specific cell surface binding (Figure 6), indicating that Ser/Thr residues in both parts of the C-terminal domain are involved in receptor internalization. In cells expressing the hEP4-ST335–354,389–484A-R mutant, which was phosphorylated to a similar extent as the wild-type receptor (Figure 2) but did not interact with β -arrestin1–GFP (Figure 5), internalization was severely impaired (Figure 6). In contrast, agonist-induced receptor internalization was not significantly different from that in cells expressing wild-type

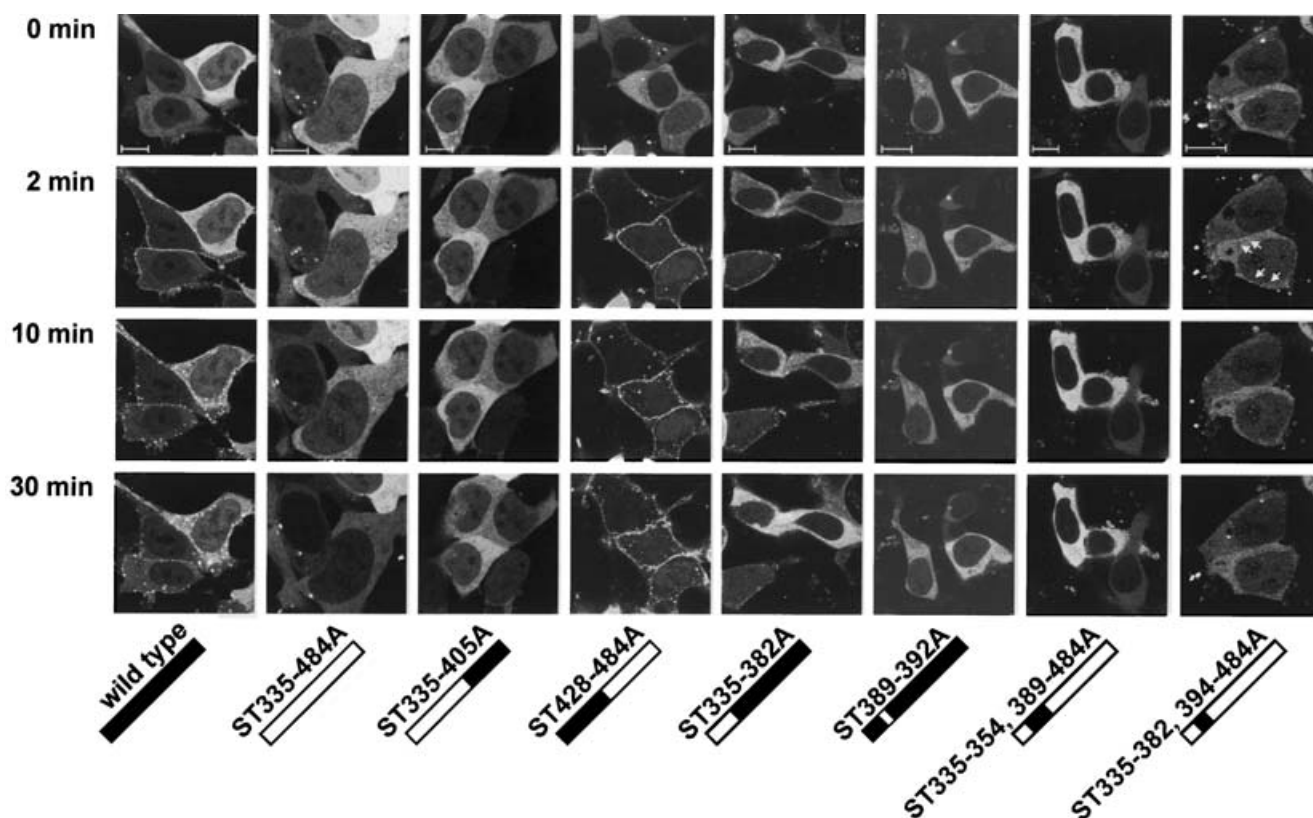


Figure 5 PGE₂-induced subcellular redistribution of β -arrestin1-GFP in cells expressing wild-type or mutant hEP4-Rs

HEK293 cells stably expressing wild-type or mutant hEP4-Rs were transiently transfected with an expression vector for β -arrestin1-GFP. After 24 h, cells were transferred to a heated (37 °C) microscope stage filled with 1 ml of Krebs/Hepes buffer, pH 7.4, and the distribution of β -arrestin1-GFP was visualized before and after treatment with 1 μ M PGE₂ on a Zeiss 510 inverted laser scanning microscope (excitation = 488 nm, emission = 515 nm). Shown are representative confocal microscopic images of β -arrestin1-GFP fluorescence in the same cells treated with PGE₂ for 0, 2, 10 or 30 min at 37 °C. White arrows highlight β -arrestin1-GFP recruitment in cells expressing the mutant hEP4-ST335-389,394-484A-R. Scale bars = 10 μ m.

hEP4-R in cells expressing the hEP4-ST335-382A-R mutant (Figure 6), which upon agonist stimulation was phosphorylated to a significantly lesser extent than the wild-type receptor (Figure 2), but interacted with β -arrestin1-GFP (Figure 5). Selective elimination of the Ser/Thr 389-392 cluster, which appeared to be essential for the agonist-induced β -arrestin1-GFP interaction, reduced agonist-induced internalization to a similar extent as the complete elimination of all Ser/Thr residues in the proximal half of the C-terminal domain. Agonist-induced internalization was almost abolished in cells expressing a receptor that contained only the Ser/Thr 389-392 cluster (i.e. no other Ser/Thr residues) (Figure 6).

In conclusion, agonist-induced internalization of the hEP4-R seems not to depend primarily on its phosphorylation. Rather, the interaction with β -arrestin1 is a prerequisite. In order to obtain maximal agonist-induced internalization, however, additional Ser/Thr residues distal to those responsible for the interaction with β -arrestin are required.

DISCUSSION

The signalling of PGE₂ via the EP4-R is modulated by desensitization of this PGE₂ receptor subtype. Previous studies showed that the C-terminal domain is necessary and sufficient to confer desensitization of the hEP4-R [7]. Desensitization of many GPCRs is linked to GRK-dependent and/or second

messenger-activated kinase-dependent receptor phosphorylation. The C-terminal domain of the hEP4-R has been shown to undergo agonist-induced phosphorylation [22], and contains 38 Ser/Thr residues that may serve as potential substrate for these kinases. Sequential truncation and site-directed mutagenesis led to the hypothesis that only some of these potential phosphorylation sites are relevant for desensitization [30]. However, no study has directly assessed which amino acids in the C-terminal domain of the hEP4-R are necessary for the interaction with β -arrestin and the internalization of the receptor, and which Ser/Thr residues are phosphorylated upon agonist stimulation.

Mapping of phosphorylation sites

Replacement of all Ser/Thr residues in the C-terminal domain of the hEP4-R with Ala resulted in a complete loss of agonist-induced phosphorylation, indicating that such phosphorylation is restricted to the C-terminal domain (Figure 2). In accordance with this result, a truncation mutant lacking the C-terminal domain was no longer phosphorylated [27]. Substitution of blocks of Ser/Thr residues revealed that the phosphorylation of the hEP4-R occurred predominantly in the proximal part of the C-terminal domain (Figure 2). Ser/Thr residues in the region comprising residues 359-382 appeared to be necessary and sufficient for maximal PGE₂-induced phosphorylation. These findings are supported indirectly by previous studies in which this cluster of potential phosphorylation sites had been shown by sequential truncation

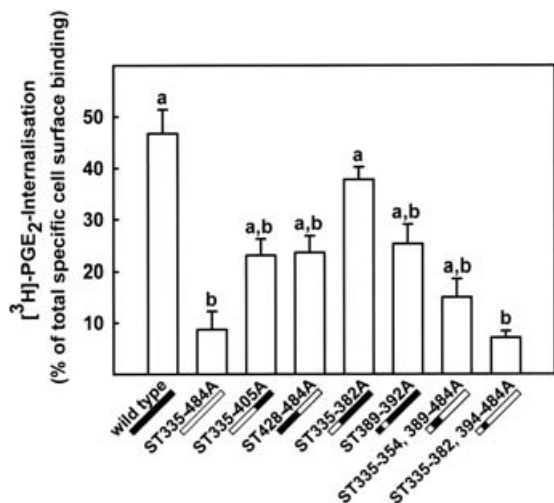


Figure 6 PGE₂-induced internalization of wild-type and mutant hEP4-Rs

HEK293 cells expressing wild-type or mutant hEP4-Rs were incubated with 5 nM [³H]PGE₂ for 2 h at 4 °C or for 30 min at 37 °C. Unspecific cell surface binding was determined in the presence of 10 μM PGE₂. Unbound ligand was removed and cell surface-bound [³H]PGE₂ was eluted by two subsequent incubations with acidic buffer (pH 3) for 5 min. The remaining cells were lysed and radioactivity in the eluate and the cell lysate was counted. Receptor internalization was calculated as the decrease in the percentage of [³H]PGE₂ cell surface binding in relation to whole-cell [³H]PGE₂ binding at 37 °C compared with 4 °C. Data are means ± S.E.M. from at least three independent experiments performed in triplicate; **P* < 0.05 for ratio of specific [³H]PGE₂ cell surface binding in relation to whole-cell specific [³H]PGE₂ binding at 37 °C compared with 4 °C; †*P* < 0.05 compared with cells expressing wild-type hEP4-R.

and block mutation to be required for PGE₂-induced hEP4-R desensitization [30].

The range of Ser/Thr residues in the C-terminal domain that was most relevant for agonist-induced phosphorylation was further narrowed by radio Edman degradation of ³²P-labelled proteolytic fragments of the wild-type hEP4-R C-terminal domain. This method also excluded potential artifacts resulting from the compensatory use of secondary phosphorylation sites after elimination of the principal phosphorylation sites (see below). One major phosphorylated peptide was identified after CNBr/GluC cleavage in which Ser-374, Ser-377, Ser-379 and Ser-382 were phosphorylated (Figure 4B). All of these phosphorylation sites are located in the potential hEP4-R wild-type CNBr fragment Ser-374–Met-421, which contained 60% of the total radioactivity incorporated into the hEP4-R upon agonist stimulation. Analysis of tryptic fragments revealed Ser-370 as an additional phosphorylation site in the wild-type hEP4-R, and confirmed the phosphorylation of Ser-379 and Ser-382 (Figure 3A). The range of amino acids that according to radio Edman degradation appeared to be phosphorylated in the ligand-occupied receptor (residues 370–382) overlapped by 100% with the block of Ser residues whose elimination had been shown previously to be essential for agonist-induced desensitization of the hEP4-R [30].

The results obtained by sequencing of the main tryptic phosphopeptide and the main CNBr/GluC phosphopeptide are somewhat discordant, in that the CNBr/GluC fragment contained four phosphorylated serine residues, Ser-374, Ser-377, Ser-379 and Ser-382, pairs of which should have been found in the tryptic fragments as well. However, tryptic peptides with a double phosphorylation of Ser-379/Ser-382 or Ser-377/Ser-379 were not detected. The main tryptic phosphopeptide appeared to contain an isolated phosphorylation of Ser-379, while only minor tryptic phosphopeptides were found with an isolated phosphorylation of

Ser-370 or Ser-382 and a double phosphorylation at Ser-370/Ser-374. Taken together, the data indicate that serine residues between Ser-370 and Ser-382 represent the predominant phosphorylation sites, but that the ratio of phosphorylation of single serines in this sequence can vary between experiments. Although radio Edman degradation did not permit the identification of single Ser/Thr residues as the exclusive substrate(s) for GRK-mediated phosphorylation of the hEP4-R, these results, together with the analysis of mutant receptors lacking groups of Ser/Thr residues, provided strong evidence that the principal phosphorylation sites for agonist-induced phosphorylation of the hEP4-R are located between Ser-370 and Ser-382.

Elimination of all potential phosphorylation sites in the proximal part of the C-terminal domain (mutant hEP4-ST335–405A-R) resulted in a marked decrease in, but not a complete loss of, agonist-induced receptor phosphorylation (Figure 2). This might indicate that potential phosphorylation sites in the distal part of the C-terminal domain may also serve as substrates for GRKs, although they contributed to the agonist-induced receptor phosphorylation to a much lesser extent than the Ser/Thr residues in the putative principal phosphorylation site. However, the possibility cannot be excluded that the residual phosphorylation in this mutant represents only a compensatory use of the distal phosphorylation sites in a receptor lacking the principal proximal phosphorylation sites. This latter possibility is supported by the finding that a tryptic digest of the agonist-exposed wild-type receptor yielded no phosphopeptide that was absent from the mutant hEP4-ST335–354,389–484A-R that lacks all phosphorylation sites in the distal part of the C-terminal domain (Figure 3A).

Agonist-induced hEP4-R-β-arrestin1 interaction

Recruitment of β-arrestin to the ligand-occupied receptor is generally a key step in initiation of the desensitization of GPCRs [28]. Phosphorylation of the receptor does not always appear to be a prerequisite for β-arrestin recruitment, but often greatly enhances the stability of the β-arrestin–receptor complex. The hEP4-R seems to follow this general rule. Apparently, two different neighbouring regions in the C-terminal domain were required for initial and sustained interaction of the hEP4-R with β-arrestin1. The cluster of Ser/Thr residues in the region 389–392 was absolutely essential for agonist-induced β-arrestin1 recruitment (Figure 5), but this cluster apparently contributed little to agonist-induced receptor phosphorylation. The presence of Ser/Thr residues in the region between Ser-370 and Ser-382, which appeared to be the main site of agonist-dependent receptor phosphorylation (Figure 2), was not necessary for the interaction of β-arrestin1 with the ligand-occupied receptor; however, phosphorylation of these residues apparently was crucial for the formation of stable receptor–β-arrestin1 complexes, since β-arrestin1 redistribution was weaker and more transient in all mutants lacking this site but retaining the Ser/Thr 389–392 cluster (Figure 5). The present results are somewhat contradictory to results published previously [30]. In that study a receptor mutant that was truncated after amino acid 383, and thus lacked the Ser/Thr 389–392 cluster but retained the putative main phosphorylation sites within region 370–382, showed the same agonist-induced desensitization as the wild-type receptor [30]. In contrast with the present study, these authors analysed the agonist-induced hEP4-R–β-arrestin1 interaction only for the wild-type receptor, but not for the truncated receptor mutant lacking the potential β-arrestin1 interaction site [31]. The possibility therefore cannot be ruled out that truncation of a main portion of the hEP4-R C-terminal domain in this mutant may have induced

an artificial conformation that allowed a receptor- β -arrestin interaction in the absence of the newly identified β -arrestin interaction site.

There are other examples of GPCRs in which phosphorylation and β -arrestin interaction are not strictly correlated. For instance, elimination of Ser/Thr residues in the C-terminal domain of rhodopsin that were not identical with the preferred target sites of GRK-mediated phosphorylation had only a small effect on rhodopsin phosphorylation, but dramatically decreased the rhodopsin- β -arrestin interaction [32]. On the other hand, translocation of β -arrestin1 to the plasma membrane was clearly observed in cells expressing a phosphorylation-deficient C5a receptor mutant lacking the preferred phosphorylation site [33].

GPCRs can be subdivided in two classes with respect to their interaction with β -arrestin. Class A receptors, such as the β_2 -adrenergic receptor and the endothelin A receptor, have a higher affinity for β -arrestin2 than for β -arrestin1, and the agonist-induced formation of β -arrestin-receptor complexes is transient, despite phosphorylation of the receptor molecule [29,34]. In contrast, class B receptors, such as the angiotensin II receptor and the substance P receptor, have similar affinities for the two β -arrestin isoforms, and activation of these receptors by agonist leads to the formation of stable complexes that are located in intracellular vesicles after prolonged agonist exposure [29,34]. The molecular determinants contributing to the formation of stable receptor- β -arrestin complexes have been shown to be clusters of Ser/Thr residues in the C-terminal receptor domains [34]. Structurally, the hEP4-R belongs to the class B receptors, containing four clusters of Ser/Thr residues: cluster 1, residues 369–371; cluster 2, 389–394; cluster 3, 428–430; cluster 4, 437–443. In cells expressing the wild-type hEP4-R, PGE₂ stimulation led to a rapid recruitment to the plasma membrane of β -arrestin1, which formed a complex with the activated receptor. After prolonged agonist treatment, β -arrestin1 was found to be located in endocytotic vesicles, indicating the formation of stable complexes with internalized hEP4-R. As expected, wild-type hEP4-R behaved as a typical class B receptor. However, despite the presence of the putative β -arrestin1 recruitment site (Ser/Thr cluster 389–392) and the putative main phosphorylation site (residues 370–382) in cells expressing the mutant hEP4-ST428–484A-R, β -arrestin1 accumulated in intracellular vesicles upon agonist stimulation to a much lesser extent than in cells expressing the wild-type receptor (Figure 5). Rather, β -arrestin1 remained at or close to the plasma membrane. No redistribution back into the cytosol was observed. This indicates that although β -arrestin1 formed stable complexes with the phosphorylated mutant receptor, these β -arrestin1-receptor complexes were not internalized efficiently. This is in line with the results of measurements of receptor internalization.

Agonist-induced hEP4-R internalization

Wild-type hEP4-R underwent agonist-induced internalization: the number of cell surface-accessible binding sites was reduced by 45% after 30 min of agonist exposure (Figure 6). This is in line with previous reports [27,35]. Substitution of all Ser/Thr residues in the C-terminal domain by Ala resulted in an almost complete loss of agonist-induced internalization (Figure 6), similar to what was observed after truncation of the C-terminal domain [27].

Internalization of GPCRs is often mediated by binding of β -arrestin to the agonist-activated receptor [17]. This was shown for the β_2 -adrenergic receptor [15], the V₂ vasopressin receptor [36] and the CCR5 receptor [37] to name but a few. Surprisingly, complete abrogation of the hEP4-R- β -arrestin1 interaction (hEP4-ST335–405A-R and hEP4-R-ST389–392A-R mutants)

did not result in the complete loss of agonist-induced receptor internalization. Rather, agonist-induced internalization amounted to approx. 50% of that of the wild-type receptor. In addition, Ser/Thr residues in the distal part of the C-terminal domain, which appeared not to contribute to β -arrestin1 recruitment or stabilization of the β -arrestin1-receptor complex, were necessary to obtain maximum agonist-induced internalization (mutant hEP4-ST428–484A-R compared with wild-type hEP4-R), indicating that a mechanism independent of β -arrestin1 complex-formation contributed to agonist-induced internalization. Notably, this β -arrestin1-independent mechanism and arrestin1 recruitment, but not stabilization of the β -arrestin1-receptor complex by phosphorylation (hEP4-ST335–382A-R), appeared to be necessary to maintain full agonist-induced receptor internalization. These results are in line with findings of a previous study reporting that elimination of residues 370–382 did not affect hEP4-R internalization, and that agonist-induced internalization was improved by co-expression of β -arrestin and inhibited by co-expression of dominant-negative β -arrestin-(319–418) or dominant-negative dynamin I K44A, suggesting a β -arrestin- and dynamin-dependent pathway of hEP4-R internalization [31,35]. The present results are, however, in striking contrast to the results of the same study [35], which showed that truncation of the hEP4-R C-terminal domain after Ser-382 did not affect agonist-induced internalization, suggesting that Ser/Thr residues distal to Ser-382 are not involved in hEP4-R internalization. A possible explanation might be that positive and negative regulatory elements are present in the hEP4-R C-terminal domain that co-operate in controlling the internalization of hEP4-R. Truncation of the C-terminal domain might have resulted in the loss of negative regulatory elements that counteract arrestin-mediated internalization in the wild-type receptor and whose action needs to be compensated by additional elements in the distal C-terminal domain. A similar situation was described for the physiological relevance of C-terminal Ser residues in bradykinin B₂ receptor internalization, where substitution of one Ser residue by Ala inhibited receptor internalization, whereas substitution of another Ser significantly enhanced bradykinin-induced internalization [38].

Conclusion

Ser/Thr residues in the C-terminal domain of the hEP4-R contribute to agonist-induced β -arrestin1 recruitment, receptor phosphorylation and receptor internalization in a complex way. Thus the Ser/Thr cluster comprising residues 389–392 appears to be crucial for the initial interaction of the ligand-occupied receptor with β -arrestin1; however, these residues appear not to contribute significantly to agonist-induced receptor phosphorylation. Phosphorylation of the putative principal phosphorylation sites within the region comprising residues 370–382, however, appears to contribute to the stabilization of the hEP4-R- β -arrestin1 complex. Additional Ser/Thr residues in the distal half of the C-terminal domain appear to be required for maximally efficient internalization of this complex. These Ser/Thr residues by themselves may confer a weaker, β -arrestin1-independent internalization of the agonist-occupied receptor. Apart from serving as potential phosphorylation sites, Ser/Thr residues distal to the 370–382 region might contribute to stabilization of the C-terminal domain in a certain conformation that is required for its function in agonist-induced hEP4-R desensitization and internalization.

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APPENDIX

Generation of mutant receptors

hEP4-ST335–484A-R

First, a *Sna*BI restriction site was introduced at position 1582 of the hEP4-R cDNA for further cloning steps. A 1000 bp 5' fragment was generated with FLAG-hEP4-f as forward primer and hEP4-SnaBI-r as a reverse primer containing the mutation (see Table 1). A 500 bp 3' fragment was produced with hEP4-SnaBI-f (Table 1) and the primer hEP4-Xba-r. A PCR product containing the entire open reading frame was generated with both overlapping fragments as template and the primers FLAG-hEP4-f and hEP4-Xba-r. The PCR product was digested with *Hind*III and *Xba*I and then ligated into the *Hind*III-*Xba*I site of pcDNA3, generating plasmid pcDNA3/hEP4-R-wt. The plasmid served as a template for PCR with primer pair hEP4-SnaBI/KpnI-f and hEP4-Xba-r (Table 1) to amplify a cDNA fragment coding for the C-terminal domain of the receptor. In addition a *Kpn*I site was added at the 5'-end of the fragment, and Ser/Thr residues in the region 335–338 were mutated to alanine. The PCR product was digested with *Kpn*I and *Xba*I and then ligated into the *Kpn*I-*Xba*I site of pBluescript (Stratagene, La Jolla, CA, U.S.A.), generating plasmid pBs/hEP4Ct-ST335–338A. Ser/Thr residues in the region 369–382 were mutated to alanine by PCR using pcDNA3/hEP4-R-wt as a template with primer pair EP4-SnaBI/KpnI-f and hEP4-3-r. The PCR product was digested with *Sna*BI and *Sma*I and inserted into pBs/hEP4Ct-ST335–338A, which was digested by the same enzymes, generating plasmid pBs/hEP4Ct-ST335–338,369–382A. For mutation of Ser/Thr residues in the region 428–443, a 1300 bp 5' fragment was generated with primer FLAG-hEP4-f as forward primer and hEP4-6-r as reverse primer containing the mutation (pcDNA3/hEP4-R-wt as a template) (Table 1). A 200 bp 3' fragment was produced with primers hEP4-6-f and hEP4-Xba-r (Table 1). The PCR products were fused by PCR with both overlapping fragments as template and the primers FLAG-hEP4-f and hEP4-Xba-r. The PCR product was digested with *Sly*I and *Xba*I and then ligated into hEP4Ct-ST335–338,369–382A, which was digested with the same enzymes, generating plasmid hEP4Ct-ST335–338,369–382,428–443A. Ser/Thr residues in the regions 354–366 and 400–405 were eliminated by PCR with the primer pair hEP4-2-f and hEP4-5-r using plasmid pBs/hEP4Ct-ST335–338,369–382A as a template. The PCR product was digested with *Dra*II and *Sly*I and ligated into the *Dra*II-*Sly*I site of hEP4Ct-ST335–338,369–382,428–443A, generating plasmid hEP4Ct-ST335–382,400–443A. The plasmid served as template to eliminate Ser/Thr cluster 389–392 using primer pair hEP4-4-f and hEP4-7-r, or to eliminate Ser/Thr residues in the region 448–484 using primer pair hEP4-7-f and hEP4-8-r. The two PCR products were fused by PCR using primer pair hEP4-4-f and hEP4-8-r, and the respective PCR product was digested with *Sma*I and *Xba*I and ligated into pBshEP4Ct-ST335–382,400–443A digested with *Sma*I and *Xba*I, generating plasmid pBs/hEP4Ct-ST335–484A. The cDNA fragment coding for the entire C-terminal domain lacking all Ser/Thr residues was digested with *Sna*BI and *Xba*I and the corresponding region of pcDNA3/hEP4-wt was substituted with the cDNA fragment, generating the expression plasmid pcDNA3/hEP4-ST335–484A-R.

hEP4-ST335–405A-R

Plasmid pcDNA3/hEP4-R-wt was digested with *Sna*BI and *Sly*I and the resultant fragment was substituted with the corresponding region of pcDNA3/hEP4-ST335–484A-R.

hEP4-ST428–484A-R

Plasmid pcDNA3/hEP4-R-wt was digested with *Sly*I and *Xba*I and the resultant fragment was substituted with the corresponding region of pcDNA3/hEP4-ST335–484A-R.

hEP4-ST335–382A-R

Plasmid pcDNA3/hEP4-R-wt was digested with *Sna*BI and *Sma*I and the resultant fragment was substituted with the corresponding region of pcDNA3/hEP4-ST335–484A-R.

hEP4-ST335–354,389–484A-R

Plasmid pcDNA3/hEP4-ST335–484A-R was digested with *Dra*II and *Sma*I and the resultant fragment was substituted with the corresponding region of pcDNA3/hEP4-R-wt.

hEP4-ST389–392A-R/hEP4-ST335–382,394–484A-R

For mutation or re-introduction of Ser/Thr residues 389–392, a 1200 bp 5' fragment was generated with primer FLAG-hEP4-f as forward primer and hEP4-9-r or hEP4-10-r as a reverse primer containing the mutation (pcDNA3/hEP4-R-wt or pcDNA3/hEP4-ST335–484A-R as a template) (Table 1). 3' Fragments of 300 bp were produced with primers hEP4-9-f or hEP4-10-f and hEP4-Xba-r (Table 1). The PCR products were fused by PCR, with both overlapping fragments as template and the primer pair FLAG-hEP4-f and hEP4-Xba-r. The PCR products were digested with *Sna*BI and *Xba*I and then ligated into pcDNA3/hEP4-R-wt or pcDNA3/hEP4-ST335–484A-R, which were digested with the same enzymes, generating plasmids pcDNA3/hEP4-ST389–392A-R and pcDNA3/hEP4-ST335-382,394-484A-R respectively.

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