Prostaglandin E₂ Induced Functional Expression of Early Growth Response Factor-1 by EP₄, but Not EP₂, Prostanoid Receptors via the Phosphatidylinositol 3-Kinase and Extracellular Signal-regulated Kinases

Hiromichi Fujino, Wei Xu, and John W. Regan

From the Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, Arizona 85721-0207

Prostaglandin E₂ (PGE₂) mediates its physiological effects by interactions with a subfamily of G-protein-coupled receptors known as EP receptors. These receptors consist of four primary subtypes named EP₁, EP₂, EP₃, and EP₄. The EP₂ and EP₄ subtypes are known to couple to Gα and stimulate intracellular cyclic 3,5-adenosine monophosphate formation, whereas the EP₁ and EP₃ receptors are known to couple to Gα and Gα, respectively. Recently we found that EP₂ and EP₄ receptors can activate T-cell factor signaling; however, EP₂ receptors did this primarily through a cAMP-dependent protein kinase-dependent pathway, whereas EP₄ receptors primarily utilized a phosphatidylinositol 3-kinase (PI3K)-dependent pathway (Fujino, H., West, K. A., and Regan, J. W. (2002) J. Biol. Chem. 277, 2014–2019). We now report that PGE₂ stimulation of EP₂ receptors, but not EP₄ receptors, leads to phosphorylation of the extracellular signal-regulated kinases (ERKs) through a PI3K-dependent mechanism. Furthermore, this activation of PI3K/ERK signaling by the EP₂ receptors induces the functional expression of early growth response factor-1 (EGR-1). Under the same conditions induction of EGR-1 protein expression was not observed following PGE₂ stimulation of EP₄ receptors. These findings point to important differences in the signaling potential of the EP₂ and EP₄ receptors, which could be significant with respect to the potential involvement of EP₂ receptors in inflammation and cancer.

The EP₂ and EP₄ prostanoid receptors are two of the four primary receptor subtypes for prostaglandin E₂ (PGE₂).¹ Both of these receptors couple to Gα and can activate adenylyl cyclase resulting in the increased formation of intracellular cyclic 3,5-adenosine monophosphate (cAMP). Prior to the molecular cloning of these receptors it was thought that the stimulation of adenylyl cyclase by PGE₂ was mediated by a single receptor subtype that was defined pharmacologically as the EP₂ subtype (1). Thus, the first adenylyl cyclase stimulatory EP receptor to be cloned was thought to be the EP₂ subtype, but it was subsequently redefined as the EP₂ subtype (2) when a second adenylyl cyclase stimulatory EP receptor was cloned, which had the expected pharmacology of the EP₂ subtype (3). Surprisingly the EP₂ and EP₄ receptors encoded by these cDNAs only shared ~30% amino acid homology and were really no more related to each other than to other prostanoid receptor subtypes (4). In fact, the EP₂ receptor shows a closer phylogenetic relationship to the DP and IP receptors than it does to the EP₄ receptor. The human EP₄ receptor is larger than the human EP₂ (488 amino acids versus 358), which is almost entirely due to a significantly longer carboxy-terminal domain (155 versus 34 amino acids).

Recently we have shown that PGE₂ stimulation of the EP₂ receptors activates a T-cell factor (Tcf) signaling pathway by a mechanism that mainly involves the activation of cAMP-dependent protein kinase (PKA) (5). PGE₂ stimulation of the EP₄ receptors also activates Tcf signaling, but the mechanism is more complex and involves the activation of phosphatidylinositol 3-kinase (PI3K) (5). The involvement of PI3K with EP₂ receptor signaling has also been suggested in studies of human colorectal carcinoma cells (6). Thus, PGE₂ stimulation of endogenous EP₄ receptors in LS-174 cells increased cellular proliferation and motility through a PI3K-dependent pathway. Given the known role of PI3K in carcinogenesis, it is interesting that a recent study of knockout mice suggested the potential involvement of EP₄ receptors in colon cancer (7).

Early growth response factor-1 (EGR-1) is a member of the zinc finger family of transcription factors and plays a key role in cell growth and differentiation. It is recognized as an immediate early gene product that regulates the expression of a number of downstream genes, such as tumor necrosis factor-α (TNF-α), interleukin-2, and p53 (8). The induction of EGR-1 expression is known to involve members of the family of mitogen-activated protein kinases (MAPKs). For example, the induction of EGR-1 expression by growth hormone was shown to depend on the phosphorylation and activation of extracellular signal-regulated kinases (ERKs), but not on the activation of either Jnk N-terminal kinase (JNK) or p38 MAPK (9). Similarly the calmodulin antagonist, trifluoroperazine, induced EGR-1 expression through the activation of ERKs and the downstream transcription factor, Elk-1 (10).

Previous studies have also shown regulation of EGR-1 expression by protein kinase C (PKC), possibly involving members of the family of prostanoid receptors. For example, in Swiss 3T3 fibroblasts increases in PGE₂ were associated with a
Expression of EGR-1 by EP4 Receptors via PI3K and ERKs

PKC-dependent increase in EGR-1 mRNA expression (11). Likewise, in mouse MC3T3 osteoblastic cell lines PGE2 was found to increase EGR-1 mRNA expression. This increase was also PKC-dependent, but did not appear to involve PKA since the induction of EGR-1 mRNA expression was not observed following stimulation with forskolin, an agent which increases the formation of intracellular cAMP (12). These findings would appear to exclude the participation of the adenyl cyclase stimulatory EP2 and EP4 receptors in the induction of EGR-1 mRNA expression. However, because neither of these studies included pharmacological characterizations, the conclusions that can be drawn are limited with respect to the specific prostanoid receptor subtypes mediating these effects. We now show that the EP4 prostanoid receptor, but not the EP2, can induce the functional expression of EGR-1 through a signaling pathway involving the sequential activation of PI3K and the ERKs. These results further strengthen the involvement of PI3K with EP4 receptor signaling and clearly differentiate the signaling potential of the EP2 and EP4 receptors.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell lines stably expressing the EP2, or EP4, receptors were prepared using HEK-293 EBNA cells and the mammalian expression vector pCEP4 (Invitrogen) as previously described (5). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 250 μg/ml genetin, 100 μg/ml gentamicin, and 200 μg/ml hygromycin B.

Western Blotting—Sixteen hours prior to the immunoblotting experiments, cells were switched from their regular culture medium to OptiMEM (Invitrogen) containing 250 μg/ml genetin and 100 μg/ml gentamicin. Cells were then incubated at 37 °C with this same medium containing 1 μM PGE2 for the times indicated in the figures. In some cases cells were pretreated with either vehicle (0.1% Me2SO) or 100 nM wortmannin (Sigma) for 15 min or 10 μM PI3K inhibitor (PI3K Inhibitor) for 30 min at 37 °C. Cells were resuspended into a lysis buffer consisting of 150 mM NaCl, 50 mM Tris-Cl (pH 8.0), 5 mM EDTA (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM sodium fluoride, 10 mM disodium pyrophosphate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotonin and transferred to microcentrifuge tubes. The samples were rotated for 30 min at 4 °C and were centrifuged at 16,000 × g for 15 min. Aliquots of the supernatants containing 20–100 μg of protein were electrophoresed on 7.5% or 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes as described previously (5). Membranes were incubated in 5% nonfat milk for 1 h and were then washed and incubated for 16 h at 4 °C with primary antibodies using the following conditions, which varied according to the antibody being used. For the ERKs, incubations were done in 3% nonfat milk containing either a 1:1,000 dilution of anti-phospho-ERK1/2 antibody (9106, Cell Signalling); or mixture of a 1:500 dilution of anti-ERK1 antibody and a 1:10,000 dilution of anti-ERK2 antibody (sc-95 and sc-154, Santa Cruz Biotechnology). For JNK, incubations were done in 3% nonfat milk containing a 1,100 dilution of anti-phospho-JNK antibody (9255, Cell Signalling) or 0.1% nonfat milk containing a 1:1,000 dilution of anti-JNK antibody (9252, Cell Signalling). For p38 MAPK, incubations were done in 5% bovine serum albumin containing a 1:1,000 dilution of anti-phospho-p38 MAPK antibody (9211, Cell Signalling); or 1% nonfat milk containing a 1:1,000 dilution of anti-p38 MAPK antibody (sc-847, Santa Cruz Biotechnology). For EGR-1, incubations were done in 3% nonfat milk containing a 1:1,000 dilution of anti-EGR-1 antibody (sc-110, Santa Cruz Biotechnology). After incubating with the primary antibody, membranes were washed three times and incubated for 1 h at room temperature with a 1:10,000 dilution of the appropriate secondary antibodies conjugated with horse-radish peroxidase using the same conditions as described above for each of the primary antibodies. After washing three times, immunoreactivity was detected by chemiluminescence as described previously (5). To ensure equal loading of proteins, the membranes were stripped and re-probed with appropriate antibodies under the same conditions as described above.

Electrophoresis Mobility Shift Assay—Cells, grown in 10-cm plates, were pretreated with either vehicle (0.1% Me2SO) or 10 μM PD98059 for 10 min at 37 °C followed by treatment with either vehicle or 1 μM PGE2 for 60 min at 37 °C. Cells were washed, trypsinized, and centrifuged at 500 × g for 1 min, and the supernatant was removed. The pellet was resuspended in approximately three times the original pellet volume using buffer A, containing 10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.5 mM dithiothreitol. The sample was transferred into a new tube, placed on ice for 15 min, and then centrifuged at 16,000 × g for 30 s at 4 °C. The supernatant was discarded, and the pellet was resuspended in ~2 volumes of buffer A with seven rapid strokes of a 1-ml syringe with a 25-gauge needle. The sample was centrifuged at 16,000 × g for 5 min at 4 °C, and the supernatant was transferred to another tube (S100 fraction). The pellet was rinsed with buffer A and resuspended with buffer C, consisting of 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 0.5 mM dithiothreitol. After a 30-min incubation on ice, the sample was centrifuged at 16,000 × g for 5 min at 4 °C, and the supernatant (nuclear extract) was dialyzed for 2 h using a 3,000 molecular weight cutoff membrane (Pierce) against buffer D containing 20 mM HEPES-KOH (pH 7.9), 20% glycerol, 20 mM KCl, 2 mM MgCl2, 0.2 mM EDTA, and 0.5 mM dithiothreitol.

A double-stranded DNA oligonucleotide probe was designed corresponding to the GC box-1 and GC box-2 sequences in the human prostaglandin E2 synthase gene promoter (13). The sense strand of this oligonucleotide sequence is 5’-GGGGGGGGGGGGCTTGGGGGCTGCT-3’, where the underlined sequence represents the consensus binding site for EGR-1 (8). The sense and antisense oligonucleotides were annealed and labeled with [γ-32P]ATP (Amersham Biosciences) using T4 kinase (Invitrogen). The reaction mixture was applied to a spin column to remove free [γ-32P]ATP and run on an 8% native polyacrylamide gel to separate any single-stranded DNA from the double-stranded probe. The binding reaction was performed for 15 min at room temperature using ~10,000 cpm of the probe and 10 μg of nuclear extract in 15 μl of a buffer containing 4% Ficoll 400, 1 mM EDTA (pH 8.1), 1 mM dithiothreitol, 4 mg/ml bovine serum albumin, 0.1 mM KCl, and 2 μg of poly-(deoxyinosine-deoxyctydylide) (Sigma). The mobility shift assay was done with a 5% native polyacrylamide gel followed by drying and visualization by autoradiography using Hyperfilm MP (Amersham Biosciences). The supershift assay was performed using 3 μg of the anti-EGR-1 antibody in which the antibody was incubated with the nuclear extract for an additional 15 min following the initial binding reaction.

RESULTS

PGE2 Stimulated Phosphorylation of ERKs in EP4 Receptor-expressing HEK Cells—We have previously reported that EP4 prostanoid receptor can activate Tcf signaling by a mechanism involving PI3K (5). Further characterization of this interaction between the EP4 receptor and PI3K was of interest given the important role of PI3K in both normal and aberrant cell signaling pathways. One of the downstream effectors of PI3K signaling is the activation of members of the MAPKs. To determine if EP4 receptors could potentially activate MAPK signaling, we examined the ability of PGE2 to stimulate the phosphorylation of three MAPKs in HEK-293 cells stably transfected with the human EP2 and EP4 prostanoid receptors. For these experiments untransfected HEK cells and cells stably expressing the EP2 and EP4 receptors were incubated with 1 μM PGE2 for various times and were then immunoblotted with antibodies to the phosphorylated and nonphosphorylated forms of ERK1 and ERK2, p54 JNK, and p38 MAPK. As shown in panel A of Fig. 1, treatment with PGE2 resulted in a time-dependent phosphorylation of ERKs 1 and 2 in EP4-expressing cells, but not in EP2-expressing cells or the control HEK cells. The PGE2-stimulated phosphorylation of ERKs in EP4 cells was maximal at 5–10 min and by 60 min had nearly returned to the original, unstimulated, levels (0 min). These same blots were stripped and re-probed with antibodies to the nonphosphorylated forms of ERK1 and ERK2, and as shown in panel B of Fig. 1 nearly identical amounts of ERKs 1 and 2 were present throughout the time course of treatment and among the three cell lines.

Panels C–F of Fig. 1 show the corresponding results for p54 JNK and p38 MAPK, respectively. In contrast to the results obtained with the ERKs, there were no substantive differences between the EP2- and EP4-expressing cells with respect to the
Expression of EGR-1 by EP$_4$ Receptors via PI3K and ERKs

Fig. 1. Immunoblots of the time course of PGE$_2$-stimulated phosphorylation of ERKs, JNK, and p38 MAPK in untransfected HEK-293 cells and in HEK-293 cells stably transfected with either the human EP$_2$ or EP$_4$ prostanoid receptors. Cells were incubated with 1 $\mu$M PGE$_2$ for the indicated times and were subjected to immunoblot analysis as described under “Experimental Procedures.” Panel A, immunoblotting with antibodies against phospho-ERKs 1 and 2 (p-ERK1/2). Panel B, the blots shown in panel A were stripped and re-probed with antibodies against ERKs 1 and 2 (ERK1/2). Panel C, immunoblotting with antibodies against phospho-JNK (p-p54 JNK). Panel D, the blots shown in panel C were stripped and re-probed with antibodies against p54 JNK. Panel E, immunoblotting with antibodies against phospho-p38 MAPK (p-p38 MAPK). Panel F, the blots shown in panel E were stripped and re-probed with antibodies against p38 MAPK. Results are representative of at least three independent experiments with each antibody and condition.

Fig. 2. The effects of wortmannin on PGE$_2$-stimulated phosphorylation of ERKs in HEK-293 cells transfected with either the EP$_2$ or EP$_4$ prostanoid receptors. Cells were pretreated with either vehicle or 100 nM wortmannin (wort) for 15 min followed by treatment with either vehicle (v) or 1 $\mu$M PGE$_2$ (P) for 10 min at 37°C and were subjected to immunoblot analysis as described under “Experimental Procedures.” Upper panel, immunoblotting with antibodies against phospho-ERKs 1 and 2 (p-ERK1/2). Lower panel, the blots shown in the upper panel were stripped and re-probed with antibodies against ERKs 1 and 2 (ERK1/2). Results are representative of three independent experiments with each antibody and condition.

PI3K in this process. The lower panel of Fig. 2 shows that nearly identical amounts of ERKs 1 and 2 were present under all conditions in both cell lines.

Induction of EGR-1 Expression by PGE$_2$ in EP$_4$-expressing Cells but Not in EP$_2$-expressing Cells—Given the phosphorylation of ERKs 1 and 2 following PGE$_2$ stimulation of the human EP$_4$ prostanoid receptor, it was of interest to explore the possible modulation of downstream effectors that could reflect this selective activation of the ERK signaling pathway. One such effecter that is known to be induced following the activation of ERKs is the early growth response factor-1 (EGR-1). Immunoblot analysis was, therefore, used to examine the time course of expression of EGR-1 following treatment of EP$_4$ and EP$_2$-expressing cells with 1 $\mu$M PGE$_2$. As shown in the upper panels of Fig. 3, there was a modest induction of EGR-1 expression in EP$_2$-expressing cells following 30 min of incubation with PGE$_2$, which was markedly increased at the 60-min time point. On the other hand, in EP$_4$-expressing cells the expression of EGR-1 was barely detectable even at the 60-min time point. To check for the equal loading of protein these blots were stripped and re-probed with antibodies to ERKs 1 and 2. As shown in the lower panels of Fig. 3 nearly identical amounts of ERKs 1 and 2 were present in both cell lines and throughout the time course of treatment with PGE$_2$.

Induction of EGR-1 Expression by PGE$_2$ in EP$_4$-expressing Cells Involves Activation of PI3K and the ERKs—Pretreatment of EP$_2$- and EP$_4$-expressing cells with either the PI3K inhibitor, wortmannin, or the MAPK/ERK kinase inhibitor, PD98059, was used to examine the potential involvement of these kinases on the PGE$_2$ induced expression of EGR-1. As shown in the upper panel of Fig. 4A, the robust induction of EGR-1 expression in EP$_4$-expressing cells following 60 min of exposure to 1
μM PGE₂ was completely blocked by pretreatment with wortmannin. EGR-1 expression in EP₂-expressing cells was not detectable either before or after treatment with PGE₂, or following pretreatment with wortmannin. The lower panel of Fig. 4A shows that nearly equal amounts of ERKs 1 and 2 were present under all conditions and indicates that the observed differences in EGR-1 expression are not because of differences in overall protein expression or in the amount of protein loaded on the gels.

![Image](image_url)

**Fig. 4.** Immunoblots of the effects of wortmannin (A) and PD98059 (B) on PGE₂-stimulated expression of early growth response factor-1 (EGR-1) in HEK293 cells transfected with either the EP₂ or EP₄ prostanoid receptors. Cells were pretreated with either vehicle or 100 nM wortmannin (wort) for 15 min (A) or were pretreated with either vehicle or 10 μM PD98059 for 10 min (B) followed by treatment with either vehicle (v) or 1 μM PGE₂ (P) for 60 min at 37 °C. The cells were then subjected to immunoblot analysis as described under “Experimental Procedures.” Upper panels, immunoblotting with antibodies against EGR-1. Lower panels, the blots shown in the upper panels were stripped and re-probed with antibodies against ERKs 1 and 2 (ERK1/2). Results are representative of three independent experiments with each antibody and condition.

The MAPK/ERK kinase inhibitor, PD98059, was then used to explore whether this specific induction of EGR-1 expression in EP₂-expressing cells involved the sequential activation of PI3K and the ERKs. For these experiments EP₂- and EP₄-expressing cell lines were pretreated with either vehicle or 10 μM PD98059 for 10 min followed by treatment with either vehicle or 1 μM PGE₂ for 60 min. As shown in the upper panel of Fig. 4B, in the absence of pretreatment with PD98059 there was a strong PGE₂-mediated induction of EGR-1 expression in EP₂-expressing cells that was completely absent in EP₄-expressing cells. Following pretreatment with PD98059, however, there was a complete block of PGE₂-mediated EGR-1 expression in EP₂-expressing cells. To confirm equal loading of protein, this blot was stripped and re-probed with antibodies to ERKs 1 and 2. The lower panel of Fig. 4B shows that nearly identical amounts of ERKs 1 and 2 were present under all conditions in both cell lines. Together these results strongly suggest that the sequential activation of PI3K and the ERKs is required for the induction of EGR-1 expression by PGE₂ in EP₂-expressing cells.

**Induction of EGR-1 Expression in EP₄-expressing Cells Leads to Functional Interactions with a DNA Promoter Sequence Containing an EGR-1 Binding Site**—The potential for PGE₂ to stimulate EGR-1 mediated transcriptional activation was examined in EP₂- and EP₄-expressing cells using electrophoresis mobility shift assays. The probe used for these experiments consisted of an oligonucleotide designed from the promoter region of the human gene for prostaglandin E₂ synthase containing a consensus binding site for EGR-1 (13). EP₂- and EP₄-expressing cells were pretreated with either vehicle or 10 μM PD98059 for 10 min followed by treatment with either vehicle or 1 μM PGE₂ for 60 min. In Fig. 5 a comparison of the “free” lane with the other lanes shows that the probe migrated with relative mobilities of 0.26 and 0.40 under all conditions in which a nuclear extract was present. Following treatment with PGE₂, however, there was a selective shift in the mobility of the probe in EP₄-expressing cells, but not in EP₂-expressing cells, yielding a unique band with a relative mobility of 0.16. The identity of the protein causing this shift was investigated in a supershift assay by an additional incubation with antibodies to

![Image](image_url)

**Fig. 5.** Electrophoresis mobility shift assay and supershift assay of EP₂ and EP₄ expressing HEK cells following treatment with PGE₂ in the absence or presence of pretreatment with PD98059. Cells were pretreated with either vehicle or 10 μM PD98059 for 10 min followed by treatment with either vehicle (v) or 1 μM PGE₂ (P) for 60 min at 37 °C. The mobility shift assay was done as described under “Experimental Procedures” with a [³²P]labeled oligonucleotide probe containing a consensus binding site for early growth response factor-1 (EGR-1). The supershift assay was done by incubating the probe and nuclear extract for an additional 15 min with antibodies to EGR-1 (EGR-Ab). Relative mobility is defined as the distance traveled from the origin by the bound probe, normalized to the distance traveled by the free probe. Results are representative of four independent experiments.
Expression of EGR-1 by EP4 Receptors via PI3K and ERKs

EGR-1. As shown in the last two lanes of Fig. 5, incubation with the antibodies to EGR-1 diminished the intensity of the 0.16-band following treatment with PGE2 and resulted in the appearance of a new band with a mobility of 0.08. This supershift confirms the interaction of the probe with the EGR-1 following the treatment of EP4 expressing cells with PGE2. Fig. 5 also shows that pretreatment of EP4-expressing cells with the MAPK/ERK kinase inhibitor, PD98059, prevented the PGE2-induced mobility shift and appearance of 0.16 band. Thus, as predicted from our previous experiments with PD98059, the PGE2-induced functional expression of EGR-1 involves the activation of a MAPK/ERK signaling pathway.

**DISCUSSION**

We have previously shown that stimulation of EP3 prostanoid receptors by PGE2 can activate a Tcf signaling pathway by a mechanism that mainly involves the activation of PKA (5). PGE2 stimulation of EP3 prostanoid receptors can also activate a Tcf signaling pathway, but the mechanism is more complex and involves the activation of PI3K (5). We now show that PGE2 stimulation of the EP4 receptor activates an additional signaling pathway involving PI3K. Thus, PGE2 treatment of cells expressing EP4 receptor, leads to a PI3K-dependent phosphorylation of ERKs 1 and 2 followed by a de novo increase in the functional expression of EGR-1. The activation of this PI3K signaling cascade was unique for the EP4 receptor and was not observed in cells expressing EP3 receptors.

One of the ways in which the EP2 and EP4 receptors are known to differ is in the characteristics of their agonist induced desensitization and internalization. Thus, EP3 receptors undergo rapid, PGE2-mediated desensitization (14) and internalization (15); whereas EP2 receptors do not. Since the internalization of some G-protein-coupled receptors is associated with a transactivation of the MAPK pathway (16, 17), it may be supposed that such a mechanism could explain the present findings of selective activation of ERK signaling by the EP3 receptors. However, in a previous study using human EP4 receptors expressed in HEK293 cells, it was found that the phosphorylation of ERKs 1 and 2 was independent of PGE2-mediated receptor internalization (18).

Another way in which the EP2 and EP4 prostanoid receptors appear to differ is in their ability to stimulate intracellular cAMP formation. Thus, despite nearly identical levels of receptor expression, the maximal levels of PGE2-stimulated cAMP formation in EP2-expressing cells was only 20% of the level obtained in EP4-expressing cells (5). However, under the same conditions the ability of PGE2 to stimulate Tcf signaling was ~50% greater in EP4-expressing cells as compared with EP2-expressing cells (5). This indicates that the lower amounts of PGE2-stimulated cAMP formation in EP2-expressing cells is because of less efficient coupling to this pathway and not because of an overall impairment in the signaling potential of these receptors.

The more efficient coupling of the EP4 receptor to intracellular cAMP formation; however, may be significant with respect to the present findings. Thus, it has been reported that the phosphorylation of Raf kinase by PKA inhibits the activity of Raf kinase and subsequently decreases Raf mediated MAPK signaling (19, 20). In EP2-expressing cells, therefore, a robust activation of PKA may inhibit Raf kinase and block the phosphorylation and activation of ERKs.

Our findings of a PGE2-mediated induction of EGR-1 expression by the EP3 receptor is interesting light of recent studies with knockout mice that show a potential involvement of the EP3 receptor with colon cancer and rheumatoid arthritis. For example EP4 knockout mice, but not EP3 knockout or control mice, show a reduced formation of preneoplastic lesions following treatment with azoxymethane, a known colon carcinogen (7). EP3 knockout mice, but not EP2 knockout or control mice, also show a significantly decreased incidence and severity of collagen antibody induced arthritis, an animal model of rheumatoid arthritis (21). In addition, in both colon cancer and rheumatoid arthritis prostaglandin levels are elevated and both conditions benefit to some extent by treatment with inhibitors of the cyclooxygenases. In colon cancer, the expression of cyclin D1, a key regulator of cell cycle progression, is known to be regulated by Tcf signaling (22). However, it has also been reported that the expression of cyclin D1 is regulated by EGR-1 through a PI3K- and ERK-dependent pathway (23). Furthermore it has also been shown that PGE2 synthase is up-regulated by the binding of EGR-1 to the promoter region of the mouse gene encoding PGE2 synthase (13). Signaling through an EP4 receptor would have the potential, therefore, to increase the expression of cyclin D1 and PGE2 synthase through a PGE2-mediated induction of EGR-1 expression. Since the product of PGE2 synthase is PGE2 itself, the potential for a positive feedback loop is obvious.

A similar situation could also explain the increased levels of PGE2 observed in rheumatoid arthritis. However, in this disease in addition to an up-regulation of the expression of PGE2 synthase, signaling through EP4 receptors could also potentially up regulate levels of TNF-α (21) whose expression is under the control of EGR-1 (24). Inhibitors of TNF-α, such as etanercept and infliximab, are used therapeutically in the treatment of rheumatoid arthritis because they slow the progression of this disease (25). It is possible that the reduced incidence and severity of collagen antibody induced arthritis in EP4 knockout mice (21) is related to the loss of EP4 receptor-mediated signaling through a PI3K/ERKs/PI3K/ERKs-1 pathway.

Recently we have shown that the FP receptor can signal through a β-catenin/Tcf signaling pathway (26) and interact with PI3K (27); and thus, has potential to be involved in the pathophysiology of colon cancer. It has also been reported that EP3 receptors (28) and EP2 receptors (29) may have roles in colon cancer. In addition, gene knockout studies with the cyclooxygenases indicate that the synthesis of prostaglandins by these enzymes contributes to the pathophysiology of colon cancer (30). Interestingly, the expression of cyclooxygenase-2 in intestinal polyps appears to be under a positive feedback through the EP2 receptor (29). Furthermore, it is clear from the findings of Sonoshita et al. (29) that the effects of the cyclooxygenase-2 knockout on the number and size of intestinal polyps was greater than the effects of the EP2 knockout and appear to require the involvement of another prostanoid receptor. Potential involvement of the EP3 receptor could work in concert with the EP2 receptor to increase PGE2 levels by increased expression of both cyclooxygenase-2 and PGE2 synthase. Our present findings with the EP3 receptors further strengthen an association of the prostaglandins and their receptors with cancer and inflammation; further knowledge of which could have practical therapeutic benefits.

**REFERENCES**

1. Bastien, L., Sawyer, N., Grygorczyk, R., Metters, K. M., and Adams, M. (1994) *J. Biol. Chem.* 269, 11873–11877
2. Nishigaki, N., Negishi, M., Honda, A., Sugimoto, Y., Namba, T., Narumiya, S., and Ichikawa, A. (1995) *FEBS Lett.* 364, 339–341
3. Regan, J. W., Bailey, T. J., Pepperell, D. J., Pierce, K. L., Bogardus, A. M., Cyclacelle, J. K., Fairbairn, C. E., Fodor, K. M., Woodward, D. F., and Gil, D. W. (1994) *Mol. Pharmacol.* 46, 213–220
4. Pierce, K. L., and Regan, J. W. (1998) *Life Sci.* 62, 1479–1483
5. Fujino, H., West, K. A., and Regan, J. W. (2000) *J. Biol. Chem.* 275, 2614–2619
6. Sheng, H., Shao, J., Washington, M. K., and DuBois, R. N. (2001) *J. Biol. Chem.* 276, 18075–18081
7. Mutoh, M., Watanabe, K., Kita, K., Shoji, Y., Takahashi, M., Kawamori, T., Tani, K., Kobayashi, M., Murayama, T., Kobayashi, K., Ohuchida, S., Sugimoto, Y., Narumiya, S., Sugimura, T., and Wakabayashi, K. (2002) *Cancer Res.* 62, 28–32
Expression of EGR-1 by EP<sub>4</sub> Receptors via PI3K and ERKs

8. Silverman, E. S., and Collins, T. (1999) Am. J. Pathol. 154, 665–670
9. Hodge, C., Liao, J., Stofega, M., Guan, K., Carter-Su, C., and Schwartz, J. (1998) J. Biol. Chem. 273, 31327–31336
10. Shin S. Y., Kim, S. Y., Kim, J.-H., Min, D. S., Ko, J., Kang, U.-G., Kim, Y. S., Kwon, T. K., Han, M. Y., Kim, Y. H., and Lee, Y. H. (2001) J. Biol. Chem. 276, 7797–7805
11. Danesch, U., Weber, P. C., and Sellmayer, A. (1994) J. Biol. Chem. 269, 27258–27263
12. Fang, M. A., Noguchi, G. M., and McDougall, S. (1996) Prostaglandins Leukot. Essent. Fatty Acids 54, 109–114
13. Naraba, H., Yokoyama, C., Tago, N., Murakami, M., Kudo, I., Fueki, M., Oh-ishi, S., and Tanabe, T. (2002) J. Biol. Chem. 277, 28601–28608
14. Ishigak, N., Negishi, M., and Ichikawa, A. (1996) Mol. Pharmacol. 50, 1031–1037
15. Desai, S., April, H., Nwaneshiudu, C., and Ashby, B. (2000) Mol. Pharmacol. 58, 1279–1286
16. Rodriguez-Viciana, P., Warne, P. H., Kihara, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) Cell 89, 457–467
17. Naga Prasad, S. V., Barak, L. S., Rapacciuolo, A., Caron, M. G., and Rockman, H. A. (2001) J. Biol. Chem. 276, 18953–18959
18. Desai, S., and Ashby, B. (2001) FEBS Lett. 501, 156–160
19. Dhillon, A. S., Pullock, C., Steen, H., Shaw, P. E., Miachak, H., and Kolch, W. (2002) Mol. Cell. Biol. 22, 3237–3246
20. Sidevar, M. F., Kozlowski, P., Lee, J. W., Collins, M. A., He, Y., and Graves, L. M. (2000) J. Biol. Chem. 275, 28688–28694
21. McCoy, J. M., Wicks, J. R., and Audoly, L. P. (2002) J. Clin. Invest. 110, 651–658
22. Tetsu, O., and McCormick, F. (1999) Nature 398, 422–426
23. Guilleminot, L., Levy, A., Raymondjean, M., and Rothinut, B. (2001) J. Biol. Chem. 276, 33934–33943
24. Yao, J., Mackman, N., Edgington, T. S., and Fan, S.-T. (1997) J. Biol. Chem. 272, 17755–17801
25. Acherli, D., Oertle, S., Mauron, H., Reichenbach, S., Fordi, B., and Villiger, P. M. (2002) Swiss Med. Wkly. 132, 414–422
26. Fuso, H., and Regan J. W. (2001) J. Biol. Chem. 276, 12489–12492
27. Fuso, H., Srinivasan, D., and Regan, J. W. (2002) J. Biol. Chem. 277, 48786–48795
28. Watanabe, K., Kawamori, T., Nakatsugi, S., Ohta, T., Obuchida, S., Yamamoto, H., Maruyama, Y., Kondo, K., Ushikubi, F., Narumiya, S., Sugimoto, T., and Wakabayashi, R. (1999) Cancer Res. 59, 5093–5096
29. Soneshita, M., Takaku, K., Sasaki, N., Sugimoto, Y., Ushikubi, F., Narumiya, S., Oshima, M., and Taketa, M. M. (2001) Nat. Med. 7, 1048–1051
30. Chulda, P. C., Thompson, M. B., Mahler, J. F., Doyle, C. M., Gaul, B. W., Lee, C., Tian, H. F., Morham, S. G., Smithies, O., and Langenbach, R. (2000) Cancer Res. 60, 4705–4708