Human prostaglandin EP3 receptor isoforms show different agonist-induced internalization patterns

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Abstract The human prostaglandin EP3 receptor comprises eight isoforms that differ in carboxyl-tail. We show here that the isoforms are trafficked differently. When expressed in HEK293 cells, the isoforms located to the cell surface, although a fraction of some remained in the cell. Upon prostaglandin E_2 stimulation, EP3.I internalized almost completely, EP3.II, EP3.V, EP3.VI and EP3.f internalized to a lesser extent and EP3.III and EP3.IV did not internalize. Both EP3.I and EP3.f internalized with β-arrestin and internalization were blocked by a dominant negative form of Eps15, a clathrin-associated protein. Although EP3.II internalized, β-arrestin did not translocate with the receptor and internalization was not blocked by mutant Eps15. EP3.V and EP3.VI internalized to discrete areas of the cell with β-arrestin.

Keywords: Prostaglandin E_2; EP3 receptor isoforms; Endocytosis; Clathrin; Eps15

1. Introduction

The G protein-coupled receptor (GPCR) superfamily contains many receptor subtypes that are stimulated by the same endogenous ligand. The subtypes often couple to different G proteins and have different distribution patterns, allowing the same ligand to exert multiple effects. The GPCR superfamily is further complicated by the existence of isoforms of a particular subtype generated by alternative splicing. Splice variants for over 30 GPCRs have been discovered, including those for the thromboxane A2 [1], μ opioid [2], serotonin 5HT4 [3] and prostaglandin EP3 receptors [4], and a review of the literature indicates that splice variants occur mainly at the carboxyl-terminus.

In terms of molecular regulation, splice variants show differences in desensitization and internalization. For example, a splice variant of the turkey β1-adrenergic receptor, with an additional 59 amino acids, is resistant to internalization compared to its shorter counterpart [5]. The tachykinin NK1 receptor has a short variant that is resistant to internalization [6]. In the case of the metabotropic glutamate receptor, mGluR1, the b isoform undergoes rapid constitutive internalization [7]. Two C-tail splice variants of the thromboxane A_2 receptor (TP) display differences in trafficking [8]. TPb, the longer isoform, undergoes arrestin-dependent agonist-induced sequestration, while its shorter counterpart, TPa, does not, suggesting that alternative splicing of the receptor is important for regulation of arrestin binding and internalization. Although some splice variants have increased numbers of serine and threonine phosphorylation sites in their cytoplasmic tails, this seems to have only modest effects on desensitization and internalization of the receptors [9].

Prostaglandin E_2 (PGE_2) exerts its actions through four distinct GPCR subtypes, designated EP1, EP2, EP3 and EP4. The EP1 receptor couples to phospholipase C, the EP2 and EP4 receptors couple to stimulation of adenylyl cyclase, and the EP3 receptor couples to inhibition of adenylyl cyclase. The human EP3 receptor exists as eight isoforms that differ in carboxyl-tail region [4,10,11]. The isoforms may serve different functions and contribute to the diversity of the actions of PGE_2. The isoforms are identical over the first 359 amino acids up to the end of the seventh transmembrane helix, but differ in carboxyl-tail, which ranges in length from 6 to 66 amino acids.

1.1. Carboxyl-terminal domains of isoforms of the human EP3 receptor

EP3.I	IRYHTNNYASSSTSLPCQCSSTLMWSDH-LER
EP3.II	VANAVSSCSNDGQKGQPISLSNEIIQTEA
EP3.III	EEFWGN
EP3.IV	MRKRRLREQEEFWGN
EP3.V	MRKRRLREQEMGPDGRCFCHAWRQVP-RTWCSSHDREPCSVQLS
EP3.VI	EMGPDGRCFCHAWRQVPRTWCSSHDRE-PCSVQLS
EP3.e	MRKRRLREQLICSLRTLRYRGQLHIVGK-YKPIVC
EP3.f	MRKRRLREQAPLLPTPTVIDPSRFCAQPF-RWFLDLSFPAMSSSHPQLPLTLASFKLLR-EPCSVQLS

When expressed in a variety of cells lines, the isoforms display similar affinity for PGE_2 but differ in coupling to G proteins and in other properties including constitutive activity [12–14].

In this study, we examined agonist-induced internalization of the isoforms. Our results indicate that the C-tail of the

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isoforms is important in determining their degree of PGE_2-induced internalization.

2. Materials and methods

2.1. Materials

HA monoclonal antibody (HA 11) was from Babco (Richmond, CA, USA). Alexa 594 goat anti-mouse antibody was from Molecular Probes (Eugene, OR, USA). HEK293 cells and Zeocin were from Invitrogen (Carlsbad, CA, USA). DMEM, Lipofectamine 2000, and geneticin were from Gibco BRL (Grand Island, NY, USA). PGE_2 was from Biomol (Plymouth Meeting, PA, USA). The cDNA for GFP-arrestin 2 was provided by Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA, USA). The cDNA for GFP-tagged dominant negative Eps15 (EΔ95/295) was provided by Dr. Alexandre Benmerah (Institut Cochin, Paris, France).

2.2. Cell-lines expressing the EP3 receptor isoforms

cDNAs for EP3 receptor isoforms I, II, III, IV, V, VI, e and f were prepared by PCR from human uterus cDNA. Each isoform was epitope-tagged with a hemagglutinin (HA) tag. A common forward primer incorporating an *Eco*RI restriction site and the HA tag, encoding the amino acid sequence YPYDVPDYA following the N-terminal methionine, was used to amplify each isoform. The sequence of the forward primer was ATG GAA TTC AAC ATG *TAC CCA TAC GAC GTC CCA GAC TAC* GCT AAG GAG ACC CGG GGC TAC GGA, with the HA tag indicated in italics. The reverse primer for EP3.I was AGT AAG CTT TCA TCT TTC CAA ATG GTC GCT CCA. The reverse primer for EP3.II was AGT AAG CTT TCA TCA TGC TTC TGT CTG TAT TAT TTC ATT. The reverse primer for EP3.III was AGT AAG CTT TTA ATT TCC CCA AAA TTC CTC CTG. The reverse primer for EP3.IV was TTA AAG CTT TTA ATT TCC CCA AAA TTC CTC TTG CTC. The reverse primer for EP3.V, VI, and f was TCA AAG CTT TCA GCT TAG CTG GAC ACT GCA GGG TTC. The products were ligated into the expression vector pcDNA3.1zeo(−). Isoforms were sequenced and found to be identical to those published [4,11], except for isoform e, which showed a single base change coding for a different carboxyl-tail from that published. Our sequence is consistent with that from the human genome sequence so that the original sequence may be in error. Because of the difference in sequence, we did not study isoform e.

The isoforms were expressed in HEK293 cells cultured in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum. Cells were transfected using Lipofectamine 2000 in a 6-well dish, according to the manufacturer's instructions. Stable cells were selected with zeocin and single clones obtained by serial dilution.

The HA-tagged isoforms were also transiently expressed in HEK293 cells, together with GFP-labeled dominant negative mutant form of Eps15, a clathrin binding protein previously shown to inhibit clathrin-dependent endocytosis [15].

2.3. Immunoblot analysis

HEK293 cells stably expressing HA-tagged EP3 isoforms I, II, III, IV, V, VI, and f were grown to75% confluence and lysed with SDS sample buffer containing 50 mM dithiothreitol. HEK293 cells transfected with vector alone (mock transfected) were treated similarly. Each sample was boiled for 5 min, centrifuged, and resolved by SDS–PAGE. Each lane contained 30 μg of protein. Protein from the gels were transferred to nitrocellulose membrane. Membranes was blocked with 5% milk in TBST, probed with the HA.11 monoclonal antibody at a 1:2000 dilution overnight at 4 °C. After washing, the membranes were incubated with an IgG goat anti-rabbit HRP-linked secondary antibody (1:5000) for 1 h at room temperature. Blots were visualized using enhanced chemiluminescence by use of Super Signal (Pierce, Rockford, IL) and developed on X-ray film.

2.4. Microscopy

HEK293 cells expressing HA-tagged EP3 isoforms were grown to 50% confluence in 6-well plates on poly-L-lysine coated coverslips. Cells were treated with anti-HA monoclonal antibody at a 1:500 dilution for 60 min at 25 °C in DMEM with 1% BSA. Cells were washed and treated with or without PGE_2 for 60 min. Cells were fixed with 2% paraformaldehyde (PFA), permeabilized with 0.01% Triton X-100 and blocked with 5% non-fat dried milk in PBS at 37 °C for 30 min. Cells were incubated with goat anti-mouse Alexa-594-conjugated antibody at a 1:150 dilution at 37 °C for 30 min. Cells were washed and the coverslips mounted on slides. Images were obtained using a Leica TCS-NT confocal microscope.

For experiments using β-arrestin 2, HEK293 cells expressing the EP3 receptor were grown to confluence in 6-well plates on glass coverslips. The cells were transfected with 0.25 μg of GFP-arrestin 2 using Lipofectamine 2000. For experiments using EΔ95/295, HEK293 cells were transiently transfected with 0.25 μg of GFP-EΔ95/295 and 4 μg of HA-tagged EP3 receptor cDNA. Studies with GFP-EΔ95/295 were performed with a Nikon Eclipse TE300 fluorescence microscope.

2.5. ELISA assay

HEK293 cells stably expressing EP3 isoforms were grown to confluence in 12-well plates. Cells were treated with 1 μM PGE_2 or vehicle at 37 °C for various times. Reactions were stopped with ice cold 2% PFA. Cells were washed, blocked with 1% BSA in PBS for 45 min and incubated with anti-HA.11 monoclonal antibody at a 1:1000 dilution for 30 min. Cells were blocked again for 15 min and incubated with horseradish peroxidase-conjugated antibody at a 1:1000 dilution for 30 min. Cells were washed and exposed to substrate *o*-phenylenediamine and hydrogen peroxide, to allow color development, and the reaction terminated using sulfuric acid. Absorbance was measured using a Perkin–Elmer microplate reader at 450 nm.

3. Results

3.1. Immunoblotting of EP3 isoform transfected cells

The specificity of the HA-antibody with regard to the HA-tagged isoforms was demonstrated by comparing immunoblots of solubilized membranes from EP3 transfected HEK293 cells with a blot from cells transfected with empty vector (mock transfected). Samples were loaded into the gel to give 15 μg of protein in each well. Fig. 1 shows that, whereas cells transfected with the isoforms gave a band close to the predicted molecular mass of the receptors (47 kDa), no band was observed with mock transfected cells. Further, the blot demonstrates that each of the isoforms is expressed at a similar level, because the intensity of each band is similar.

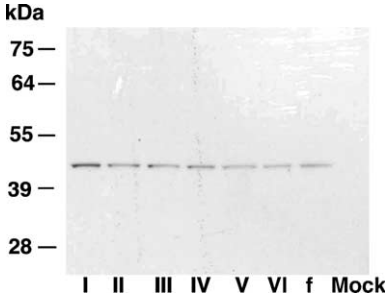

Fig. 1. Western blot showing the specificity of the HA-antibody for the EP3 HA-constructs. HEK293 cells stably expressing HA-tagged EP3 isoforms I, II, III, IV, V, VI, and f were grown to 75% confluence and lysed with SDS sample buffer. HEK293 cells transfected with vector alone (mock transfected) were treated similarly. 30 μg samples were resolved by SDS–PAGE and transferred to nitrocellulose membrane. Membranes were probed with the HA.11 monoclonal antibody followed by goat anti-mouse HRP-conjugated secondary antibody. Antibody labeling was detected by chemiluminescence. Each isoform was detected at around 47 kDa, the calculated molecular weight of the isoforms. Molecular mass is indicated on the left.

3.2. Confocal imaging of labeled isoforms and β-arrestin

Several different modes of agonist-induced internalization are utilized by GPCRs. A common pathway, mediated by β-arrestin, involves endocytosis into clathrin-coated pits. To determine the mechanisms involved in internalization of EP3 isoforms, HEK293 cells expressing the isoforms were co-transfected with β-arrestin 2 fused with GFP. Images obtained with each isoform are presented in Fig. 2. Under basal conditions (in the absence of PGE_2), EP3.I was localized to the cell surface and β-arrestin was dispersed throughout the cytoplasm. In the absence of agonist, all of the other isoforms were primarily located on the cell surface. Of these, isoforms III, IV, and f are located just below the cellular surface, whereas isoform V is more centrally located in the intracellular compartment. The specificity of the anti-HA antibody in immunofluorescence studies was apparent from the observation that mock transfected cells showed no fluorescence following treatment (not shown). In other experiments, we fixed the cells prior to labeling with the anti-HA antibody. No difference was seen in the distribution of receptors under basal conditions, indicating that the antibody itself does not cause endocytosis (not shown).

When stimulated with PGE_2, the EP3.I isoform translocated to large, punctate vesicles below the cell surface and β-arrestin migrated into the same vesicles. Isoforms II, V, VI, and f internalized to a lesser extent than EP3.I, whereas isoforms III and IV did not internalize.

The distribution pattern of β-arrestin varied with each isoform and in each case was distinct from that observed with EP3.I. Under basal conditions, EP3.II was present along the cellular surface and the β-arrestin was dispersed throughout the cytoplasm. When treated with PGE_2, some of the receptor internalized; however, the β-arrestin did not translocate with the receptor. The experiments were performed four times with different clones of EP3.II. In each experiment, β-arrestin consistently failed to colocalize with the receptor.

When the same experiment was performed on isoforms III and IV, receptors that do not internalize, β-arrestin remained dispersed throughout the cytoplasm.

Isoforms V and VI yielded similar results when co-expressed with β-arrestin 2, probably due to their similar C-tail composition. Under basal conditions, both receptors were primarily located on the cell surface, with a small amount of EP3.V in the intracellular compartment. β-Arrestin was distributed throughout the cytoplasm, except for an overlap with internalized EP3.V. When stimulated with PGE_2, both isoforms partially internalized to discrete areas of the cells and β-arrestin translocated with the internalized receptors.

Isoform f behaved mostly like isoform I. Under basal conditions, the receptor was located on the cell surface and β-arrestin was evenly distributed throughout the cytoplasm. Upon agonist challenge, the two co-localized into endosomes.

3.3. ELISA analysis of internalization

To confirm differences in the extent of internalization, we used ELISA. We studied isoforms I, II and III because they were representative of receptors that internalize robustly (isoform I), receptors that internalize to a lesser degree (isoforms II, V, VI, and f), and receptors that fail to internalize at all (isoforms III and IV). Cell-lines expressing each of the isoforms were challenged with PGE_2 for 0–60 min and the amount of remaining surface HA antigen was determined.

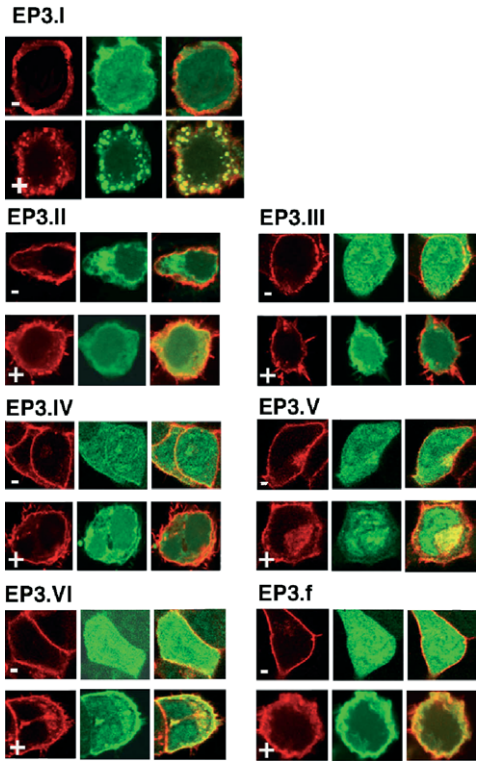

Fig. 2. Localization and trafficking of isoform, EP3.I. EP3.II, III, IV, V, VI and f. HEK293 cells stably expressing HA-tagged EP3 receptor isoforms were co-transfected with 0.25 μg GFP-arrestin. 48 h post-transfection, cells were treated with vehicle (−) or 1 μM PGE_2 (+) and fixed. The cells were then processed for simultaneous detection of surface and internalized receptors and GFP-arrestin. For each isoform, the left column shows the localization of each isoform, the middle columns show the distribution of GFP-arrestin, and the right column shows the superimposed image. Regions of overlap appear yellow. Magnification 400×. Results were representatives of four separate experiments.

EP3.I underwent rapid internalization to the extent of greater than 60% (Fig. 3). These data correlate with the images of EP.I obtained by confocal microscopy. EP3.II internalized to a lesser degree and EP3.III did not internalize in ELISA analysis, confirming prior confocal results.

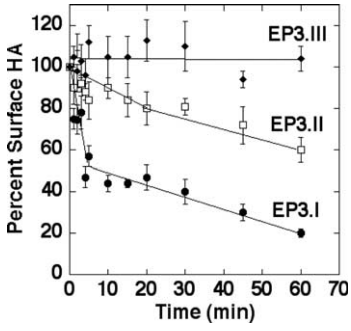

Fig. 3. Agonist-induced internalization of EP3 receptor isoforms I, II, and III. Cells expressing EP3.I, EP3.II or EP3.III were stimulated with 1 μM PGE_2 for the indicated time periods. The amount of surface HA immunoreactivity for each sample was measured by ELISA analysis. Values are representatives of means ± S.E. of at least six independent experiments, each done in triplicate. The amount of surface receptor is expressed as a percentage of the sample not treated with agonist.

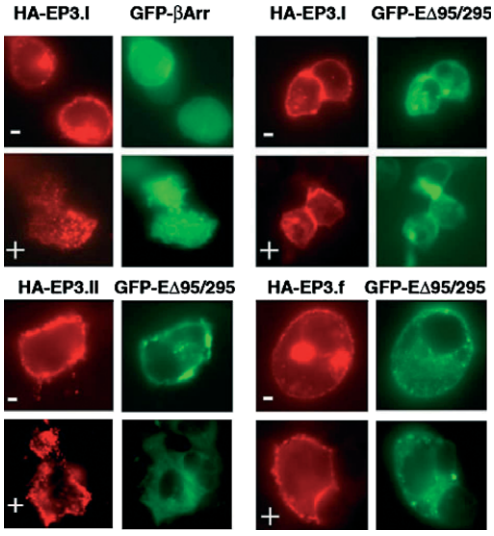

Fig. 4. Effect of dominant negative Eps15 mutant GFP-EΔ95/295 on endocytosis of isoforms EP3.I, EP3.II and EP3.f. HEK293 cells were transiently transfected with HA-tagged EP3 receptor isoforms together with GFP-ED95/295 cDNA. 48 h post-transfection, cells were treated with vehicle (−) or 1 μM PGE_2 (+) and fixed. The cells were then processed for simultaneous detection of surface and internalized receptors. As a control, the upper left group of images shows internalization of EP3.I in the presence of GFP-arrestin. The upper right group of images shows that GFP-ED95/295 prevents internalization of HA-EP3.I. The lower left series of images show that GFP-ED95/295 has no effect on EP3.II internalization. The lower right series of images show that GFP-ED95/295 inhibits internalization of EP3.f. Images were obtained using a Nikon Eclipse TE300 fluorescence microscope. Magnification 400×. The experiments were repeated three times with similar results.

3.4. Effect of Eps15 mutant EΔ95/295 on internalization

To further examine the mechanism of internalization, we co-transfected HEK 293 cells with EP3.I and a dominant negative mutant of Eps15. Eps15 (EGFR pathway substrate clone 15) is a protein component of plasma membrane clathrin-coated pits that is associated with AP-2 [15]. Overexpression of dominant negative mutants of Eps15 has been shown to inhibit clathrin-dependent endocytosis [15].

Fig. 4 compares the distribution of HA-EP3.I and GFP-arrestin with dominant negative Eps15 mutant GFP-EΔ95/295 in transfected cells in the absence or presence of PGE_2. Micrographs presented in the upper left panels show that EP3.I internalizes in the presence of PGE_2 and co-localizes with β-arrestin as described above. In contrast, EΔ95/295 prevented PGE_2-mediated internalization of EP3.I, indicating that EP3.I endocytosis involves clathrin-coated pits. Fig. 4 also shows that internalization of HA-EP3.II is not affected by GFP-EΔ95/295 (lower left series), whereas internalization of EP3.f is abolished by GFP-EΔ95/295 (lower right series). The results correlate with the observation in Fig. 2 that EP3.I and EP3.f internalize in a β-arrestin-dependent manner, whereas EP3.II internalizes independently of β-arrestin.

4. Discussion

The different C-tails of the EP3 receptor isoforms appear to be involved in controlling receptor localization and trafficking. Our findings are consistent with studies of other GPCRs. Membrane-targeting studies on the three mouse EP3 receptor isoforms show that they differ in subcellular localization depending on their C-tails [14]. In fact, a truncated form of the mouse EP3 isoforms was retained in the intracellular compartment, suggesting that it is the C-tail that directs targeting of the receptors to the cell surface. Other studies have also implicated the C-tail of GPCRs in receptor localization. The α1 adrenergic receptor subtypes A, B, and D all showed differences in cellular localization. Hence, subtype 1B was located on the plasma membrane, subtype 1A was present both on the plasma membrane and in the intracellular compartment, and subtype 1D exhibited a perinuclear orientation [16].

All human EP3 isoforms were located primarily on the cell surface under non-stimulated conditions. Isoforms III, IV, V, and f were also present in the intracellular compartment, which may be related to the fact that some of the isoforms are constitutively active [12]. The α1D-adrenergic subtype is thought to be localized intracellularly because it is constitutively active and, therefore, constitutively internalized [12].

Upon PGE_2 stimulation, EP3.I internalized rapidly and robustly into punctate vesicles. Isoforms II, V, VI, and f also internalized but to a lesser extent. Their internalization was unusual in two ways. First, a large amount of receptor was retained on the cell surface. Second, the internalized receptor appeared to migrate laterally to one quadrant of the cells. It may be that these isoforms are being downregulated and are entering lysosomes or proteosomes.

Isoforms III and IV, the shortest isoforms, do not internalize in response to agonist. Moreover, isoforms III and IV contain no serine or threonine residues, which have been implicated in internalization of other GPCRs including the m2 muscarininc acetylcholine receptor [17], the chemokine CXCR4 receptor [18] and the bradykinin B2 receptor [19].

Internalization frequently depends on the nature of the carboxyl-tail and its relation to adaptor proteins such as β-arrestin [20]. In general, carboxyl-tails with a significant number of serines and threonines may be phosphorylated, allowing the binding of β-arrestin. β-Arrestin is crucial in the regulation of GPCR trafficking and would be expected to co-localize with an internalized receptor such that the two are associated with a clathrin-coated pit.

Co-expression of EP3.I with β-arrestin showed that the internalized receptor and arrestin co-localize into the same endosomes, indicating that this isoform is likely internalized via a mechanism involving clathrin-coated pits. This conclusion is confirmed by the lack of internalization of EP3.I in the presence of the dominant negative mutant form of the clathrin associated protein Eps15, EΔ95/295, which has been shown to inhibit clathrin-coated pit assembly. Isoform EP3.f also appears to internalize by a β-arrestin, clathrin-dependent mechanism. By contrast, internalization of isoform EP3.II proceeds independently of β-arrestin and is not affected by EΔ95/295, indicating that EP3.II may internalize by an alternate mechanism. Isoforms V and VI both partially internalized to discrete areas of the cells when stimulated with PGE_2. However, only a small fraction of β-arrestin co-migrated with the internalized receptors. Upon stimulation with agonist, neither isoform III nor IV internalized and β-arrestin remained throughout the cell.

In summary, the isoforms of the human prostaglandin EP3 receptor show different degrees of internalization in response to PGE_2. In addition, the isoforms appear to internalize by dif-

ferent mechanisms from one another. The findings may indicate differences in physiological function among the isoforms, with some responding rapidly to changes in PGE_2 concentration and others involved in prolonged responses to PGE_2.

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