Role of Cyclooxygenase 2 in Protein Kinase C βII-mediated Colon Carcinogenesis*

Wangsheng Yu, Nicole R. Murray, Capella Weems, Lu Chen, Huiping Guo, Richard Ethridge, Jeffrey D. Ceci, B. Mark Evers, E. Aubrey Thompson, and Alan P. Fields‡

From the Sealy Center for Cancer Cell Biology and the Departments of Pharmacology and Toxicology, Human Biological Chemistry and Genetics and Surgery, The University of Texas Medical Branch, Galveston, Texas 77555-1048

Elevated expression of protein kinase C βII (PKCβII) is an early promotive event in colon carcinogenesis (Gokmen-Polar, Y., Murray, N. R., Velasco, M. A., Gatalica, Z., and Fields, A. P. (2001) Cancer Res. 61, 1375–1381). Expression of PKCβII in the colon of transgenic mice leads to hyperproliferation and increased susceptibility to colon carcinogenesis due, at least in part, to repression of transforming growth factor β type II receptor (TGF-βRII) expression (Murray, N. R., Davidson, L. A., Chapkin, R. S., Gustafson, W. C., Schattenberg, D. G., and Fields, A. P. (1999) J. Cell Biol., 145, 699–711). Here we report that PKCβII induces the expression of cyclooxygenase type 2 (Cox-2) in rat intestinal epithelial (RIE) cells in vitro and in transgenic PKCβII mice in vivo. Cox-2 mRNA increases more than 10-fold with corresponding increases in Cox-2 protein and PGE2 production in RIE/PKCβII cells. PKCβII activates the Cox-2 promoter by 2- to 3-fold and stabilizes Cox-2 mRNA by at least 4-fold. The selective Cox-2 inhibitor Celecoxib restores expression of TGF-βRII both in vitro and in vivo and restores TGF-β-mediated transcription in RIE/PKCβII cells. Likewise, the ω-3 fatty acid eicosapentaenoic acid (EPA), which inhibits PKCβII activity and colon carcinogenesis, causes inhibition of Cox-2 protein expression, re-expression of TGF-βRII, and restoration of TGF-β-mediated transcription in RIE/PKCβII cells. Our data demonstrate that PKCβII promotes colon cancer, at least in part, through induction of Cox-2, suppression of TGF-β signaling, and establishment of a TGF-β-resistant, hyperproliferative state in the colon epithelium. Our data define a procarcinogenic PKCβII → Cox-2 → TGF-β signaling axis within the colon epithelium, and provide a molecular mechanism by which dietary ω-3 fatty acids and nonsteroidal antiinflammatory agents such as Celecoxib suppress colon carcinogenesis.

Cancer has been described as a disease of aberrant signal transduction (1). Carcinogenesis is a multistep process characterized by progressive changes in the amounts or activity of proteins that regulate cellular proliferation, differentiation, and survival (1, 2). These changes can be mediated through both genetic and epigenetic mechanisms. Protein kinase C (PKC) is a family of ubiquitously expressed serine/threonine protein kinases whose members play central roles in cell proliferation, differentiation, and apoptosis (reviewed in Ref. 3). The discovery that PKC is a major cellular target for the transforming growth factor β (TGF-β) type II receptor (TGF-βRII) (Gokmen-Polar, Y., Murray, N. R., Velasco, M. A., Gatalica, Z., and Fields, A. P. (2001) Cancer Res. 61, 1375–1381) and accounts, at least in part, for the colonic hyperplasia and increased susceptibility to colon carcinogenesis characteristic of transgenic PKCβII mice (6, 7). Our studies to date indicate that PKCβII plays a critical role in the early stages of colon carcinogenesis by inducing the loss of TGF-β responsiveness, thereby imposing a hyperproliferative phenotype, two prominent characteristics of colon cancer. We have shown that the cellular phenotype induced by PKCβII is mediated through changes in gene expression. Thus, we have initiated a genomic analysis to identify PKCβII target genes in rat intestinal epithelial (RIE-1) cells. Among the gene targets induced by PKCβII is the inducible form of cyclooxygenase, Cox-2. Cox-2 was originally cloned as a phorbol ester-inducible protein that accounts for the increased PKC signaling in tumor initiation and progression (4). However, the relative contribution of individual PKC isozymes to carcinogenesis is not well understood. Ultimately, the role of individual PKC isozymes in carcinogenesis will be understood through identification of downstream targets that participate in specific aspects of the transformed phenotype.

We have focused our recent efforts on deciphering the role of specific PKC isozymes in the development of colon cancer (5–7). We have shown that colon carcinogenesis is accompanied by changes in PKC isozyme expression, including a dramatic increase in the level of PKCβII expression (5). PKCβII protein levels are elevated in preneoplastic lesions in the colon, aberrant crypt foci, and are further elevated in colon tumors (5). To determine whether elevated PKCβII levels contribute to colon carcinogenesis, we developed transgenic PKCβII mice that express elevated PKCβII in the colon epithelium to levels comparable with those observed in carcinogen-induced colon tumors (6, 7). These animals exhibit hyperproliferation of the colonic epithelium and enhanced susceptibility to carcinogen-induced carcinogenesis (6). We recently characterized the transforming growth factor β receptor type II (TGF-βRII) as a target for PKCβII-mediated transcriptional repression in intestinal epithelial cells and in the colonic epithelium of transgenic PKCβII mice (7). PKCβII-induced inhibition of TGF-βRII renders intestinal epithelial cells insensitive to growth inhibition by TGF-β and accounts, at least in part, for the colonic hyperproliferation and increased sensitivity to colon carcinogenesis characteristic of transgenic PKCβII mice (6, 7). Our studies to date indicate that PKCβII plays a critical role in the early stages of colon carcinogenesis by inducing the loss of TGF-β responsiveness, thereby imposing a hyperproliferative phenotype, two prominent characteristics of colon cancer. We have shown that the cellular phenotype induced by PKCβII is mediated through changes in gene expression. Thus, we have initiated a genomic analysis to identify PKCβII target genes in rat intestinal epithelial (RIE-1) cells. Among the gene targets induced by PKCβII is the inducible form of cyclooxygenase, Cox-2. Cox-2 was originally cloned as a phorbol ester-inducible gene (8, 9), and it has been implicated in the etiology of colon cancer in rodents and humans (10–13). Our present data demonstrate that Cox-2 is a specific genomic target of PKCβII and

*This work was supported by grants from the National Cancer Institute (to A. P. F.) (CA81436 and CA56869). 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.

‡To whom correspondence should be addressed: The Sealy Center for Cancer Cell Biology, The University of Texas Medical Branch, 301 University Blvd., MBB 9.104, Galveston, TX 77555-1048, Tel.: 409-747-1935; Fax: 409-747-1938; E-mail: afields@utmb.edu.

Published, JBC Papers in Press, December 11, 2002, DOI 10.1074/jbc.M211424200

This paper is available online at http://www.jbc.org
that PKCβII-mediated repression of TGF-βIII depends on Cox-2. Finally, we show that the chemopreventive ω-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA), a known PKCβII inhibitor in vivo and in vitro (7), inhibits Cox-2 expression, induces TGF-βIII expression, and restores TGF-β responsiveness in RIE/Cox-2 cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Treatments—**RIE-1 cells and derivatives were grown in 5% fetal bovine serum in Dulbecco’s modified Eagle’s medium as previously described (14). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. After removing the cultures were incubated in all experiments otherwise specified. Construction of RIE/PKCβII cells has been described elsewhere (7). RIE/PKC cells were produced by infection of RIE-1 cells with a retrovirus containing the full-length human PKCβII cDNA. RIE/H-Ras and RIE/Cox-2 cells were generous gifts of Drs. Hongmiao Sheng and Ray DuBois, Vanderbilt University (11). In some experiments, cells were incubated with ω-3 fatty acid EPA (Cayman Chemicals) at the concentrations and for the times indicated in the figure legends. In some cases, cells were incubated with 25 μM Cexolizib (UTMB Pharmacy) and/or 120 μM TGF-βIII (BD Biosciences) in the culture medium for the times indicated in the figure legends. EPA and Cexolizib were solubilized in dimethyl sulfoxide (Me2SO). A final Me2SO concentration of 0.1% was used for all treatments, and 0.1% Me2SO was used as a diluent control. The stability of the Cox-2 mRNA was determined in RIE-1 and RIE/PKCβII cells by treatment of cells with 25 μM 5,6-dichlorobenzimidazole ribose to inhibit RNA polymerase II. Total cellular RNA was isolated as described previously (14) at various times after dichlorobenzimidazole ribose exposure and subjected to real-time RT-PCR analysis for Cox-2 mRNA expression as described below.

**Immunoblot Analysis—**For immunoblot analysis, cells were washed twice with ice-cold phosphate-buffered saline and lysed in protein lysis buffer consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 0.1% SDS, and protease inhibitor mixture (Sigma) for 30 min on ice. Lysates were centrifuged at 10,000 g for 20 min, aliquots of total cellular protein (50 μg) were electrophoresed in 10% acrylamide Tris-glycine gels (Invitrogen) and electrophoretically transferred to polyvinylidene difluoride membrane (Bio-Rad). The membranes were incubated with 5% nonfat dried milk in 10 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.05% Tween 20 (TBST) overnight at 4°C to block excess protein sites. Membranes were incubated with rabbit polyclonal antibodies against PKCβII (Santa Cruz; 1:2,000 dilution), PKCβII (Santa Cruz; 1:6,000 dilution), Cox-2 (Cayman; 1:1,000 dilution), TGF-βIII (Santa Cruz; 1:1,000 dilution), or actin (Santa Cruz; 1:10,000 dilution) in TBST at room temperature for 1 h, after which the membranes were washed in TBST three times for 15 min each. The membranes were incubated in 1:1,000–1:10,000 (for Cox-2) conditions prior to conjugated goat anti-rabbit antibody (Kirkegaard and Perry Laboratories, 1:125,000 dilution) in TBST for 1 h at room temperature. The membranes were washed three times in TBST for 15 min each, and antigen-antibody complexes were detected using chemiluminescence (Amersham Biosciences) according to the manufacturer’s instructions.

**Treatment of Transgenic Mice with Celecoxib and Isolation of Colonic Epithelium—**Transgenic PKCβII mice expressing PKCβII in the colonic epithelium were characterized previously (6, 7). Transgenic PKCβII mice and nontransgenic littermates were administered 6 mg/kg Celecoxib by oral gavage twice daily for 3 days. As controls, some mice were administered an equivalent volume of diluent (0.5% carboxymethylkellulose). Mice were terminated on the morning of the fourth day. The membranes were incubated with rabbit polyclonal antibodies against PKCβII and PKCβIII (Santa Cruz; 1:2,000 dilution) in TBST (1:6,000 dilution), Cox-2 (Cayman; 1:1,000 dilution), TGF-βIII (Santa Cruz; 1:1,000 dilution), or actin (Santa Cruz; 1:10,000 dilution) in TBST at room temperature for 1 h, after which the membranes were washed in TBST three times for 15 min each. The membranes were incubated in 1:1,000–1:10,000 (for Cox-2) conditions prior to conjugated goat anti-rabbit antibody (Kirkegaard and Perry Laboratories, 1:125,000 dilution) in TBST for 1 h at room temperature. The membranes were washed three times in TBST for 15 min each, and antigen-antibody complexes were detected using chemiluminescence (Amersham Biosciences) according to the manufacturer’s instructions.

**Gene Microarray Analysis—**Gene profiling analysis was performed on total RNA from RIE-1, RIE/PKCβII, and RIE/Cox-2 cells using BG-U34A Gene Chips® microarrays (Affymetrix). Total RNA (25 μg) was used for first-strand cDNA synthesis using a T7-dT24 oligomer (5′-GGCCAGGTAGATGGTAA-GCGTACCTATAGGGAGCGCGGTT-3′) and SuperScript II reverse transcriptase (Invitrogen). The cDNA was converted to double-stranded DNA by transcription in vitro. cDNAs were synthesized using bacteriophage T7 RNA polymerase in the presence of biotinylated nucleotides. Biotin-labeled target RNAs were fragmented to a mean size of 200 bases according to the manufacturer’s protocol. Hybridization of the rat RG-U34A microarrays was performed at 45°C for 16 h in 0.1× 2-morpholinoethanesulfonic acid (MES) pH 6.8, 1 mM NaCl, 0.02 mM EDTA, and 0.05% Tween 20. Microarrays were washed using both nonstringent (1× 2×SSPE, 0.1% SDS for 20 min at 55°C) and stringent (1× 2×SSPE, 0.1% SDS for 20 min at 55°C) conditions prior to hybridization with phycoerythrin-labeled streptavidin (10 μg/ml final concentration). Data were collected using a Gene Array Scanner (Hewlett Packard) and analyzed using the Affymetrix GeneChip Analysis Suite 5.0 software.

**Real-time Reverse Transcriptase-Polymerase Chain Reaction Analysis of Gene Expression—**Real-time reverse transcriptase-polymerase chain reaction (real time RT-PCR) assays were used to determine gene expression using TaqMan technology on an Applied Biosystems 7000 sequence detection system. Applied Biosystems Assays-By-Design containing a 20× assay mix of primers and TaqMan MGB probes (FAMTM dye-labeled) were used for all target genes and the endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were designed using primers that span exon junctions so as not to detect genomic DNA. All primer and probe sequences were searched against the Celera data base to confirm specificity. The primer and probe sequences used were as follows: rat PAI-1 probe spanning exon8 CCAAGAGAGACAATCC, forward primer AGCATCTTCTTCTTGTGTTT, reverse primer CATCACTGGCCATCTGAAGAC, human KCII probe spanning the PKCβII/PKCβIII alternative splice junction TGGCAGAGACAGT, forward primer AAATCTGGAACCGAAGAGATCCA, reverse primer ATCCGTCAGTGTTCACGATT.
A Procarcinogenic PKCβII → Cox-2 → TGF-βRII Signaling Axis

11169

Fig. 1. Establishment of RIE cells expressing human PKCβII. RIE-1 cells were transfected with a retrovirus containing the full-length human PKCβII cDNA as described under “Experimental Procedures.”

A. immunoblot analysis of RIE-1, RIE/PKCβII, and HEK293 cells with antibodies to PKCβI, PKCβII, and actin. B. quantitative real-time RT-PCR assay for human PKCβII mRNA in HEK293, RIE-1, and RIE/PKCβII cells. Results are expressed as relative PKCβII mRNA abundance and represent the mean of triplicate determinations ± S.E.

RESULTS

Our recent studies demonstrated that elevated expression of PKCβII in the colonic epithelium is an early, promotive event in colon carcinogenesis (5–7). To elucidate the molecular mechanisms by which PKCβII mediates increased colon carcinogenesis, we established a cell model system in which to explore PKCβII-mediated signaling. RIE-1 cells are immortalized but not transformed and consequently express abundant PKCβII but little or no detectable PKCBII protein (Fig. 1A). This pattern of expression of PKCβI and PKCβII is consistent with that observed in the colonic epithelium in vivo (5). To assess the cellular and genomic effects of PKCβII expression, we created the RIE/PKCβII cell line that expresses abundant human PKCβII (Fig. 1A). A real-time RT-PCR assay for human PKCβII mRNA demonstrated that RIE/PKCβII cells expressed human PKCβII mRNA at levels somewhat lower than those observed in human HEK293 cells, which express abundant endogenous PKCβII protein (Fig. 1B). We previously demonstrated that the growth rate of RIE/PKCβII cells is indistinguishable from that of RIE-1 cells (7), indicating that overexpression of PKCβII has no significant effect on proliferation or apoptosis of RIE-1 cells in culture. Similarly, no changes in gross cellular morphology were noted in RIE/PKCβII cells, nor were these cells able to form colonies in soft agar, indicating that expression of PKCβII is not sufficient to cause cellular transformation (data not shown).

Fig. 2. The Cox-2 gene is induced by PKCβII. A. total RNA from RIE-1 and RIE/PKCβII cells was subjected to microarray analysis using Affymetrix Gene Chips as described under “Experimental Procedures.” Rat Cox-2 mRNA abundance was independently measured by real-time RT-PCR as described under “Experimental Procedures.” Results are plotted as signal intensity ± S.D. B. Northern blot analysis for Cox-2 mRNA was performed on total RNA isolated from RIE-1, RIE/PKCβII, and RIE/Cox2 cells as described under “Experimental Procedures.” An 18S RNA probe was used to assess RNA loading on the gel.

Affymetrix Gene Chips were used to identify genes that are either induced or inhibited in RIE/PKCβII cells when compared with RIE-1 cells. Significance analysis of microarrays (17) was used to compare expression profiles of RNA extracted from control RIE-1 cells (n = 7) and RIE/PKCβII cells (n = 3). Among the probe sets whose expression changed by >2.0-fold in RIE/PKCβII cells was that encoding the inducible form of cyclooxygenase, Cox-2. Because Cox-2 expression can be induced by phorbol esters (8, 9), and because of the strong association between Cox-2 expression and colon cancer (10–13), we focused our analysis on this gene. We confirmed our microarray analysis using a quantitative real-time RT-PCR assay specific for Cox-2 mRNA (Fig. 2A). The mean signal intensities obtained from Cox-2 probe sets in microarrays from RIE-1 (n = 7) and RIE/PKCβII (n = 3) cells (gray bars) correlated well with the level of Cox-2 mRNA detected by real-time RT-PCR (black bars), providing independent confirmation of elevated expression of Cox-2 RNA in RIE/PKCβII cells. Northern blot analysis indicated that Cox-2 mRNA expression was increased >10-fold in RIE/PKCβII cells when compared with RIE-1 cells (Fig. 2B), providing an independent confirmation of the microarray and real-time RT-PCR data. As a positive control, RIE/Cox2 cells, which express a Cox-2 transgene that is deleted of the 3′-untranslated region (18), was used to compare the abundance of Cox-2 mRNA in RIE/PKCβII cells expressing the endogenous, full-length Cox-2 transcript. The data in Fig. 2B indicate that Cox-2 mRNA is even more abundant in RIE/PKCβII cells

mix reagent kit (Applied Biosystems). The cycling parameters were as follows: reverse transcription, 48 °C for 30 min; AmpliTaq activation, 95 °C for 10 min; denaturation, 95 °C for 15 s; and annealing/extension, 60 °C for 1 min for 40 cycles. Duplicate CT values were analyzed in Microsoft Excel using the comparative CT(ΔΔCT) method as described by the manufacturer (Applied Biosystems).
Fig. 3. Cox-2 protein and enzyme activity is induced in RIE/PKCβII cells and in the colons of transgenic PKCβII mice. A, total cell lysates from RIE-1, RIE/PKCβII, and RIE/Cox2 cells were subjected to immunoblot analysis for PKCβII, Cox-2, and actin as described under “Experimental Procedures.” B, total cell lysates from RIE-1 and RIE/PKCβII cells were subjected to immunoblot analysis for Cox-2 and actin. C, PGE2 levels from culture supernatants from RIE-1, RIE/PKCβII, and RIE/Cox2 cells were measured by enzyme-linked immunosorbent assay as described under “Experimental Procedures.” Results are expressed as pg/ml PGE2 ± S.E. for three independent measurements. *p-values were determined by Student’s t test. D, lysates from colonic epithelium from transgenic PKCβII mice (6, 7) and nontransgenic littermates were subjected to immunoblot analysis for PKCβII, Cox-2, and actin as described under “Experimental Procedures.” Than in RIE/Cox2 cells. Real-time RT-PCR analysis confirmed that RIE/PKCβII cells express 1.4-fold more PKCβII mRNA than RIE/Cox2 cells. Taken together, these data provide conclusive evidence that Cox-2 mRNA is induced by PKCβII in RIE-1 cells.

Immunoblot analysis was used to assess the level of Cox-2 protein expression in RIE-1, RIE/PKCβII, and RIE/Cox2 cells (Fig. 3A). Cox-2 protein was expressed at nearly undetectable levels in RIE-1 cells. In contrast, RIE/PKCβII cells expressed abundant Cox-2 protein comparable with the amount of Cox-2 protein expressed in RIE/Cox2 cells. To assess whether induction of Cox-2 expression is specific for PKCβII expression, we assayed Cox-2 protein levels in RIE/PKCβII cells, which were engineered to overexpress transgenic human PKCβII (Fig. 3B). RIE/PKCβII cells expressed very low levels of Cox-2 (which could be observed only after very long exposures of the immunoblots) comparable with those observed in RIE-1 cells. These results are consistent with our microarray analysis of RIE/PKCβII cells, which did not identify Cox-2 as a potential transcriptional target of PKCβII (data not shown). These results indicate that induction of Cox-2 is not a general response to the expression of any PKC isozyme, but rather is a specific response to PKCβII expression.

To assess whether the Cox-2 protein expressed in RIE/PKCβII cells was functional, RIE-1, RIE/PKCβII, and RIE/Cox2 cells were assayed for production of PGE2, a product of Cox-2 enzyme activity (Fig. 3C). Enzyme-linked immunosorbent assay analysis of culture supernatants demonstrated that RIE/PKCβII cells, like RIE/Cox2 cells, secreted 3- to 4-fold higher levels of PGE2 than RIE-1 cells. These results demonstrate that PKCβII induced the expression of active Cox-2 enzyme in RIE/PKCβII cells.

We next wished to determine whether Cox-2 is also a target for PKCβII regulation in the colonic epithelium in vivo. We have developed transgenic PKCβII mice overexpressing PKCβII in the colonic epithelium that exhibit an increased sensitivity to azoxymethane-mediated colon carcinogenesis (6, 7). Immunoblot analysis of colonic epithelium from nontransgenic and transgenic PKCβII mice demonstrate that transgenic PKCβII mice expressed significantly more PKCβII and Cox-2 protein than their nontransgenic littermates (Fig. 3D). These data demonstrate that Cox-2 is a significant genomic target of PKCβII both in RIE-1 cells in culture and in the colonic epithelium in vivo.

We next assessed the mechanism by which PKCβII leads to elevated Cox-2 mRNA levels in RIE-1 cells. For this purpose, a Cox-2 promoter/luciferase reporter gene was transiently co-transfected into RIE-1 cells along with a PKCβII expression vector to assess the effect of PKCβII on Cox-2 promoter activity (Fig. 4A). The activity of the Cox-2/luciferase reporter was increased by 2- to 3-fold in RIE-1 cells in which a PKCβII expression vector was simultaneously introduced. These experiments utilized a human Cox-2 promoter construct consisting of 4 kb of 5’-flanking sequence, but consistent results were also obtained using promoters containing 7 kb or 1.4 kb of 5’-flanking sequence (data not shown). To independently assess the effect of PKCβII expression on Cox-2 promoter activity, the Cox-2 promoter/luciferase reporter was transfected into RIE-1, RIE/PKCβII, and RIE/Ras cells, and the activity of the reporter was assessed by luciferase assay (Fig. 4B). RIE/Ras cells express an activated H-Ras allele previously shown to activate Cox-2 transcription (11, 19) and served as a positive control for activation of Cox-2 promoter activity. Consistent with the transient cotransfection data shown in Fig. 4A, the Cox-2 promoter was 2–3 times more active in RIE/PKCβII cells than in RIE-1 cells. As expected, the Cox-2 promoter was also more active, by about 5-fold, in RIE/Ras cells, which are known to express high levels of Cox-2 (11). These data demonstrate that PKCβII causes a 2- to 3-fold increase in Cox-2 promoter activity in RIE cells. Although this represents a significant and reproducible increase in Cox-2 promoter activity, the magnitude of the effect was clearly not sufficient to account for the dramatic increase in Cox-2 mRNA expression observed in RIE/PKCβII cells. Taken together, these data indicate that an additional mechanism(s) may be responsible for the effects of PKCβII on Cox-2 mRNA levels in RIE-1 cells.

The Cox-2 gene is known to be regulated not only at the transcripational level but also at the level of mRNA stability (20–22). Therefore, Cox-2 mRNA stability was compared in RIE-1 and RIE/PKCβII cells by measuring mRNA abundance by quantitative real-time RT-PCR as a function of time after addition of the nonspecific RNA polymerase II inhibitor dichlorobenzimidazole riboside (Fig. 4C). Cox-2 mRNA in RIE-1 cells was relatively unstable with an estimated $t_{1/2}$ of degradation of $\sim 12–16$ min. In contrast, the apparent $t_{1/2}$ of degradation of Cox-2 mRNA in RIE/PKCβII cells was greater than 50 min, indicating that PKCβII expression results in significant stabilization of Cox-2 mRNA. Thus, PKCβII-mediated elevation of Cox-2 mRNA levels result from a combined effect of PKCβII on Cox-2 gene transcription and Cox-2 mRNA stability.

We recently showed that PKCβII inhibits expression of TGF-βRII both in the colonic epithelium of transgenic PKCβII mice and in RIE/PKCβII cells (7). Therefore, we assessed whether the effect of PKCβII on TGFβRII expression is mediated through Cox-2 activation (Fig. 5). RIE-1 cells expressed abundant TGFβRII protein as assessed by immunoblot analysis...
ment of RIE/PKC values were calculated by Student repress of TGF-

Procedures.

times, cells were lysed, and the amount of Cox-2 mRNA was assessed by quantitative real-time-RT-PCR as described under "Experimental Procedures." B, RIE-1, RIE/PKCII, and RIE/H-Ras cells were transiently transfected with the Cox-2 promoter construct, and promoter activity was assessed by luciferase assay as described in under "Experimental Procedures." Results in A and B are expressed as relative luciferase activity and represent the means from triplicate determinations ± S.E. C, RIE-1 and RIE/PKCII cells were incubated with the RNA polymerase inhibitor dichlorobenzimidazole riboside. At the indicated times, cells were lysed, and the amount of Cox-2 mRNA was assessed by quantitative real-time-RT-PCR as described under "Experimental Procedures."

Fig. 4. PKCβII induces Cox-2 gene transcription and stabilizes the Cox-2 mRNA. A, RIE-1 cells were transiently transfected with a luciferase reporter construct containing the Cox-2 promoter along with the indicated amount of a PKCβII expression vector. The activity of the Cox-2 promoter was determined by luciferase assay as described under "Experimental Procedures." B, RIE-1, RIE/PKCII, and RIE/H-Ras cells were transiently transfected with the Cox-2 promoter construct, and promoter activity was assessed by luciferase assay as described under "Experimental Procedures." Results in A and B are expressed as relative luciferase activity and represent the means from triplicate determinations ± S.E. C, RIE-1 and RIE/PKCII cells were incubated with the RNA polymerase inhibitor dichlorobenzimidazole riboside. At the indicated times, cells were lysed, and the amount of Cox-2 mRNA was assessed by quantitative real-time-RT-PCR as described under "Experimental Procedures."

Fig. 5. PKCβII-mediated repression of TGF-βRII expression depends on Cox-2. A, RIE-1 (lane 1) and RIE/PKCβII (lanes 2–5) cells were subjected to immunoblot analysis for TGF-βRII and actin as described under “Experimental Procedures.” RIE/PKCβII cells were incubated in the absence (lane 2) or presence (lanes 3–5) of 25 μM Celecoxib for 0 h (lane 3), 24 h (lane 4), or 48 h (lane 5) prior to lysis and immunoblot analysis. B, RIE/PKCβII cells were transiently transfected with a Cox-2 promoter construct and treated with either nothing, TGF-β1, Celecoxib, or both as indicated in the figure. Cox-2 promoter activity was measured by luciferase assay as described under “Experimental Procedures." Results are expressed as relative luciferase activity and represent the mean of three determinations ± S.E. p values were calculated by Student's t test. C, the abundance of endogenous PAI-1 mRNA was assessed by real-time RT-PCR analysis of RNA isolated from RIE/PKCII cells treated with nothing, TGF-β1, or TGF-β1 and Celecoxib for 24 h or 48 h as indicated. Results are expressed as the mean of three independent determinations ± S.E. p values were calculated by Student’s t test. D, transgenic PKCβII mice were treated with either vehicle or Celecoxib and isolated colonic epithelium was subjected to immunoblot analysis for TGF-βRII and actin as described under “Experimental Procedures.”

(Fig. 5A, lane 1), whereas RIE/PKCβII cells expressed significantly lower levels of TGF/βRII protein (Fig. 5A, lane 2). Treatment of RIE/PKCβII cells with 25 μM Celecoxib for 0, 24, or 48 h led to a time-dependent increase in TGF-βRII protein expression (Fig. 5A, lanes 3–5). We have also observed that treatment of RIE-1 cells with Celecoxib leads to increased TGF-βRII protein expression, indicating that Cox-2 exerts a tonic suppressive effect on TGF-βRII expression in RIE-1 cells that can be reversed by inhibition of the enzyme(data not shown).

RIE/PKCβII cells exhibit a profound loss of TGF-β-mediated transcriptional activity as a consequence of PKCβII-mediated repression of TGF-βRII expression (7). We therefore assessed the ability of Celecoxib to restore TGF-β-mediated transcriptional activity in RIE/PKCβII cells (Fig. 5, B and C). RIE/ PKCβII cells were transiently transfected with a TGF-β-responsive luciferase reporter plasmid and then treated with either TGF-β1, Celecoxib, or both (Fig. 5B). RIE/PKCβII cells exhibited little or no transcriptional response to TGF-β1, consistent with our previous results (7). However, when these cells were treated with Celecoxib prior to exposure to TGF-β1, they exhibited a robust transcriptional response. Celecoxib treatment had no effect in the absence of TGF-β1, demonstrating that the observed transcriptional effects of Celecoxib are TGF-β-dependent. Consistent with these results, the level of the mRNA for the endogenous TGF-β1-responsive gene, plasminogen activator inhibitor-1 (PAI-1) (23), was dramatically induced when RIE/PKCβII cells were treated with Celecoxib for 24 or 48 h prior to exposure to TGF-β1 (Fig. 5C).
A Procarcinogenic PKCβII → Cox-2 → TGF-βRII Signaling Axis

To determine whether the PKCβII-mediated repression of TGF-βRII expression in Cox-2-dependent in vivo, we assessed the effect of treating transgenic PKCβII mice with Celecoxib on TGF-βRII expression in the colonic epithelium (Fig. 5D). Treatment of transgenic PKCβII mice with Celecoxib led to reexpression of TGF-βRII as assessed by immunoblot analysis. Therefore, PKCβII-mediated repression of TGF-βRII expression and TGF-β-responsiveness are dependent on Cox-2 activity both in intestinal epithelial cells in vitro and in the colonic epithelium in vivo.

Chemopreventive dietary ω-3 fatty acids such as EPA block Cox-2 induction in azoxymethane-treated mice (24). We and others have shown that azoxymethane induces colonic PKCβII expression (5, 25). We have found that a diet high in ω-3 fatty acids inhibits colonic PKCβII activity, induces TGF-βRII expression, and blocks PKCβII-mediated colon carcinogenesis in transgenic PKCβII mice (7). These observations lead to the hypothesis that the chemopreventive effects of ω-3 fatty acids are mediated through inhibition of a PKCβII → Cox-2 → TGF-β signaling pathway. To test this hypothesis, we treated RIE-1 and RIE/PKCβII cells with EPA, an ω-3 fatty acid found in fish oil, and measured TGF-βRII and Cox-2 expression by immunoblot analysis (Fig. 6A). Treatment of RIE/PKCβII cells with EPA led to a dose-dependent increase in TGF-βRII expression and a concomitant decrease in Cox-2 expression (Fig. 6A, left panel). This effect was dependent on PKCβII expression because no significant changes in either TGF-βRII or Cox-2 expression were observed in RIE-1 cells treated with EPA (Fig. 6A, right panel). Quantitative analysis of these data revealed that Cox-2 expression was inhibited by >50% in RIE/PKCβII cells but was unaffected in RIE-1 cells (Fig. 6B). On the other hand, TGF-βRII was induced in RIE/PKCβII cells but not in RIE-1 cells treated with EPA (Fig. 6C). These data are consistent with our recent observation that a diet high in ω-3 fatty acids induces TGF-βRII expression in the colonic epithelium of transgenic PKCβII mice and blocks PKCβII-mediated colon carcinogenesis (7). They also establish a direct link between cancer-preventive dietary ω-3 fatty acids, PKCβII activity, Cox-2 expression, and TGF-β signaling in vitro and in vivo.

DISCUSSION

We have focused our recent attention on the role of individual PKC isozymes in the development of colon cancer (5–7). We have found that PKCβII, which is expressed at very low levels in the proliferative zone of normal colonic epithelium, is rapidly induced in the colonic epithelium of azoxymethane-treated rodents and is abundantly expressed in both preneoplastic aberrant crypt foci and colon tumors that form following azoxymethane treatment (5). Transgenic PKCβII mice overexpressing PKCβII in the colonic epithelium exhibit hyperproliferation and increased susceptibility to azoxymethane-induced colon carcinogenesis (6, 7), demonstrating that PKCβII plays a critical promotive role in colon carcinogenesis.

We also recently demonstrated that PKCβII is an important cellular target for the cancer-preventive activity of dietary ω-3 fatty acids (7). Diets high in ω-3 fatty acids have been shown to block colon carcinogenesis in rodent models (7, 26–29), and epidemiologic studies indicate that ω-3 fatty acids have chemopreventive effects against colon cancer in humans (30–33). We found that ω-3 fatty acids, which are abundant in dietary fish oils, inhibit colonic PKCβII activity and suppress the hyperproliferative and cancer-prone phenotype of transgenic PKCβII mice (7). In those studies, we also established that the TGF-βRII gene is a target for repression by PKCβII (7).

The present studies were initiated in an effort to further elucidate the molecular mechanism(s) by which PKCβII promotes colon carcinogenesis. The data presented herein identify the Cox-2 gene as a prominent target for PKCβII-mediated regulation in intestinal epithelial cells in vitro and the colonic epithelium in vivo.

Fig. 6. EPA inhibits Cox-2 expression and restores TGF-βRII expression in RIE/PKCβII cells. A, RIE-1 and RIE/PKCβII cells were cultured in the presence of the indicated concentration of EPA for 4.5 h prior to harvest and immunoblot analysis for TGF-βRII, Cox-2 and actin as described under “Experimental Procedures.” B and C, quantitative analysis of the immunoblot analysis in A for Cox-2 (B) and TGF-βRII (C) expression.
colon carcinogenesis. Based on our current and previously published data, we propose a model for how PKCβII and Cox-2 promote colon cancer (Fig. 7). In this model, the tissue-selective carcinogen azoxymethane induces PKCβII expression in the colonic epithelium, an event that has been well documented by our group and others (5, 25). Our current data show that PKCβII induces Cox-2 expression in intestinal epithelial cells in vitro and in the colonic epithelium in vivo. We have also demonstrated that PKCβII leads to repression of TGF-βII expression and TGF-β1 signaling in RIE cells and to a loss of TGF-βII expression in the colonic epithelium of transgenic PKCβII mice (7). In the present study, we demonstrate that PKCβII-mediated repression of TGF-βII expression and signaling can be reversed by the Cox-2 inhibitor Celecoxib, indicating that PKCβII mediates its effects on TGF-β signaling through Cox-2. These data are consistent with the recent association between elevated Cox-2 expression and loss of TGF-βII expression in RIE cell variants selected for loss of TGF-β1 responses (36).

The effects of Cox-2 in intestinal epithelial cells and in colon tumors are thought to be mediated through production of prostanoids, particularly PGE2 (37). PGE2 in turn signals through binding to specific PGE2 receptors, of which there are four well characterized members, termed EP1 through EP4 (reviewed in Ref. 38). The EPs are members of the G-protein-coupled receptor family whose downstream effectors include adenylate cyclase and phosphatidylinositol-phospholipase C (38). Accumulating evidence demonstrates that EP1 plays a pivotal role in colon carcinogenesis (39–41). Specifically, two different pharmacologic inhibitors selective for EP1 have been shown to inhibit formation of preneoplastic aberrant cryptic foci in azoxymethane-treated mice and of intestinal polyps in APCmin mice (39–41). Furthermore, mice that are nullizygous for EP1 exhibit suppressed colon carcinogenesis (39).

It is well documented that EP1 signals through activation of PI-PLC and generation of the second messengers diacylglycerol and inositol trisphosphate, which in turn lead to intracellular calcium mobilization and PKC activation (38). Based on these observations, and our present data, it is attractive to suggest that PGE2-mediated activation of EP1 leads to activation of PKCβII, a classical PKC isozyme, generating an autocrine positive-feedback loop. In this model, activated PKCβII in turn causes repression of TGF-βRII function by an as-yet-unidentified mechanism. It should be noted, however, that the role of EPs in colon cancer development appears to be complex. In this regard, both EP2 (42) and EP4 (43) have also been implicated in colon carcinogenesis, indicating that PGE2 can activate multiple signaling pathways in the colonic epithelium. The complex role of EPs, as well as other possible Cox-2 products, in PKCβII-mediated effects in intestinal epithelial cells will require further experimentation.

In recent studies, we demonstrated that the chemopreventive effects of dietary ω-3 fatty acids are mediated, at least in part, through inhibition of PKCβII activity in the colonic epithelium (7). Furthermore, we demonstrated that ω-3 fatty acids can block the hyperproliferation and enhanced colon carcinogenesis exhibited by transgenic PKCβII mice through inhibition of colonic PKCβII activity (7). In this study we show that ω-3 fatty acids inhibit Cox-2 expression and induce TGF-βRII by a mechanism that depends on PKCβII expression. These data are consistent with the model proposed in Fig. 7, because there is abundant evidence that dietary ω-3 fatty acids prevent azoxymethane-induced elevation of Cox-2 expression in the colonic epithelium (24). ω-3 fatty acids such as EPA have been shown in some cell systems to inhibit Cox-2 activity (44, 45). However, our data indicate that EPA does not induce TGF-βRII expression in RIE/PKCβII cells through inhibition of Cox-2 for the following reasons. First, both RIE-1 and RIE/PKCβII cells express active Cox-2 enzyme (Fig. 3C). Despite that fact, EPA induces TGF-βRII expression in RIE/PKCβII cells, but not in RIE-1 cells (Fig. 6). Treatment of RIE-1 cells with Celecoxib however, leads to elevated expression of TGF-βRII in both RIE-1 and RIE/PKCβII cells. If EPA were acting through inhibition of Cox-2 activity, it should induce TGF-βRII expression in both cell lines. Therefore, it is unlikely that the effects of EPA on TGF-βIIR expression in RIE/PKCβII cells are caused by Cox-2 inhibition.

The accumulating evidence that PKCβII and Cox-2 expression and activity can be regulated in a similar fashion by dietary components that modulate colon cancer risk further suggests a mechanistic link between these two cancer-promoting genes. Our model is attractive in that it reconciles many seemingly disparate observations in the literature regarding the role of PKCβII, Cox-2, and TGF-β signaling in colon carcinogenesis. Furthermore, it provides a paradigm within which to understand the mechanism(s) by which dietary compounds can modulate colon cancer risk. We are currently using our transgenic cell and animal models to explore the mechanism(s) by which azoxymethane and dietary factors such as EPA regulate PKCβII expression and activity and to assess whether PKCβII expression is required for Cox-2 gene induction and colon carcinogenesis in vivo.

In summary, we have shown that PKCβII induces Cox-2 expression both in vitro and in vivo. Cox-2 is intimately linked to the development of colon cancer, and our studies provide a molecular mechanism by which induction of PKCβII expression during azoxymethane-induced carcinogenesis, or overexpression of PKCβII in transgenic mice, predisposes mice to colon cancer. The elucidation of a PKCβII → Cox-2 → TGF-β signaling axis, which is operative in both intestinal epithelial cells in culture, and the colonic epithelium in vivo, provides an important mechanistic link that can explain how changes in PKCβII expression promotes colon carcinogenesis and how dietary lipids and nonsteroidal antiinflammatory drugs can modulate colon cancer risk.
Acknowledgments—We thank the members of the Fields laboratory for helpful discussions.

REFERENCES

1. Weinstein, I. B. (1991) Environ. Health Perspect., 93, 175–179
2. Fearon, E. R., and Vogelstein, B. (1990) Cell 61, 759–767
3. Murray, N. R., Thompson, L. J., and Fields, A. P. (1997) in Molecular Biology Intelligence Unit (Parker, P. J., and Dekker, L., eds) pp. 97–120, R. G. Landes Press, Austin, TX.
4. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851
5. Gokmen-Polar, Y., Murray, N. R., Velasco, M. A., Gatalica, Z., and Fields, A. P. (2003) Cancer Res. 63, 1375–1381
6. Murray, N. R., Davidson, L. A., Chapkin, R. S., Gustafson, W. C., Schattenberg, D. G., and Fields, A. P. (1999) J. Cell Biol. 145, 699–711
7. Murray, N. R., Weems, C., Chen, L., Leon, J., Yu, W., Davidson, L. A., Jamieson, L., Chapkin, R. S., Thompson, E. A., and Fields, A. P. (2002) J. Cell Biol. 157, 915–920
8. Hla, T., and Nelson, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7384–7388
9. Jones, D. A., Carlson, D. P., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1993) J. Biol. Chem. 268, 9049–9054
10. Kutcher, W., Jones, D. A., Matsunami, N., Groden, J., McIntyre, T. M., Zimmerman, G. A., White, R. L., and Prescott, S. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4816–4820
11. Sheng, G. G., Shao, J., Sheng, H., Hooton, E. B., Isaacson, P. C., Morrow, J. D., Coffey, R. J., Dubois, R. N., and Beauchamp, R. D. (1997) Gastroenterology 113, 1883–1891
12. Kargman, S. L., O'Neill, G. P., Vickers, P. J., Evans, J. F., Mancini, J. A., and Jothy, S. (1995) Cancer Res. 55, 2356–2359
13. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trasakos, J. M., Evans, J. F., and Taketo, M. M. (1996) Cell 87, 803–809
14. Ko, T. C., Yu, W., Sakai, T., Sheng, H. M., Beauchamp, R. D., and Thompson, E. A. (1998) Oncogene 16, 3445–3454
15. Chomyczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
16. Inoue, H., Nanayama, T., Hara, S., Yokoyama, C., and Tanabe, T. (1994) FEBS Lett. 350, 51–54
17. Tusher, V., Tibshirani, R., and Chu, C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5116–5121
18. O'Mahony, C. A., Beauchamp, R. D., Albo, D., Tsujii, M., Sheng, H. M., Shao, J., Dubois, R. N., and Berger, D. H. (1999) Surgery 126, 364–370
19. Sheng, H., Williams, C. S., Shao, J., Liang, P., Dubois, R. N., and Beauchamp, R. D. (1996) J. Biol. Chem. 271, 12210–12217
20. Zhang, Z., Sheng, H., Shao, J., Beauchamp, R. D., and Dubois, R. N. (2000) Neoplasia 2, 523–530
21. Sheng, H., Shao, J., Dinchuk, J. E., Williams, C. S., Prescott, S. M., Dubois, R. N., and Beauchamp, R. D. (2000) J. Biol. Chem. 275, 6628–8835
22. Monick, M. M., Roheff, P. K., Butler, N. S., Flaherty, D. M., Carter, A. B., Peterson, M. W., and Hunninghake, G. W. (2002) J. Biol. Chem. 277, 32992–33000
23. Gerwin, B. I., Koski-Oja, J., Seddon, M., Lechner, J. F., and Harris, C. C. (1990) Am. J. Pathol. 139, 1262–1269
24. Singh, J., Hamid, R., and Reddy, B. S. (1997) Cancer Res. 57, 3465–3470
25. Wai, R. F., Krawly, B. P. Jr., Hartmann, S., Roy, H. K., Khare, S., Seagulli-Sewell, B. A., Earnest, D. L., Sirin, M. D., Brasitus, T. A., and Bissauanette, M. (1995) Cancer Res. 55, 5257–5264
26. Deschner, E. E., Lytle, J. S., Wong, G., Ruperto, J. F., and Newmark, H. L. (1990) Cancer 68, 2350–2356
27. Reddy, B. S., Burill, C., and Rigotti, J. (1991) Cancer 51, 487–491
28. Takahashi, M., Minamot, T., Yamashita, N., Yazawa, K., Sugimura, T., and Esumi, H. (1993) Cancer Res. 53, 2786–2789
29. Chang, W. C., Chapkin, R. S., and Lupton, J. R. (1997) Carcinogenesis 18, 721–730
30. Bang, H. O., Dyerberg, J., and Hjorne, H. (1976) Acta Med. Scand. 200, 69–73
31. Caygill, C. P. J., and Hill, M. J. (1995) Eur. J. Cancer Prev. 4, 329–332
32. Caygill, C. P. J., Charlett, A., and Hill, M. J. (1996) Br. J. Cancer 74, 159–164
33. Bartsch, H., Nair, J., and Owen, R. W. (1999) Carcinogenesis 20, 2209–2218
34. Williams, C. S., Luongo, C., Radhika, A., Zhang, T., Lamps, L. W., Nanney, L. B., Beauchamp, R. D., and Dubois, R. N. (1996) Gastroenterology 111, 1134–1140
35. DuBois, R. N., Radhika, A., Reddy, B. S., and Entingh, A. J. (1996) Gastroenterology 110, 1259–1262
36. Sheng, H., Shao, J., O'Mahony, C. A., Lamps, L., Albo, D., Isaacson, P. C., Berger, D. H., Dubois, R. N., and Beauchamp, R. D. (1999) Oncogene 18, 855–867
37. Hansen-Petricek, M. B., McEntee, M. F., Jull, B., Shi, H., Zemel, M. B., and Whelan, J. (2002) Cancer Res. 62, 403–408
38. Breyer, R. M., Bagdassarian, C. K., Myers, S. A., and Breyer, M. D. (2001) Annu. Rev. Pharmacol. Toxicol. 41, 661–690
39. Watanabe, K., Kawamori, T., Nakatsugi, S., Ohta, T., Ohuchi, S., Yamamoto, H., Maruyama, T., Kondo, K., Ushikubi, F., Narumiya, S., Sugimura, T., and Wakabayashi, K. (1999) Cancer Res. 59, 5093–5096
40. Watanabe, K., Kawamori, T., Nakatsugi, S., Ohta, T., Ohuchi, S., Yamamoto, H., Maruyama, T., Kondo, K., Narumiya, S., Sugimura, T., and Wakabayashi, K. (2000) Cancer Lett. 156, 57–61
41. Kawamori, T., Uchiya, N., Kitamura, T., Ohuchi, S., Yamamoto, H., Maruyama, T., Sugimura, T., and Wakabayashi, K. (2001) Anticancer Res. 21, 3865–3869
42. Sonehista, M., Takaku, K., Sasaki, N., Sugimoto, Y., Ushikubi, F., Narumiya, S., Oshima, M., Takato, M. M. (2001) Nut. Med. 7, 1048–1051
43. Mutoh, M., Watanabe, K., Kitamura, T., Shoji, Y., Takahashi, M., Kawamori, T., Tani, K., Kobayashi, M., Maruyama, T., Kobayashi, K., Ohuchi, S., Sugimoto, Y., Narumiya, S., Sugimura, T., and Wakabayashi, K. (2002) Cancer Res. 62, 28–22