Cloning of a Carboxyl-terminal Isoform of the Prostanoid FP Receptor

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An FP prostanoid receptor isoform, which appears to arise from alternative mRNA splicing, has been cloned from a mid-cycle ovine large cell corpus luteum library. The isoform, named the FPβ receptor, is identical to the original isoform, the FPα, throughout the seven transmembrane domains, but diverges nine amino acids into the carboxyl terminus. In contrast to FPα, whose carboxyl terminus continues for another 46 amino acids beyond the nine shared residues, the FPβ terminates after only one amino acid. The FPα isoform appears to arise by the failure to utilize a potential splice site, while a 3.2-kilobase pair intron is spliced out from the FP gene to generate the FPβ isoform mRNA. The two isoforms have indistinguishable radioligand binding properties, but seem to differ in functional coupling to phosphatidylinositol hydrolysis. Thus, in COS-7 cells transiently transfected with either the FPα or the FPβ receptor cDNAs, prostaglandin F2α (PGF2α) stimulates inositol phosphate (IP) accumulation to the same absolute maximum, but the basal level of inositol phosphate accumulation is approximately 1.3-fold higher in cells transfected with the FPβ as compared with cells transfected with the FPα isoform. Using the polymerase chain reaction, mRNA encoding the FPβ isoform was identified in the ovine corpus luteum.

Prostanoids, autacoids formed from arachidonic acid by the actions of cyclooxygenases, exert diverse physiological effects throughout the body. For example, in sheep and in many other species, prostaglandin F2α (PGF2α) is the trigger that initiates luteolysis or regression of the corpus luteum in the absence of pregnancy (1). There are five primary prostanoids, prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), PGF2α, prostaglandin I2 (PGI2), and thromboxane A2 (TXA2). Unique G-protein-coupled receptors have been cloned for each prostanoid, including four receptors specific for PGE2 (EP1–EP4) (2). The protein-coupled receptors have been cloned for each prostanoid, with carboxyl-terminal splice variants. The isolated cloned receptors are named DP, FP, IP, and TP and bind PGD2, PGF2α, PGL2, and thromboxane A2, respectively. In addition, alternative mRNA splice variants, have been cloned for the EP3 receptor (3, 4) and for the TP receptor (5, 6). In each case, the splice variants are identical throughout the seven-transmembrane domains, but diverge approximately 9–12 amino acids into the carboxyl terminus. The significance of this carboxyl-terminal alternative splicing is not well understood, but for the EP3 receptor, differences among the isoforms have been found in localization (7), receptor/G-protein coupling (3, 8–10), and desensitization (11).

Phylogenetic analysis of the prostanoid receptors shows that the receptors segregate into two branches (12, 13). One branch contains the DP, IP, EP3, and EP4 receptors. The second branch contains the EP1, EP2, FP, and TP receptors. Since isoforms have been found for two of the four receptors in the second branch, the EP3 and TP receptors, we hypothesized that isoforms might also exist for the FP receptor.

To examine this possibility, we screened an ovine mid-cycle large cell corpus luteum library. Previously, we used this library to clone the ovine homologue (14) of the human FP receptor (15) and found that the message for the FP receptor was highly abundant (~0.1% of the total message). Using a combination of homology-based screening with the ovine FP receptor as a probe and PCR, we cloned a novel isoform of the FP receptor. This isoform, the FPβ, is identical to the original (termed here the FPα isoform) throughout the seven-transmembrane domains, but diverges nine amino acids into the carboxyl terminus. Functionally, both isoforms are able to stimulate inositol phosphate (IP) accumulation to the same maximum, but the basal level of hydrolysis is 130% higher for the novel FPβ isoform than for the original FPα isoform.

EXPERIMENTAL PROCEDURES

cDNA Cloning—A cDNA containing the complete coding sequence of the ovine FPα receptor (14) was labeled with 32P by nick translation (Life Technologies, Inc.) and an ovine large cell corpus luteum library screened as described previously (14). The library was plated at a density of ~3400 plaques/plate (15 cm), and five plates were screened. From a total of 24 positives, 18 were isolated and each was placed in 250 µL of M13.

To differentiate possible carboxyl-terminal splice variants from the original FPα isoform, two rounds of PCR were used. In the first round, sense (nt 258–275) and antisense (nt 638–654) primers upstream of the putative sixth transmembrane domain were used. These primers were predicted to be specific for the common region of all FP isoforms. In the second round of PCR, a sense primer (nt 793–810) upstream of the sixth transmembrane domain and an antisense primer (nt 1168–1185) specific for the carboxyl terminus were used that could amplify the original FPα isoform but would be unlikely to yield products (at least of the predicted size) with carboxyl-terminal splice variants. The isolated positive plaques were vortexed for 15 s and lysed by three freeze/thaw cycles consisting of freezing in a dry ice/ethanol bath and thawing at 55 °C for 5 min. For each 50 µL PCR, 26 µL of the phage lysate was added to a 50 µL reaction containing final concentrations of 1 × PCR buffer I (Perkin-Elmer), 10% dimethyl sulfoxide (Sigma), 200 µM dNTPs (Perkin-Elmer), and 1 µM each primer. The samples were heated to 95 °C for
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RESULTS

We screened an ovine mid-cycle large cell luteal library with the previously cloned ovine FP receptor (14) and used PCR to identify possible carboxyl-terminal alternative mRNA splice variants. From a total of 17,000 plaques, one putative alternative splice variant was identified. We have designated this clone FPB, in contrast to the original isoform which we now define as FPA. Fig. 1 shows the cDNA and deduced amino acid sequences of the cloned FPA and FPB receptor isoforms. The sequences of the putative transmembrane domains are double underlined, and potential sites for N-linked glycosylation are in bold (amino acids 4 and 19). The unique cDNA and deduced amino acid sequences of the isoforms are separated with the putative amino acid sequences in bold. The HindIII site (nt. 638) used to construct the full-length FPB isoform and for restriction enzyme analysis is indicated with single underlining.

To determine how the two FP receptor isoforms arose, PCR was performed on sheep genomic DNA using primers that spanned the proposed splice site. The same sense primer (nt 1078–1104) was used for both the FPA and FPB receptor isoforms. The FPB-specific primer, however, yielded a 3200-bp product. This product was used to construct the full-length FPB isoform and for restriction enzyme analysis is indicated with single underlining.

The FPB receptor, the antisense primer corresponding to nt 1291–1317, and the small fragment containing the 3' end of FPB from the FPB digestion were isolated using GeneClean II (BIO 101, Inc.), ligated, subcloned, and KS+ isolated. To make an expression vector, KS+FPB/fucod was restricted with Nael, which cuts at nt 80, and Nco1, which cuts at nt 1213, filled in using Klenow and subcloned into the blunt-ended BamHI/HindIII sites of pBluescript (Stratagene) and sequenced. One clone (KS+FPB/fulcod) was identified. We have designated this clone FPB, in contrast to the original isoform which we now define as FPA. Fig. 1 shows the cDNA and deduced amino acid sequences of the cloned FPA and FPB receptor isoforms. The sequences of the putative transmembrane domains are double underlined, and potential sites for N-linked glycosylation are in bold (amino acids 4 and 19). The unique cDNA and deduced amino acid sequences of the isoforms are separated with the putative amino acid sequences in bold. The HindIII site (nt. 638) used to construct the full-length FPB isoform and for restriction enzyme analysis is indicated with single underlining.

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To determine how the two FP receptor isoforms arose, PCR was performed on sheep genomic DNA using primers that spanned the proposed splice site. The same sense primer (nt 1078–1104) was used in both reactions in combination with an antisense primer specific to each isoform (FPA, nt 1291–1317; FPB, nt 1078–1104). A picture of the agarose gel on which the reactions were electrophoresed is shown in Fig. 2. Lane 1 shows the size of the product obtained with the FPA-specific primer, and lane 2 shows the size of the product obtained with the FPB-specific primer. Thus, amplification with the FPA-specific primer yielded a product of ~300 bp, which is identical to the size predicted from the cDNA. Amplification with the FPB-specific primer, however, yielded a 3200-bp product. This product is much larger than the 86-bp product that would be expected from cDNA.
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To verify that the message for the FPB was expressed in the ovine corpus luteum, RT-PCR was performed on mRNA obtained from a mid-cycle corpus luteum using primers flanking the stop codon. Fig. 3 shows the products obtained from an RT-PCR reaction using a common sense primer and an antisense primer specific for the FPB isoform. Lane 1 shows the products obtained from PCR using a common sense primer with an antisense primer specific for the FPB isoform. The size of the products obtained is indicated to the right of the gel.

To examine the pharmacology of the FPB clone, radioligand binding was performed using membranes from COS-7 cells that were transiently transfected with pBcFPB/fulcod. Fig. 4 shows radioligand binding competition curves using 17-[3H]phenyl-trinor-PGF2α as the radioligand. The ability of a series of natural and synthetic prostanoids to displace 17-[3H]phenyl-trinor-PGF2α, binding was measured, and the rank order of potencies of the competitors was as follows: 17-phenyl-trinor-PGF2α, fluprostenol, 8-epi-PGF2α. Thus, the synthetic prostanoid, 17-phenyl-trinor-PGF2α, is the most potent with an EC50 of ~10 nM, while PGF2α is the most potent natural prostanoid, with an EC50 of ~40 nM, although both PGD2 and PGE2 were also able to displace 17-[3H]phenyl-trinor-PGF2α binding. These results are nearly indistinguishable from those obtained for the FPα under similar conditions.

Since the FP receptor cloned from other species activates PI hydrolysis (17, 18), we wanted to determine whether the ovine FPα and FPB isoforms could also stimulate IP accumulation and whether there were any differences between the isoforms. Fig. 5 shows the results of experiments performed on COS-7 cells that were transiently transfected with the FPα (14) or the FPB and treated with different concentrations of PGF2α. IP accumulation was normalized to the basal level of IP accumulation of FPα expressing cells. These data are the mean ± S.E. of three separate experiments performed in duplicate.

FIG. 2. Photograph of an agarose gel after electrophoresis of PCR on sheep genomic DNA using primers specific for the FPα and FPB receptor isoforms. The sizes of the λ phage/HindIII and φX174/HaeIII molecular weight standards electrophoresed in the lane S are indicated to the left of the gel. Lane 1 shows the products obtained from PCR using a common sense primer and an antisense primer specific for the FPα isoform. Lane 2 shows the products obtained from PCR using the same sense primer with an antisense primer specific for the FPB isoform. The size of the products obtained is indicated to the right of the gel.

FIG. 3. Photograph of an ethidium-stained agarose gel after electrophoresis of a PCR using primers specific to the FPB receptor on cDNA obtained from corpus luteum. The standards are in lane S and the size (kilobase pairs) indicated to the left of the gel. Lane 1 shows the products obtained from an RT-PCR reaction using mid-cycle ovine corpus luteum mRNA as the template, lane 2 shows the positive control using KS+/FPB as the template, and lane 3 shows the negative control without added template. The primer pair used (see “Experimental Procedures”) was expected to amplify a product of 1010 bp, as indicated under "Results.”

FIG. 4. Radioligand binding competition studies for the binding of 17-[3H]phenyl-trinor-PGF2α. Data are normalized to the basal level of IP accumulation of FPα expressing cells. These data are the mean ± S.E. of three separate experiments performed in duplicate.

FIG. 5. PGF2α stimulated IP accumulation in cells transiently expressing either the FPα (○) or FPB (●). Data are normalized to the basal level of IP accumulation of FPα expressing cells. The experiment has been repeated with similar results both in COS-7 and COS-P cells.

Expected from amplification of the FPB cDNA.
PGF$_{2a}$ was able to elicit the same maximal level of IP accumulation, 220% over basal, and the EC$_{50}$ was similar for the two isoforms, 8 nM for the FPA and 11 nM for the FPB isoform. However, the basal level of IP accumulation was ~130% higher in cells expressing the FPB isoform than in cells expressing the FPA, which in turn was ~140% higher than the basal level of hydrolysis in untransfected cells (data not shown). Based on radioligand binding competition studies, the level of receptor expression was higher for cells expressing the FPA receptor than for cells expressing the FPB receptor. In addition, similar experiments were repeated in COS-P cells, with essentially the same findings.

**DISCUSSION**

Previously, we and others reported the cloning of the FP prostanoioid receptor from bovine (17), human (15), mouse (18), and ovine (14). Here we report the cloning of the FPB, an isoform of the ovine FP receptor. Isoforms have been reported for the EP$_3$ and TP prostanooid receptors, but this is the first report of isoforms of the FP receptor. Like the EP$_3$ and TP receptor isoforms, the FP receptor isoforms diverge in the carboxyl termini. Thus far, two isoforms of the human TP receptor (6) have been cloned, while a total of 13 different isoforms of the EP$_3$ receptor have been cloned among bovine, human, rabbit, and mouse (2). The existence of FP receptor isoforms was hypothesized based on previously cloned EP$_3$ and TP receptor isoforms and relationships established by the phylogenetic analysis of the prostanooid receptors. This analysis places the DP, EF$_1$, EF$_2$, and IP receptors in one branch and the remaining EP$_1$, EP$_3$, FP$_1$, and TP receptors in the second. With the cloning of the FPB isoforms, there have been found for three of the four receptors in the second branch. Isoforms have not yet been reported for the EP$_1$ receptor, but the phylogenetic relationships suggest that they, too, may exist. Interestingly, the existence of isoforms also seems to correlate with second messenger coupling. Thus, the receptors in the second branch where isoforms have been reported are all primarily coupled to either PI hydrolysis or inhibition of adenylyl cyclase, while isoforms have not been obtained for the receptors in the first branch, which are all coupled to stimulation of adenylyl cyclase.

The TP and EP$_3$ receptor isoforms arise by alternative mRNA splicing at a location that is homologous to the site at which the FP isoforms diverge, suggesting that the FP receptor isoforms are also generated by alternative mRNA splicing. In the case of the TP receptor isoforms, the isoforms arise by the failure to utilize a potential splice site (6). Thus, the sequence of the carboxyl terminus of the endothelial variant is identical to the genomic sequence, while this sequence is spliced out to form the placentatic variant. It appears that the same mechanism generates the two FP receptor isoforms. Using the primers specific for the FPA receptor, the PCR product obtained from the genomic DNA is identical to the size of the product predicted from the cDNA sequence, indicating that no sequence is spliced out to generate this isoform. However, when primers specific for the FPB are utilized in a PCR on genomic DNA, the product obtained is much larger than predicted from the cDNA. Thus it appears that a ~3.2-kilobase pair intron is spliced out to generate the FPB receptor message. Consistent with this mechanism for the generation of these two isoforms, the highly conserved dinucleotide (GT) from the splice donor site is retained in the sequence of the FPB isoform (nt 1048–1049) (19).

The functional significance of the carboxyl-terminal isoforms has been most well characterized for the α, β, and γ isoforms of the mouse EP$_3$ receptor. Agonist binding among the isoforms is virtually indistinguishable (8), but differences among the isoforms have been found for receptor/G-protein coupling. Traditionally, the mouse EP$_3$ isoforms are coupled to inhibition of adenylyl cyclase, but some of the isoforms have been found to stimulate cAMP formation and/or activate PI hydrolysis (3, 9, 20). In addition, two isoforms, the EP$_3a$ and EP$_3b$, and a recombinant mutant that is truncated at the splice site have been shown to have agonist-independent constitutive activity (21). Thus, in the absence of agonist, the level of forskolin-stimulated cAMP accumulation is greater in cells expressing the EP$_{3b}$ isoform than in cells expressing either the EP$_{3a}$ or EP$_{3b}$ isoforms or the truncation mutant. However, maximal inhibition of forskolin-stimulated cAMP formation in the presence of agonist is the same for all three isoforms and for the truncation mutant. For the EP$_{3a}$ isoform, there is agonist-independent constitutive activity and agonist-dependent inhibition of cAMP formation, while for the truncation mutant, there is only agonist-independent inhibition of cAMP formation. Data obtained with the EP$_{3a}$ isoform suggest that there is agonist-independent stimulation of the G-protein, G$_a$, but agonist-dependent stimulation of the G-protein, G$_b$ (22).

Our data with the FP receptor isoforms indicate that the receptor/G-protein coupling for the FP receptor isoforms are analogous to the data found for the mouse EP$_{3a}$ and EP$_{3b}$ isoforms. There is agonist-independent constitutive IP accumulation in cells expressing either the FPA or FPB receptor isoforms, but this level is approximately 130% higher for cells expressing the truncated FPB isoform than for cells expressing the FPA receptor isoform. In addition, both the FPA and FPB isoforms, there is agonist-stimulated IP accumulation which reaches the same maximal level. This suggests that the FPB isoform is a naturally occurring truncated receptor that shows agonist-independent constitutive activity, but is still responsive to agonist. This is in contrast to the truncation mutant of the EP$_3$ receptor, which shows only agonist-independent constitutive activity and is unresponsive to agonist. The data with both the EP$_3$ and FP receptor isoforms are consistent with mutations made in other G-protein coupled receptors, including the avian β-adrenergic receptor, which suggests that sequences in the carboxyl-terminal tail may suppress G-protein interactions in the absence of ligand (23, 24).

More work will be needed to establish if additional biochemical differences exist between the FP receptor isoforms. Of particular interest are possible differences in desensitization, localization, and ability to activate other downstream effectors. The physiological significance of the FPB isoform, and of other prostanooid receptor alternative splice variants, is also intriguing. In the mid-cycle large cell corpus luteum library from which the FPB was cloned, the FPA is far more abundant than the FPB. This, however, may not be true throughout the luteal cycle and in other tissues that are also responsive to PGF$_{2a}$.

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