Cyclooxygenase and its derived prostaglandin E₂ (PGE₂) have been shown to stimulate the growth of cancer cells and promote tumor angiogenesis. Here, we show that PGE₂ activated the β-catenin/T cell factor-dependent transcription in colon cancer cells through the cAMP/protein kinase A pathway. The expression of cyclin D1 and vascular endothelial growth factor was induced by PGE₂ in LS-174T cells. Moreover, PGE₂ and mutated β-catenin stimulated the transcription of cyclin D1 and vascular endothelial growth factor in a synergistic fashion. Mechanistically, PGE₂ increased the phosphorylation of glycogen synthase kinase-3 and consequently accumulated β-catenin. In addition, PGE₂ induced the expression of T cell factor-4 transcription factor, which formed transcriptionally active complex with β-catenin. In animal experiments, administration of 16,16-dimethyl PGE₂ strongly increased the expression of cyclin D1 and vascular endothelial growth factor in APC<sup>min</sup>/ mouse polyps. Thus, our results provide a novel mechanism, suggesting that cyclooxygenase-2/PGE₂ may exert pro-oncogenic actions through stimulating the β-catenin/T cell factor-mediated transcription, which plays critical roles in colorectal carcinogenesis.

A large body of studies demonstrates a strong link between COX-1-2/PGE₂ signaling and the APC/β-catenin/TCF pathway in intestinal neoplasia. The most convincing evidence was provided by clinical trials conducted in familial adenomatous polyposis patients that result from germ line mutations of the APC gene. Administration of sulindac or celecoxib, which inhibit COX enzyme activity, significantly reduces the number and size of polyps in these patients (1, 2). A murine model for familial adenomatous polyposis patients containing a germ line mutation in APC alleles is a primary animal model for investigation of pro-oncogenic actions of COX-2/PGE₂ (3–5). Disruption of COX-2 gene, which encodes the key enzyme for conversion of arachidonic acid to prostaglandins (PGs), results in a ~60% reduction of the number of polyps in APC<sup>−/−</sup> mice (3). Disruption of the E type prostaglandin receptor EP₂, which mediates PGE₂ signaling, significantly reduces the number and size of adenomas in APC<sup>−/−</sup> mice as well (6). These data are supported by pharmacologic inhibition of the COX enzyme that achieves similar anti-neoplastic effects in APC-mutated mice (7). Greater than 95% of APC mutations in colorectal cancers are truncations of the C terminus (8), which is required for degradation of β-catenin (9). Stabilized β-catenin binds to members of the TCF/LEF family of transcription factors and results in inappropriate onco- genic Wnt signaling (10). The regulation of a number of pro-oncogenic genes involves β-catenin/TCF-dependent transcription that include cyclin D1 (11), c-myc (12), vascular endothelia growth factor (VEGF) (13), and matrixin (14).

Cumulative evidence indicates that COX-2-derived PGE₂ provides growth advantage to colorectal carcinomas through transactivation of the epidermal growth factor receptor (EGFR) signaling system (15–17). Further evidence demonstrates that COX-2/PGE₂ promotes the growth of colon cancers through enhancing tumor angiogenesis (18, 19). Growth of solid tumors requires a blood supply that is achieved through neoangiogenesis, which is controlled by a number of growth factors, including VEGF. Knock-out of EP₂ receptor results in a decrease in the number and size of intestinal polyps in APC<sup>−/−</sup> mice that is associated with a dramatic reduction of VEGF expression (6).

Treatment with selective COX-2 inhibitors reduces levels of VEGF, particularly the membrane-bound form, in polyps of APC<sup>−/−</sup> mice (4). On the other hand, PGE₂ exposure induces the expression of VEGF in colon cancer cells (20). These observations suggest a critical interaction between the COX-2/PGE₂ pro-oncogenic pathway and the oncogenic APC/β-catenin/TCF pathway in colorectal neoplasia. In the present study, we demonstrated that PGE₂ transactivated the β-catenin/TCF-dependent transcription through inhibition of glycogen synthase kinase (GSK)-3 and induction of TCF-4 in colon cancer cells. In addition, PGE₂ and mutated β-catenin induced the transcription of TCF target genes cyclin D1 and VEGF in a synergistic manner. These results suggest that COX-2/PGE₂ may robustly enhance the oncogenic activity of the APC/β-catenin/TCF pathway, which play key roles in colorectal carcinogenesis.

**MATERIALS AND METHODS**

Cell Culture and Chemicals—LS-174T cells were purchased from ATCC (Manassas, VA), and the HCA-7 cell line was a generous gift from Susan Kirkland (University of London). Human colon cancer cells were maintained in McCoy’s 5A medium containing 10% fetal bovine serum. PGE₂ was purchased from Cayman Chemicals (Ann Arbor, MI). H-89, LY-294002, PD-98059, and PD-153035 were purchased from Calbiochem. For the GSK-3 inhibitors, LiCl was from Sigma, SB-216763 was from Cayman Chemicals (Ann Arbor, MI). H-89, LY-294002, PD-98059, and PD-153035 were purchased from Calbiochem. From the Departments of Surgery, Urology, and Cancer Center, Indiana University School of Medicine, Indianapolis, Indiana 46202 and the Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555

This paper is available on line at http://www.jbc.org

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*This work was supported by Grants DK-065615 and DK-64593 (to H. S.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; TCF, T-cell factor; PKA, protein kinase A; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; GSK, glycogen synthase kinase; MEK, MAPK/ERK kinase; SB-216763, (3,2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrrole-2,5-dione; BIO, (6-bromo-2-oxindolind-3-oxime; dnTCF-4, dominant negative TCF-4; LEF, lymphoid enhancer-binding factor.

Received for publication, November 18, 2004, and in revised form, May 12, 2005
Published, JBC Papers in Press, May 17, 2005, DOI 10.1074/jbc.M413056200
Celecoxib was a generous gift from Pharmacia (St. Louis, MO).

RNA Extraction and Northern Blot Analysis—Extraction of total cellular RNA was carried out as described previously (16). RNA samples (20 μg/lane) were separated on formaldehyde-agarose gels and blotted onto nitrocellulose membranes. The blots were hybridized with cDNA probes labeled with [α-32p]dCTP by random primer extension (Stratagene, La Jolla, CA). After hybridization and washes, the blots were subjected to autoradiography.

Enzyme-linked Immunosorbent Assay—Levels of VEGF protein in cell culture media were quantified using an enzyme-linked immunosorbent assay kit (R & D System, Minneapolis, MN). Cells (2 × 10^5) were seeded in 24-well plate, and serum was deprived for 48 h prior to PGE2 treatment. Culture media were collected and stored at −80 °C for assays.

Transient Transfection and Luciferase Assay—Assays to determine transcriptional activity were described previously (21). The TCF report plasmid, TOPflash, containing two sets (with the second set in the reverse orientation) of three copies of the TCF site (ATCAAAG) upstream of the thymidine kinase minimal promoter and luciferase open reading frame was purchased from Upstate (Lake Placid, NY). The FOPflash construct containing mutated TCF elements was obtained from Upstate also. The reporter construct for cyclin D1 promoter (−1745 to +134 in PASLuc vector) was kindly provided by Dr. Richard Pestell (22). The reporter construct for the VEGF promoter containing the 5′-flanking sequence of the human VEGF gene between −2279 and +54 in pGL-2 vector was kindly provided by Dr. Hideo Kimura (23). Truncated β-catenin (∆89) was provided by Dr. Paul Polakis (24). LEF-1 expression vector is a kind gift from Dr. Elaine Fuchs (25). Wild type and dominant negative TCF-4 expression vectors were purchased from Upstate. The active MAPK/ERK (MEK-1) expression plasmid was purchased from Upstate as well.

For transient transfection, cells were co-transfected with 0.5 μg of reporter plasmid and 0.5 μg of the pRL-thymidine kinase plasmid, containing the Renilla luciferase gene (Promega), using the FuGENE 6 procedure (Roche Applied Science) as described in the manufacturer’s protocol. Transfected cells were lysed at the indicated times for luciferase assay. Firefly and Renilla luciferase activities were measured using a dual-luciferase reporter assay system (Promega) and a luminometer. Firefly luciferase values were standardized to Renilla values.

Immunoblot Analysis—Immunoblot analysis was performed as previously described (26). The antibodies used were cyclin D1 and TCF-4 (BD Transduction Laboratories).

Animal Study—APCmin/+ mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and were housed in the Animal Care Facility according to National Institutes of Health and institutional guidelines for laboratory animals. 16,16-Dimethyl PGE2 (Cayman Chemicals, Ann Arbor, MI) was dissolved in 10% EtOH and was administered by intraperitoneal injection (100 μg/site) to 8-12-week-old male and female mice for laboratory animals. 16,16-Dimethyl PGE2 (Cayman Chemicals, Ann Arbor, MI) was dissolved in 10% EtOH and was administered by intraperitoneal injection (100 μg/kg of body weight). Internal controls were collected for preparation of RNA and protein.

Data Analysis—All statistical analyses were performed on a personal computer with the StatView 5.0.1 software (SAS Institute Inc. Cary, NC). Analyses between multiple groups were determined by analysis of variance (ANOVA). Analyses between two groups were determined using the unpaired Student’s t test. Differences with a p value of <0.05 were considered as statistically significant.

RESULTS

PGE2 Induced TCF-dependent Transcription—The Wnt signaling pathway stabilizes cytosolic β-catenin. Accumulated β-catenin binds and activates transcription factors of the TCF/LEF family, which, in turn, regulates target genes by binding to TCF/LEF elements within the promoter of the gene (27). To determine whether PGE2 was able to stimulate TCF transcription, LS-174T cells were transfected with a TCF reporter vector, TOPflash, which contains a combination of TCF binding elements. Exposure to PGE2 increased the transcriptional activity of TOPflash ~10-fold (Fig. 1A). As a positive control, co-transfection with a LEF-1 expression vector increased the activity of TOPflash ~40-fold in LS-174T cells. In contrast, the reporter construct containing mutated TCF sites, FOPflash, had very low activity in LS-174T cells; treatment with PGE2 did not significantly increase luciferase activity. Furthermore, PGE2 induced TCF activity in a concentration-dependent fashion.
ion; as low as 1 nm, PGE₂ clearly activated TCF elements and, at 0.1 μM, PGE₂ reached the maximum effect (Fig. 1B). To investigate the effect of endogenous PGE₂ on TCF activation, we treated HCA-7 cells with celecoxib at low concentrations. Previous studies have shown that HCA-7 cells express high levels of COX-2 and release PGE₂ into culture media (26). Treatment with 0.1 μM celecoxib is able to completely block PGE₂ production but does not affect HCA-7 cell growth (28). TOPflash reporter vector was introduced into HCA-7 cells. 0.1 or 1 μM celecoxib was added immediately after the transfection procedure. Inhibition of COX-2 activity reduced TCF activity by ~50% (Fig. 1C), suggesting that endogenous PGE₂ was partially responsible for the TCF-mediated transcription in HCA-7 cells.

PGE₂ signals via activation of the cAMP/protein kinase A (PKA) pathway in LS-174T cells (21). As demonstrated in Fig. 1D, a specific PKA inhibitor, H-89, inhibited the PGE₂-induced TCF activation in a concentration-dependent manner. Because PGE₂ may transactivate the epidermal growth factor receptor (EGFR) signaling pathway (17), we next tested whether PGE₂-stimulated TCF activation involved EGFR-mediated signaling pathways. LS-174T cells were treated with PD-153035, an EGFR tyrosine kinase inhibitor, LY-294002, a phosphatidylinositol 3-kinase inhibitor, or PD-98059, a MAPK/ERK kinase (MEK) inhibitor, along with PGE₂ exposure. Inhibition of EGFR signaling did not attenuate the PGE₂-induced TCF transactivation (Fig. 1E). Furthermore, inhibition of the phosphatidylinositol 3-kinase pathway or the MEK/ERK MAPK pathway did not reduce the PGE₂-induced TCF activation as well.

**PGE₂ Induced the Expression of Cyclin D1**—Although PGE₂ greatly activated the artificial TCF reporter system; it was of importance to determine whether PGE₂ truly induced the expression of TCF target genes. *Cyclin D1* has been determined as a TCF-dependent gene, which contains a number of TCF elements within its promoter region; the expression of mutated β-catenin increases the activity of *cyclin D1* promoter and levels of cyclin D1 mRNA (11). We treated LS-174T cells with PGE₂ and found that levels of cyclin D1 protein were rapidly increased (Fig. 2A). To determine whether transcriptional regulation was involved in PGE₂-induced cyclin D1 expression, LS-174T cells were transfected with a *cyclin D1* promoter driving reporter vector. Exposure to PGE₂ increased the luciferase activity ~4.5-fold (Fig. 2B), indicating that PGE₂ regulated cyclin D1 expression, at least partially, at a transcriptional level. In addition, activation of cAMP/PKA was required for PGE₂-stimulated *cyclin D1* transcription (Fig. 2C); treatment with H-89 completely attenuated the PGE₂-induced activation of the *cyclin D1* promoter. In contrast, PD-153035, LY-294002, and PD-98059 did not significantly alter the PGE₂-induced *cyclin D1* transcription. To further elucidate the potential interaction between the APC/β-catenin/TCF pathway and PGE₂ signaling, we introduced wild type β-catenin or truncated β-catenin (∆-89) into LS-174T cells. Although LS-174T cells express mutated β-catenin (29), expression of stabilized β-catenin (∆-89) increased the activity of the *cyclin D1* promoter ~2-fold (Fig. 2D). Interestingly, PGE₂ and mutated β-catenin (∆-89) induced *cyclin D1* transcription in a synergistic manner (Fig. 2D).

**PGE₂ Induced the Expression of VEGF**—Tumor angiogenesis plays critical roles in tumor growth, and VEGF is one of the major regulators for neoangiogenesis. The regulation of VEGF is thought to involve TCF activation, and there are seven TCF binding elements within the promoter of VEGF1 (13). Treatment with PGE₂ increased VEGF1 mRNA in LS-174T cells, as analyzed by Northern blot (Fig. 3A). To determine whether PGE₂ increased VEGF protein and its secretion in colon cancer cells, LS-174T cells were treated with either vehicle or PGE₂. Levels of VEGF in cell culture medium were determined by enzyme-linked immunosorbent assay. The basal level of VEGF protein in LS-174T cell culture medium was ~120 pg/ml. PGE₂ stimulation increased the levels of VEGF ~2-fold (Fig. 3B). To determine whether PGE₂ regulated VEGF1 expression at the transcriptional level, LS-174T cells were transfected with a VEGF1 promoter-driving reporter vector. As shown in Fig. 3C, PGE₂ treatment increased the transcription of VEGF1 ~5-fold (Fig. 3C). Although inhibition of PKA activity strongly blocked the PGE₂-induced VEGF transcription, PD-153035 and PD-98059 also significantly reduced the PGE₂-stimulated VEGF promoter activity. These results suggested that PGE₂ induced VEGF transcription predominantly through activation of the cAMP/PKA pathway; however, transactivation of the EGFR system also contributed to PGE₂-induced VEGF expression. Furthermore, the ectopic expression of stabilized β-catenin (∆89) increased the activity of the VEGF1 promoter ~3-fold, compared with the cells transfected with empty vector or wild...
shown are representative of three separate experiments. *, p < 0.05. Results shown are representative of three separate experiments. A, induction of VEGF mRNA by PGE2. LS-174T cells were treated with 0.5 μM PGE2 for the indicated times; total RNA was extracted. Levels of VEGF were analyzed by Northern blot. B, induction of VEGF protein by PGE2. LS-174T cells (2 × 10^5) were seeded in a 24-well plate, and serum was deprived for 48 h prior to PGE2 treatment. After cells were treated with 0.5 μM PGE2, culture media were collected for enzyme-linked immunosorbent assay. Plotted is the mean ± S.D. of VEGF (pg/ml) performed in triplicate. *, p < 0.05. Results shown are representative of three separate experiments. C, roles of PKA in PGE2 induction of VEGF. LS-174T cells were transiently transfected with VEGF reporter vector. Cells were treated with 0.5 μM PGE2 in the presence of vehicle (V), 5 μM H-89 (H89), 1 μM PD-153035 (PD15), 10 μM LY-294002 (LY), or 25 μM PD-98059 (PD98) for 6 h. Plotted is the mean ± S.D. of Renilla-adjusted luciferase values performed in quadruplicate. *, p < 0.05. Results shown are representative of three separate experiments. D, synergy between PGE2 and β-catenin on VEGF transcription. LS-174T cells were transiently co-transfected with VEGF promoter (-2279 to +54) reporter vector along with empty vector, wild type β-catenin (WT), or Δ89 β-catenin (Δ89). PGE2 (0.5 μM) was added 6 h prior to harvest. Plotted is the mean ± S.D. of Renilla-adjusted luciferase values performed in quadruplicate. *, p < 0.05. Results shown are representative of three separate experiments.

*PGE2* Stimulated GSK-3 Phosphorylation—PGE2 enhances the phosphorylation of GSK-3α in human embryonic kidney cells that ectopically express E-prostanoid receptors (30) and in human neuronal cells (31). The phosphorylation of GSK-3 inhibits its kinase activity, which is required for phosphorylation and degradation of β-catenin (32). To determine whether PGE2 increases the phosphorylation of GSK-3 in human colon cancer cells, LS-174T cells were treated with PGE2. Levels of phosphorylated GSK-3α were rapidly elevated (Fig. 3A). An increase in the level of β-catenin was detected as well. The function of inhibition of the GSK-3 was next investigated. LS-174T cells were treated with LiCl, which is an established inhibitor of both GSK-3α and GSK-3β (33). Treatment with LiCl at 10–20 mM increased the transcriptional activity of TOPflash reporter, cyclin D1 promoter, and VEGF promoter 2–4-fold (Fig. 3B). Because LiCl is not a selective inhibitor of GSK-3 (34), we tested two more specific GSK-3 inhibitors for their ability to induce TCF-dependent transcription in LS-174T cells. SB-216763 and BIO are structurally distinct inhibitors of GSK-3α and GSK-3β (35, 36). Both SB-216763 and BIO at relatively low concentrations (0.1–1 μM) significantly induced the transcription of TOPflash reporter, cyclin D1 promoter, and VEGF promoter (Fig. 3C).

**PGE2** Induced TCF-4 Expression—The stimulatory effect of PGE2 on TCF-dependent transcription was significantly stronger than the effects of GSK-3 inhibitors, suggesting that PGE2 transactivation of TCF-mediated transcription involved additional mechanisms. TCF-4 is a member of the TCF transcription factor family, which is expressed in intestinal epithelial cells (37). TCF-4 activates transcription of target genes when associated with β-catenin. Cyclin D1 and c-myc have been identified as targets of TCF-4 (11, 12). To determine whether PGE2-stimulated TCF transcription involved the regulation of TCF-4 expression, levels of TCF-4 protein were examined in LS-174T cells. Exposure to PGE2 strongly increased the levels of TCF-4.
of TCF-4 protein in LS-174T cells (Fig. 5A). Previous studies have shown that TCF-4 plays critical role in TCF-dependent transcription in LS-174T cells (29). We found that ectopic expression of wild type TCF-4 protein dramatically elevated the transcription of TOPflash; addition of PGE2 further increased TCF transcription in a synergistic manner (Fig. 5B, left panel). Similar results were observed when LS-174T cells were co-transfected with the TCF-4 expression vector along with the cyclin D1 promoter reporter (Fig. 5B, middle panel). Expression of TCF-4 increased cyclin D1 transcription $\sim$5-fold; addition of PGE2 further increased the TCF-4-induced cyclin D1 transcription in an additive manner. In contrast, expression of TCF-4 did not significantly alter VEGF transcription, and there were no collaborative actions between TCF-4 and PGE2 on VEGF transcription as well (Fig. 5B, right panel). It has been demonstrated that expression of a dominant negative TCF-4 protein (dnTCF-4), which does not bind to $\beta$-catenin and acts as a potent inhibitor of the $\beta$-catenin-TCF complex, strongly inhibits TCF-dependent transcription in LS-174T cells (29). The expression of dnTCF-4 reduced the basal activity of TOPflash by $\sim$70%. Interestingly, dnTCF-4 almost completely attenuated the PGE2-induced TCF activation in LS-174T cells (Fig. 5C, left panel). Moreover, dnTCF-4 decreased the basal activity of cyclin D1 promoter by $\sim$50% but completely attenuated the PGE2-induced cyclin D1 transcription (Fig. 5C, middle panel). In contrast, expression of dnTCF-4 did not alter the basal transcriptional activity of VEGF and only slightly reduced the PGE2-induced VEGF transcription (Fig. 5C, right panel).

Synergistic Action of $\beta$-Catenin and MEK-1 on VEGF Transcription—To further elucidate the molecular mechanism by which PGE2 synergistically enhanced $\beta$-catenin-induced VEGF transcription, we investigated the interaction between $\beta$-catenin-TCF signaling and other signaling pathways. Because PGE2-induced VEGF transcription involved the transactivation of EGFR/MEK signaling (Fig. 3C), collaborative action between $\beta$-catenin and MEK-1 was evaluated. LS-174T cells were co-transfected with $\beta$-catenin ($\Delta$-89) and active MEK-1 expression vectors along with the VEGF promoter reporter. Expression of either $\beta$-catenin ($\Delta$-89) or MEK-1 increased VEGF transcription; however, in combination, they stimulated the activation of VEGF promoter in a synergistic manner (Fig. 6).

PGE2 Induced Cyclin D1 and VEGF in Vivo—Numerous studies demonstrate that inhibition of COX-2 enzyme results in decreased tumor cell proliferation in APC-/- mouse polyps, suggesting that COX-2-derived PGs are critical for tumor cell proliferation in germ line APC mutation-induced neoplasia (3). To determine whether PGE2 was able to activate $\beta$-catenin/TCF signaling and stimulate the expression of TCF target genes in vivo, 16,16-dimethyl PGE2 was administered to APC-/- mice. Protein was extracted from the polyps of an APC-/- mouse intestine. The expression of cyclin D1 protein was barely detected in vehicle-treated APC-/- mouse tumors. In contrast, cyclin D1 was strongly expressed in all tumors collected from PGE2-treated mouse intestine (Fig. 7A).
experiments. Group included three mice, and three polyps were collected from each mouse 2 h after 16,16-dimethyl PGE2 treatment. Protein was extracted from polyps, and the levels of cyclin D1 were analyzed. Both Western blots (V and PGE2) were subjected to a completely identical procedure. Results shown are representative of two separate experiments. B, PGE2 induction of VEGF mRNA. APC<sup>min/+</sup> mice were treated with vehicle or 16,16-dimethyl PGE2 for the indicated times (2 mice/time point). Levels of VEGF mRNA from pooled intestinal polyps were determined by Northern analyses. Results were similar in two independent experiments. C, PGE2 induction of VEGF protein. APC<sup>min/+</sup> mice were treated with vehicle or 16,16-dimethyl PGE2. Each group included three mice, and three polyps were collected from each mouse 2 h after the treatment. The levels of VEGF protein were analyzed by Western blot. Results were similar in two separate experiments.

Disruption of COX-2 gene or inhibition of COX-2 enzyme decreases tumor angiogenesis and VEGF expression in APC<sup>Δ716</sup> mouse adenomas (6). To determine whether PGE2 stimulated the expression of VEGF in vivo, 16,16-dimethyl PGE2 was administered to APC<sup>min/+</sup> mice. RNA and protein were extracted from polyps of APC<sup>min/+</sup> mouse ileum. Levels of VEGF mRNA (Fig. 7B) and protein (Fig. 7C) were dramatically elevated in APC<sup>min/+</sup> mouse tumors following administration of 16,16-dimethyl PGE2.

**DISCUSSION**

COX-2 is expressed at low levels in normal intestinal mucosa; its activity increases dramatically following mutations of the APC gene, suggesting that COX-2-generated prostaglandins are involved in the oncogenic activity of the APC/β-catenin/TCF pathway. It has been shown that PGE2 transactivates the β-catenin/TCF pathway in human embryonic kidney-293 cells, which ectopically express EP<sub>2</sub> or EP<sub>4</sub> receptors (30). In the present study, we found that PGE2 activated TCF-dependent transcription in colon cancer cells at concentrations of 1–100 nM, which were similar to the levels of PGE2 detected either in APC<sup>min/+</sup> polyps or in colon cancer cells (5, 26). In support with these results, inhibition of COX-2 enzyme activity significantly reduced TCF activity in HCA-7 cells, suggesting that COX-2/PGE<sub>2</sub> signaling may stimulate cell proliferation through transactivation of the TCF-dependent transcription. Mechanistically, we found that PGE2-stimulated TCF transcription involved phosphorylation of GSK-3 and induction of TCF-4 transcription factor, which formed a transcriptionally active complex with β-catenin. These results suggest a positive feedback loop between the APC/β-catenin/TCF pathway and the COX-2/PGE2 signaling system. APC mutation-activated TCF transcription increases the expression of COX-2 and production of PGE<sub>2</sub> (3, 38), which then in turn enhances the β-catenin/TCF-mediated transcription through a cAMP/PKA-dependent mechanism. Apparently, PGE2 induced oncogenic activity is crucial for tumor development in APC mutation-initiated colorectal carcinogenesis, as inhibition of COX-2, a key enzyme for conversion of arachidonic acid to PGs, results in a dramatic reduction of tumor number and size in humans and mice with germ line APC mutations (2, 3).

Numerous studies demonstrate that expression of COX-2 is associated with a growth advantage in colon cancer cells (16, 17). The molecular mechanism by which COX-2 promotes proliferation and growth of cancer cells is complex. Recent studies suggest that PGE2 may transactivate the EGFR signaling system through various mechanisms (17, 21, 39, 40). In the present study, we showed that PGE2 induced the transcription of cyclin D1, a TCF-4 target gene, through induction of both β-catenin and TCF-4. Progression through the mid to late G<sub>1</sub> phase of the mammalian cell cycle is dependent upon the cyclin D1-mediated activation of cyclin-dependent kinases (41). The activated cyclin D-dependent kinases phosphorylate and inactivate the retinoblastoma protein, thereby preventing its inhibition of transcription factors including the E2Fs that are essential for DNA synthesis. The expression of cyclin D1 is elevated in both the familial adenomatous polyposis patient tumor and APC<sup>min/+</sup> mouse polyp and is thought to be critical for APC mutation-induced neoplasia (42). Given the critical roles of cyclin D1 in cell cycle progression and in colorectal carcinogenesis, our results provide an additional mechanism by which COX-2/PGE<sub>2</sub> promotes the proliferation and growth of colon cancer cells.

VEGF stimulates neangiogenesis by inducing endothelial cell proliferation, migration, and tubular organization. Seven consensus TCF binding sites have been mapped in the promoter of the human VEGF-1 gene (13). Levels of VEGF-1 mRNA are significantly elevated in primary colon cancers, which contain a mutated APC gene in comparison with tumors that have a wild type APC<sub>1</sub> gene, suggesting that VEGF-1 is an APC/β-catenin/TCF target gene. On the other hand, COX-2/PGE<sub>2</sub> signaling plays critical roles in neangiogenesis; homozygous deletion of the EP<sub>2</sub> receptor significantly reduces the number and size of intestinal polyps in APC<sup>Δ716</sup> mice, which is associated with a reduction of VEGF expression, suggesting that PGE2/EP<sub>2</sub> signaling is critical for increased levels of VEGF in intestinal neoplasm (6). In support with these observations, we found that administration of 16,16-dimethyl PGE2 strongly increased levels of VEGF mRNA and protein in APC<sup>min/+</sup> mouse polyps. Moreover, our data demonstrated that inhibition of GSK-3 or ectopic expression of mutated β-catenin significantly increased VEGF transcription, indicating that VEGF-1 is transcriptionally regulated by β-catenin/
TCF. However, expression of TCF-4 did not increase VEGF promoter activity; additional experiments are required to identify which member(s) of the TCF/LEF family mediated the activation of VEGF transcription. It has been reported that PGE2 induction of VEGF in HCT-116 colon cancer cells is mediated by the transcriptional activator hypoxia-inducible factor 1 (20). Our results showed that PGE2-induced VEGF transcription involved transactivation of the EGFR system and the MEK pathway synergistically enhanced the β-catenin-induced VEGF transcription. These findings suggest that regulation of VEGF expression by PGE2 is mediated by a complex mechanism and the synergetic actions of PGE2 on VEGF transcription involves both β-catenin/TCF-dependent and -independent mechanisms.

Cumulative evidence suggests that the COX-2/PGE2 signaling pathway plays critical roles in neoplasia of a variety of organs (43, 44); however, this pathway is not a putative oncogenic pathway, which phosphorylates GSK-3 and inhibits its kinase activity and targets transcription through regulating levels of both β-catenin and TCF-4. In addition, PGE2 may synergistically enhance the transcriptional activity of the β-catenin/TCF pathway through transactivating other signaling systems. Previously, we have shown that PGE2 and the EGFR signaling system synergistically stimulate the growth and migration of colon cancer cells (40). In addition, PGE2 and the Ras/MAPK pathway synergistically induce the expression of an EGFR-like growth factor, amphiregulin. Our studies provide an interesting hypothesis that PGE2 exerts promutagenic effects by stimulating critical oncogenic pathways, such as the β-catenin/TCF-4 pathway, the EGFR signaling system, and the Ras/MAPK pathway, in a collaborative fashion.

Fujino et al. (30) have reported that the EP2 signaling pathway induces TCF activity through a cAMP/PKA-dependent mechanism, whereas EP4-mediated TCF activation is largely dependent on the phosphatidylinositol 3-kinase pathway. Phosphorylation of β-catenin by GSK-3 is crucial for its degradation. Both PKA and phosphatidylinositol 3-kinase/Akt can phosphorylate GSK-3 and inhibit its kinase activity and thereby activate the β-catenin/TCF pathway (45). Our previous studies have shown that LS-174T cells express both EP2 and EP4 receptors and that activation of the EP4 receptor is critical for PGE2-induced cell migration (16). Additional studies are required to determine which EP receptor(s) is responsible for the PGE2-activated β-catenin/TCF-dependent transcription in colon cancer cells.

In summary, inactivation of GSK-3 by mutations of the APC gene, which stabilizes β-catenin and stimulates TCF-dependent transcription, is thought to be the earliest genetic event in colorectal carcinogenesis (46). COX-2-derived PGE2 may enhance the APC/β-catenin/TCF pathway via various mechanisms (Fig. 8). 1) PGE2 activates the cAMP/PKA pathway, which phosphorylates GSK-3 and further increases levels of cytosolic β-catenin. 2) PGE2 increases the expression of TCF-4 transcription factor, which forms transcriptionally active complex with β-catenin. 3) PGE2 transactivates other signaling pathways, which enhances the β-catenin/TCF-dependent transcription in a collaborative fashion. These findings provide a novel mechanism for the understanding of COX-2/PGE2 pro-neoplastic actions in colorectal neoplasia.

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FIG. 8. Hypothetical scheme for PGE2 transactivation of β-catenin/TCF-4-dependent transcription in colon cancer cells. Gs, stimulatory guanine nucleotide-binding protein; P, phosphorylation; APC, adenomatous polyposis coli; β-caten, β-catenin.
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\textit{J. Biol. Chem.} 2005, 280:26565-26572.
doi: 10.1074/jbc.M413056200 originally published online May 17, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M413056200

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