FP Prostanoid Receptor Activation of a T-cell Factor/β-Catenin Signaling Pathway*

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FP prostanoid receptors are G-protein-coupled receptors (GPCR) that consist of two known isoforms, FP_A and FP_B. These isoforms, which are generated by alternative mRNA splicing, are identical except for their carboxyl-terminal domains. Previously we have shown that stimulation of both isoforms with prostaglandin F₂α (PGF₂α) activates the small G-protein Rho, leading to morphological changes consisting of cell rounding and the formation of cell aggregates. Following the removal of PGF₂α, however, FP_A-expressing cells show rapid reversal of cell rounding, whereas FP_B-expressing cells do not. We now show that acute treatment of FP_A-expressing cells with PGF₂α, leads to a subcellular reorganization of β-catenin, a decrease in the phosphorylation of cytoplasmic β-catenin, and persistent stimulation of Tcf/Lef-mediated transcriptional activation. This does not occur in FP_A-expressing cells and may underlie the differences between these isoforms with respect to the reversal of cell rounding. The Tcf/β-catenin signaling pathway is known to mediate the actions of Wnt acting through the heptahelical receptor, Frizzled, and has not been associated previously with GPCR activation. Our findings expand the signaling possibilities for GPCRs and suggest novel roles for FP receptors in normal tissue development and malignant transformation.

The amino acid sequences of the ovine FP_A and FP_B prostanoid receptor isoforms are the same throughout their amino termini and seven-membrane-spanning domains, but the FP_B isoform is truncated and lacks the last 46 carboxyl-terminal amino acids present in the FP_A isoform (1). This is very similar to the EP₃ (2) and thromboxane A₂ (3) prostanoid receptors in which alternative mRNA splicing gives rise to a variety of isoforms in humans and in other species (4). The physiological significance of these receptor isoforms is not clear, although differences have been shown to exist with respect to second messenger coupling and receptor desensitization. We have found that the FP_A and FP_B receptor isoforms have similar pharmacological properties and that prostaglandin F₂α (PGF₂α) stimulates phosphoinositide turnover to a similar extent in cells expressing these isoforms (1). In addition, stimulation of FP_A or FP_B-expressing cells with PGF₂α, activates Rho leading to the formation of actin stress fibers, phosphorylation of p125 focal adhesion kinase, and cell rounding (5). Cell rounding involves the retraction of filopodia and a change from an isolated dendritic appearance to one in which the cells are rounded and form small cobblestone-like aggregates (see Fig. LA). Following the removal of PGF₂α, however, FP_A-expressing cells return to their original dendritic morphology, but the FP_B-expressing cells do not and remain rounded (6). We hypothesized that FP_A-expressing cells might remain rounded because of prolonged signaling following the removal of agonist. However, a specific mechanism of this prolonged signaling was not established. Here we show that Tcf/β-catenin-mediated transcriptional activation is elevated 16 h after an initial 1-h treatment of FP_A-expressing cells with PGF₂α. This transcriptional activation is not observed in FP_A-expressing cells and suggests that FP_B-expressing cells remain rounded because of activation of a Tcf/β-catenin signaling pathway.

EXPERIMENTAL PROCEDURES

Immunofluorescence Microscopy—HEK-293 cells stably expressing the ovine FP_A and FP_B prostanoid receptor isoforms (5) were split and grown in six-well plates containing 22-mm round glass coverslips for 3–4 days. Cells were treated with either vehicle (sodium carbonate, 0.002% final) or 1 μM PGF₂α, and were rapidly washed, fixed, and incubated with a 1:1000 dilution of a mouse monoclonal antibody to β-catenin (Transduction Laboratories). They were then washed and incubated with a 1:4000 dilution of an fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Sigma). Nuclei were visualized by phase contrast and epifluorescence microscopy as described previously (6).

Immunoprecipitation and Blotting—Cells were scraped and sonicated in a lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 2 mM EGTA, 2 mM phenylmethylsulfonylfluoride, 0.1 mg/ml leupeptin, and 2 mM sodium vanadate. Samples were centrifuged (16,000 × g) for 15 min at 4 °C, the supernatant (cytosolic fraction) was removed, and the pellet (particulate fraction) was solubilized with lysis buffer containing 0.2% Triton X-100 and then centrifuged again to remove insoluble debris. For immunoprecipitation, samples were rotated for 2 h at 4 °C with antibodies to β-catenin followed by the addition of protein G-Sepharose (Amersham Pharmacia Biotech) and rotation for another hour. The Sepharose was washed with lysis buffer and then resuspended with SDS-polyacrylamide gel electrophoresis sample buffer and boiled. Samples were electrophoresed on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with either antibodies to β-catenin or a mixture of mouse monoclonal antibodies to phosphoserine (Sigma) and phosphothreonine (Sigma). The membranes were washed, incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies, and visualized by enhanced chemiluminescence (SuperSignal, Pierce). The resulting films were scanned at high resolution (300 dpi) as positive transparencies (Microtek ScanMaker4) and saved as TIFF files. Quantitation was performed using the Gelplot2 macro in Scion Image for Windows (beta version 4.02). Nuclear extracts were prepared according to the method of Dignam as modified by Westin et al. (7).

RT-PCR—RT was done using the Superscript Preamplification System (Life Technologies, Inc.) and 1 μg of RNA/sample that had been

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1 The abbreviations used are: PGF₂α, prostaglandin F₂α; Tcf, T-cell factor; Lef, lymphoid enhancer factor; DAPI, 4',6-diamidino-2-phenylindole; APC, adenomatous polyposis coli; NSAID, nonsteroidal anti-inflammatory drug.
pretreated with DNase I. This was followed by PCR using an initial incubation at 94 °C for 5 min, followed by 20 cycles of 94 °C, 60 °C, and 68 °C each for 2 min, and a final incubation at 68 °C for 10 min. The human β-catenin and GAPDH primer pairs were exactly according to Rezvani and Liew (8). Product sizes were 521 base pairs for β-catenin and 737 base pairs for GAPDH and were resolved by electrophoresis on 1.5% agarose gels. Preliminary experiments were done to find the optimal conditions for quantitative amplification of β-catenin and GAPDH mRNA.

Tcf/Lef Reporter Gene Assay—Cells were split into 10-cm dishes and the next day were transiently transfected using FuGENE-6 (Roche Molecular Biochemicals) and either 10 μg/dish of the wildtype Tcf/Lef reporter plasmid TOPflash or the mutant plasmid FOPflash. FOPflash differs from TOPflash by the mutation of its Tcf binding sites and serves as a reporter plasmid TOPflash or the mutant plasmid FOPflash. FOPflash was prepared using the Luciferase Assay System (Promega). Luciferase activity in the extracts was measured using a Turner TD-20/20 luminometer and was corrected for background by subtraction of FOP-FLASH values from corresponding TOP-FLASH values.

RESULTS

Fig. 1A shows phase contrast microscopy of HEK cells stably expressing either the ovine FP A prostanoid receptor (panels a and b) or the ovine FP B prostanoid receptor (panels c and d) following a 1-h treatment with either vehicle (panels a and c) or 1 μM PGF 2α, (panels b and d). It can be appreciated that in both

![Image](https://example.com/image1)

**Fig. 1.** A, phase contrast microscopy (×225) of FP A and FP B-expressing cells after treatment with either vehicle (panels a and c) or 1 μM PGF 2α, (panels b and d) for 1 h at 37 °C. B, β-catenin immunofluorescence (green) and nuclear DAPI fluorescence (blue) microscopy (×225) of FP A and FP B cells after the same treatment. Cells were labeled and prepared for microscopy as described under “Experimental Procedures.” The results are representative of more than three experiments.

FP A- and FP B-expressing cells treatment with PGF 2α resulted in morphological changes consisting of a loss of filopodia and formation of cell aggregates. We have previously shown that these morphological changes involve the activation of Rho and phosphorylation of p125 focal adhesion kinase (5). However, following the removal of PGF 2α, the FP A-expressing cells show a rapid (within 1 h) reversal of these morphological changes, whereas the FP B-expressing cells remain rounded even after 48 h (6). To investigate the possible role of other adhesion proteins in this process, we used immunofluorescence microscopy to examine the localization of E-cadherin and β-catenin in HEK cells stably expressing either the FP A or FP B isoforms following treatment with 1 μM PGF 2α. Although the effects on E-cadherin localization were not apparent (data not shown), Fig. 1B shows that PGF 2α treatment resulted in a marked accumulation of β-catenin in regions of cell-to-cell contact in FP B-expressing cells (panels c and d) but not in FP A-expressing cells (panels a and b). Both cell lines, however, showed agonist-dependent cell rounding following treatment with PGF 2α, (Fig. 1A), indicating that the process of cell rounding itself was not responsible for the increased contiguous accumulation of β-catenin in the particulate fraction.

In addition to its role in cell adhesion, β-catenin is well recognized as a signaling molecule that undergoes stimulus-dependent translational from the cytosol to the nucleus where it is involved in the regulation of Tcf/Lef-mediated gene transcription (9–11). We, therefore, used immunoblotting to examine both particulate and cytosolic fractions for changes in β-catenin expression following treatment of FP A- and FP B-expressing cells with PGF 2α. Fig. 2A shows that the expression of β-catenin is ~3-fold higher in the particulate fraction and ~2-fold higher in the cytosolic fraction from FP B-expressing cells compared with FP A-expressing cells. Furthermore, treatment with PGF 2α caused a slight increase the levels of cytosolic β-catenin in both the FP A- and FP B-expressing cells but had little effect on the levels of β-catenin in the particulate fraction.
Reverse transcription (RT) followed by polymerase chain reaction (PCR) was used to determine whether there were any differences in β-catenin mRNA levels under these same experimental conditions. Fig. 2B shows that β-catenin and GAPDH mRNA levels were the same for both cell lines and were not affected by PGF2α, indicating that the observed differences in β-catenin expression appear to be the result of changes in translation and/or protein turnover.

Serine/threonine phosphorylation of β-catenin by glycogen synthase kinase-3β (GSK-3β) marks β-catenin for degradation and is a critical factor in the regulation of its signaling activity (12, 13). Thus, under most conditions cytosolic β-catenin is phosphorylated, leading to an association with the tumor suppressor protein, adenomatous polyposis coli (APC), and the scaffolding protein, axin, which is then followed by ubiquitination and proteasomal degradation (14). Using immunoprecipitation and immunoblotting, we examined serine/threonine phosphorylation of β-catenin following treatment of either FP A- or FP B-expressing cells with PGF2α. Fig. 3 shows that in FP A-expressing cells the vehicle control levels of cytosolic β-catenin are very low (lane a, middle panel) and there is little detectable phosphorylation (lane a, upper panel). Following treatment of FP A-expressing cells with PGF2α, the levels of cytosolic β-catenin increase 6-fold (lane b, middle panel) and there is a 19-fold increase in phosphorylation (lane b, upper panel). In FP B-expressing cells the vehicle control levels of cytosolic β-catenin are already higher than in the FP A-expressing cells (cf. lanes c and a, middle panel) and so is phosphorylation (cf. lanes c and a, upper panel). This would be expected to reflect endogenous GSK-3β activity and tight coupling to the elevated levels of cytoplasmic β-catenin. After treatment of FP B-expressing cells with PGF2α there is a further 2-fold increase in cytosolic β-catenin (lane d, middle panel) but an unexpected 12-fold decrease in phosphorylation (lane d, upper panel). The ratio of phosphorylated to total β-catenin in the cytoplasm, therefore, shows dramatic differences following activation of these two FP receptor isoforms. Thus, in FP A-expressing cells this phosphorylation ratio increases from 0.5 to 1.6 with agonist treatment, whereas in FP B-expressing cells it falls from 2.3 to 0.1. It would therefore be expected that degradation of cytosolic β-catenin would be favored at the expense of nuclear translocation in FP A-expressing cells, whereas the opposite would be true in FP B-expressing cells. This appears to be confirmed in Fig. 3 where immunoblotting of nuclear extracts shows a 3-fold higher level of β-catenin in FP B-expressing cells following treatment with PGF2α (lane d, bottom panel) as compared with FP A-expressing cells (lane b, bottom panel).

Following nuclear translocation, β-catenin is known to interact with members of the Tcf/Lef family of transcription factors (15). Because of this signaling potential, we were interested in the possibility that the failure of FP B-expressing cells to return to their original dendritic morphology following removal of PGF2α might represent a β-catenin-mediated switch in gene expression. To examine an effect on gene expression, we transiently transfected either FP A- or FP B-expressing cells with a Tcf/Lef-responsive reporter plasmid (16) and measured luciferase reporter gene activity following treatment with 1 μM PGF2α. Initially we found that basal levels of luciferase activity were elevated (~3-fold) in FP B-expressing cells as compared with FP A-expressing cells and that luciferase activity was not stimulated immediately following a 1-h treatment with PGF2α. 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erase activity (column d) that is roughly 6.5-fold higher than either the vehicle control (column c) or PGF$_{2\alpha}$-treated FP$_{A}$ cells (column b). The failure of PGF$_{2\alpha}$-expressing cells to show reversal of cell rounding is not because of changes in the kinetics of PGF$_{2\alpha}$ binding or in its removal during the washout procedure (6).

**DISCUSSION**

We show that FP$_{B}$-expressing cells differ in several important regards from FP$_{A}$-expressing cells in terms of their potential for activation of Tcf/β-catenin-mediated signaling. First, FP$_{A}$-expressing cells show PGF$_{2\alpha}$-stimulated accumulation of β-catenin at their contiguous cell boundaries that is not evident in FP$_{B}$-expressing cells. Second, although both FP$_{A}$ and FP$_{B}$-expressing cells show PGF$_{2\alpha}$-stimulated increases in cytosolic β-catenin, in FP$_{A}$-expressing cells this is accompanied by increased β-catenin phosphorylation and in FP$_{B}$-expressing cells by decreased β-catenin phosphorylation. Third, FP$_{B}$-expressing cells show a stimulation of Tcf/LeF reporter gene activity 16 h after agonist removal that is essentially absent in FP$_{A}$-expressing cells. A key control point could be in the differential phosphorylation of β-catenin. Thus, it is possible that the agonist-stimulated accumulation of β-catenin at the cell boundaries of FP$_{B}$ cells results in enhanced interactions with E-cadherin. In turn, this could initiate E-cadherin outside-in signaling leading to the sequential activation of phosphatidylinositol 3-kinase and Akt kinase (17). This is meaningful because phosphorylation of GSK-3β by Akt kinase is inhibitory (18) and could lead to the decreased phosphorylation of β-catenin found in agonist-treated FP$_{B}$ cells.

Recently Meigs et al. (19) reported that constitutively active mutants of G$_{a12}$ and G$_{a13}$ interact with E-cadherin resulting in a release of β-catenin and stimulation of Tcf/LeF reporter gene activity in a mutant cell line lacking APC. This link between heterotrimeric G-proteins and the Tcf/β-catenin signaling pathway is novel, but its physiological relevance might be questioned because of the altered nature of the model. In light of the present findings, however, it appears likely that both GPCRs and heterotrimeric G-proteins will be involved with activation of this signaling pathway. We have shown that FP receptors activate Rho through the probable activation of G$_{12}$ and/or G$_{13}$ (5). Both receptor isoforms were equally effective in this regard, and therefore it appears unlikely that activation of G$_{12}$ and/or G$_{13}$ could be solely responsible for the present findings because activation of Tcf/β-catenin signaling was observed only for cells expressing the FP$_{B}$ isofrom.

We believe that activation of Tcf/β-catenin signaling by PGF$_{2\alpha}$ in cells expressing the FP$_{B}$ receptor is involved with a phenotypic transformation that is morphologically similar to, but fundamentally different from, the cell rounding observed in agonist-treated FP$_{A}$ cells. Thus, maintenance of shape change in FP$_{A}$-expressing cells depends on continuous stimulation by PGF$_{2\alpha}$, and following its removal the cells revert back to their original morphology. In contrast, although shape change in FP$_{B}$-expressing cells is initiated by PGF$_{2\alpha}$, its maintenance is independent of further PGF$_{2\alpha}$ stimulation. In this manner the FP$_{B}$ prostaglandin receptor functions as one would expect of a trigger in a developmental or malignant transformation pathway.

The present findings have significance for the signaling potential of FP prostaglandin receptors and possibly for other GPCRs as well. For example, in sheep it is known that PGF$_{2\alpha}$ is the physiological signal for regression of the corpus luteum but only during a short window of the luteal cycle. Thus, if pregnanocy occurs the corpus luteum is maintained and loses sensitivity to the luteolytic actions of PGF$_{2\alpha}$ (20). Interestingly, the expression of FP$_{A}$ receptors does not change during this transition (21). Brief exposure of a small population of FP$_{A}$ receptors during the sensitive phase of the luteal cycle could explain the luteolytic actions of PGF$_{2\alpha}$.

Another condition that might involve the FP$_{B}$ isofrom or a homologue is in colorectal cancer. It is known that aberrant activation of Tcf/β-catenin signaling is associated with the development of this disease (22–24) and that inhibition of cyclooxygenase by NSAIDs can slow tumor progression (25). However, the specific mechanism of this beneficial effect is vague because of the large number of prostaglandin metabolites that are affected. Our findings support a mechanism in which NSAID-mediated decreases in PGF$_{2\alpha}$ would decrease Tcf/β-catenin signaling by FP$_{B}$ prostanoid receptors. This conclusion is supported by animal models of skin carcinogenesis in which PGF$_{2\alpha}$ reversed the anti-tumor-promoting activity of indomethacin (26). Although a human homologue of the ovine FP$_{B}$ receptor has not yet been identified other mechanisms could give rise to functional FP$_{B}$ isofroms. Thus, much like the known mutations of APC, truncation of the human FP$_{A}$ receptor by allelic variation, somatic mutations or proteolytic cleavage could give rise to receptors capable of producing activation of Tcf/β-catenin signaling. The possible role of FP$_{B}$ receptors in these and other physiological processes is intriguing and awaits future studies.

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