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Protein Kinase CβII Regulates Its Own Expression in Rat Intestinal Epithelial Cells and the Colonic Epithelium *in Vivo**

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Protein kinase C βII (PKCβII) is induced early during colon carcinogenesis. Transgenic mice expressing elevated PKCβII in the colonic epithelium (transgenic PKCβII mice) exhibit hyperproliferation and enhanced colon carcinogenesis. Here we demonstrate that nullizygous PKCβ (PKCβKO) mice are highly resistant to azoxymethane (AOM)-induced preneoplastic lesions, aberrant crypt foci. However, reexpression of PKCβII in the colon of PKCβKO mice by transgenesis restores susceptibility to AOM-induced colon carcinogenesis. Expression of human PKCβII in rat intestinal epithelial (RIE) cells induces expression of endogenous rat PKCβII mRNA and protein. Induction of PKCβII is dependent upon catalytically active PKCβII and does not appear to involve changes in alternative splicing of the PKCβ gene. Two human PKCβ promoter constructs are activated by expression of PKCβII in RIE cells. Both PKCβ promoter activity and PKCβII mRNA levels are inhibited by the MEK1 and -2 inhibitor U0126, but not the Cox-2 inhibitor celecoxib in RIE/PKCβII cells. PKCβ promoter activity correlates directly with expression of endogenous PKCβII mRNA and protein in HT29 and HCT116 human colon cancer cell lines. PKCβ promoter activity and PKCβII mRNA expression in HCT116 cells are inhibited by the selective PKCβ inhibitor LY317615 and by U0126, demonstrating autoregulation of PKCβII expression. Transgenic PKCβII mice exhibit specific induction of endogenous PKCβII, but not its splice variant PKCβI, in the colonic epithelium *in vivo*. Taken together, our results demonstrate that 1) expression of PKCβII in the colonic epithelium is both necessary and sufficient to confer susceptibility to AOM-induced colon carcinogenesis in transgenic mice, 2) PKCβII regulates its own expression in RIE and human colon cancer cells *in vitro* and in the colonic epithelium *in vivo*, and 3) PKCβII autoregulation is mediated through a MEK-dependent signaling pathway in RIE/PKCβII and HCT116 colon cancer cells.

Colon cancer is the second leading cause of cancer death in the United States (1). Colon carcinogenesis involves stepwise, progressive disruption of intestinal epithelial cell proliferation, differentiation, and survival mechanisms (2). Protein kinase C (PKC)[1] is a family of lipid-dependent serine/threonine kinases involved in the regulation of cell proliferation, differentiation, and survival (3, 4). Specific, reproducible changes in PKC isozyme expression patterns occur during carcinogen-induced colon carcinogenesis in rodents (5, 6). We recently demonstrated reduced expression of PKCα and increased expression of PKCβII and PKCι in AOM-induced mouse colon tumors (7, 8). Our subsequent studies provided direct evidence that both PKCβII and PKCι play critical, but distinct, roles in the promotion of colon carcinogenesis (8–10).

We have developed transgenic PKCβII mice that express elevated PKCβII in the colonic epithelium (9, 10). Transgenic PKCβII mice exhibit hyperproliferation of the colonic epithelium and are prone to AOM-induced colon cancer (9, 10). This cancer-prone phenotype results, at least in part, from the establishment of a PKCβII-dependent hyperproliferative phenotype (9, 10). We have also established nontransformed rat intestinal epithelial (RIE) cell lines that overexpress PKCβII (RIE/PKCβII cells) (10, 11). Genomic analysis of RIE/PKCβII cells demonstrated that PKCβII induces expression of the Cox-2 enzyme and suppresses expression of the transforming growth factor β receptor type II (TGFβRII) (11). As a result, RIE/PKCβII cells no longer respond to the growth-inhibitory effects of TGFβ (11). Further analysis revealed that PKCβII-mediated loss of TGFβ responsiveness requires the activity of both PKCβII and Cox-2 (11). Based on these data, we defined a novel, procarcinogenic PKCβII → Cox-2 → TGFβRII signaling pathway by which PKCβII confers resistance to TGFβ (11). This pathway contributes to the hyperproliferative phenotype exhibited by transgenic PKCβII mice (11). This PKCβII-mediated pathway is activated by carcinogens, whereas chemopreventive ω-3 fatty acids inhibit PKCβII activity, suppress PKCβII-mediated hyperproliferation, and attenuate the cancer-prone phenotype exhibited by transgenic PKCβII mice (10, 11).

PKCβII also induces an invasive phenotype in RIE cells through activation of a novel proinvasive PKCβII → Ras → PKCι/Rac1 → MEK signaling pathway that is distinct from that responsible for TGFβ resistance (12). Thus, PKCβII promotes AOM-induced colon cancer through activation of at least two distinct signaling pathways, one that confers TGFβ resistance and a second that induces invasion in intestinal epithelial cells. Here we resolve two critical questions regarding PKCβII and colon carcinogenesis. First, we provide direct evidence that PKCβII expression is both necessary and sufficient for AOM-induced colon carcinogenesis. Second, we identify a major

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[1] The abbreviations used are: PKC, protein kinase C; RIE, rat intestinal epithelial; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; RT, reverse transcription; QRT, quantitative real time; ACF, aberrant crypt foci; AOM, azoxymethane; TGFβ, transforming growth factor β; TGFβRII, TGFβ receptor type II.

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mechanism by which PKCβII expression is regulated in the colonic epithelium. Our results demonstrate that PKCβII induces its own expression in RIE and human colon cancer cells *in vitro* and in the colonic epithelium *in vivo*. PKCβII autoregulation is dependent upon PKCβII activity and is mediated predominantly through transcriptional activation of the PKCβ promoter through a PKCβII-, MEK-dependent signaling pathway. Our results suggest that PKCβII autoregulation plays a key promotive role in carcinogen-induced colon cancer.

EXPERIMENTAL PROCEDURES

Transgenic Mice and Carcinogenesis Studies—Mice nullizygous for PKCβ (PKCβKO mice) were generated and characterized previously (13). Transgenic PKCβII mice, which express PKCβII in the colonic epithelium, were generated and characterized previously (9, 10). PKCβKO and transgenic PKCβII mice on a C57B6 genetic background were crossed to obtain PKCβKO/PKCβII mice. Genotyping was performed as described previously (9, 13). Wild-type, PKCβKO, and PKCβKO/PKCβII mice were treated with AOM to induce colon carcinogenesis and assessed for ACF as described previously (9).

Cell Culture and Immunoblot Analysis—RIE cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum as described previously (8, 10–12). RIE cells stably expressing human PKCβII were generated and maintained as described previously (10). The established human HCT116 and HT-29 colon cancer cell lines were obtained from the American Tissue Type Culture Collection and maintained in McCoy's 5a medium (Invitrogen) with 1.5 mM L-glutamine and 10% fetal bovine serum. Whole cell extracts were prepared and subjected to immunoblot analysis as described previously (10–12). Antibodies specific to PKCβII and β-actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG antibody was from Sigma. Antigen antibody complexes were visualized using the ECL Western blotting detection system (Amersham Biosciences) as described previously (10–12). LY317615 was kindly provided by Lilly. In some cultures, LY317615 was added to a final concentration of 25 μM. This concentration was chosen based on the reported IC50 (3.5 μM) of this compound to inhibit PKCβ signaling in SW2 small cell lung carcinoma cells in culture (14). In some cultures, the selective MEK1 and -2 inhibitor U0126 (10 μM; Calbiochem), PD98059 (30 μM; Sigma), or the Cox-2 inhibitor celecoxib (25 μM) was added as described previously (12).

Isolation of Colonic Crypts, RNA Isolation, and Quantitative Real Time PCR (QRT-PCR)—Enriched populations of mouse colonic epithelial and mesenchymal cells were isolated from wild-type and transgenic PKCβII mice essentially as described (15). A detailed description of this procedure will be published elsewhere.[2] Briefly, the entire colon was isolated from mice as described previously (9). Colons were everted and washed with Hepes-buffered saline (25 mM Hepes, pH 7.5, 150 mM NaCl) supplemented with 1% fetal bovine serum. Colonic crypts enriched in epithelial cells were released by incubation in Hepes-buffered saline containing 10 mM EDTA, and isolated crypts were collected by centrifugation. Mesenchymal cells were isolated by gentle scraping from the remaining colon tissue using a plastic slide coverslip. Total RNA was isolated using RNAqueous reagent (Ambion), treated with DNase for 15–30 min, and subjected to RT-PCR analysis as described previously (8, 11).

QRT-PCR was carried out using target-specific probes and primers to detect PKCβIIa (probe spanning exons 16 and 17 of the human PKCβ gene), PKCβI (probe spanning exons 16 and 18), and PKCβI and PKCβIIb (probes internal exon 18) mRNA species. PKCβ reagents were generated using the Assay by Design program from Applied Biosystems, Inc., and were validated by demonstrating a linear relationship between ΔCt and cDNA concentration over a wide range of sample concentrations. Commercially available, validated primer and probe sets for mouse E-cadherin and vimentin were from Applied Biosystems (Assays on Demand). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA abundance to control for RNA concentration. Standard curves were established using serial dilution of a reference sample. TaqMan Universal PCR Master Mix, murine leukemia virus reverse transcriptase, and RNase inhibitor were used (PerkinElmer Life Sciences). Amplification data were collected using an Applied Biosystems Prism 7900 sequence detector and analyzed using the Sequence Detection System software from Perkin Elmer Life Sciences.

Kinase-deficient Mutant of Human PKCβII Mutation and Retrovirus Transfection—A kinase-deficient mutant of human PKCβII (kdPKCβII) was generated by PCR-based site-directed mutagenesis to mutate lysine 372 of PKCβII, a residue essential for ATP binding and kinase activity, to tryptophan using the QuikChange XL site-directed mutagenesis Kit (Stratagene) as described previously (12). The kdPKCβII mutant was inserted into the pBabe/Flag/puro retroviral expression vector, and virus stocks were produced using Phoenix-E cells. Puromycin-resistant, stable transfectants were generated as described previously (12). Expression of PKCβII or kdPKCβII was confirmed by immunoblot analysis using anti-FLAG and anti-PKCβII antibodies as described previously (12). Mid-log phase cultures were used for all experiments unless otherwise specified.

Cloning and Characterization of the Human PKCβ Promoter—Two PKCβ promoter constructs consisting of 2.3 kb (pkcβ-2.3) and 500 bp (pkcβ-0.5) of 5′-flanking sequence were cloned by PCR from a human genomic DNA library using the Genome Walker Kit (BD Biosciences, Clontech). The resultant PCR products were confirmed by sequencing and found to correspond to the sequence reported in the public human genome sequence data base. Promoter constructs were cloned into the luciferase reporter vector pGL3 (Promega) to yield pPKCβ/luc reporters. Promoter activity was assayed by co-transfection of 1 μg of pGL3-pkcβ-0.5 or pGL3-pkcβ-2.3 plus 50 ng of phRL-SV40 into RIE, HCT116, and HT-29 cells using the FuGene6 lipofection reagent (Roche Applied Science) as described by the manufacturer. 24 h after transfection, cells were assayed for firefly and *Renilla* luciferase activity using a dual luciferase assay system (Promega) as described previously (11). Results are expressed as firefly luciferase activity normalized to *Renilla* luciferase, which served as a control for transfection efficiency.

RESULTS

Expression of PKCβII in the Colonic Epithelium Is Necessary and Sufficient to Confer Susceptibility to Colon Carcinogenesis—The impetus for studying the regulation of PKCβII expression in the colonic epithelium comes from our observation that PKCβII expression is elevated in colonic preneoplastic lesions, ACF, and colon tumors of mouse exposed to the chemical carcinogen azoxymethane (7). Furthermore, transgenic PKCβII mice expressing elevated PKCβII in the colonic epithelium to levels consistent with those observed in AOM-induced colon tumors exhibit hyperproliferation of the colonic epithelium and increased sensitivity to AOM-induced colon carcinogenesis (9). Thus, PKCβII levels correlate with colon carcinogenesis, and elevation of PKCβII levels by transgenesis leads to a cancer-prone phenotype. However, a remaining question was whether PKCβII expression was necessary for AOM-induced colon carcinogenesis. To address this question, we determined the susceptibility of PKCβKO mice (13) to AOM-induced colon carcinogenesis (Fig. 1).

Wild-type and PKCβKO mice were treated with AOM to induce colon carcinogenesis and assessed for development of ACF as described previously (9). ACF are considered preneoplastic lesions, and their number is highly predictive of subsequent colon tumor formation (16, 17). PKCβKO mice exhibit a statistically significant, 3-fold decrease in ACF formation when compared with wild-type mice. These results demonstrate that expression of PKCβII is an important determinant of susceptibility to AOM-induced colon carcinogenesis. We next assessed the role of PKCβII expression in the colonic epithelium in susceptibility to colon carcinogenesis. For this purpose, PKCβKO mice were crossed to transgenic PKCβII mice to generate PKCβKO/PKCβII mice. PKCβKO/PKCβII mice are nullizygous for the PKCβ gene by virtue of the germ line disruption of the endogenous PKCβ gene (13). However, these mice are capable of expressing PKCβII only in the colonic epithelium due to the presence of transgenic human PKCβII, whose expression is restricted to the colonic epithelium (9). When PKCβKO/PKCβII mice are treated with AOM, ACF formation is restored to levels indistinguishable from wild-type mice. These data demonstrate that expression of PKCβII

[2] W. Su, N. R. Murray, A. P. Fields, and E. A. Thompson, manuscript in preparation.

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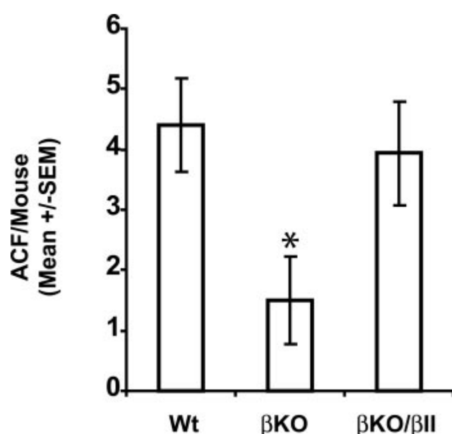

FIG. 1. **PKCβII in the colonic epithelium is necessary and sufficient to confer sensitivity to colon carcinogenesis.** Wild-type, nullizygous PKCβ (PKCβKO) and compound transgenic PKCβKO/PKCβII mice were treated with AOM to induce colon carcinogenesis as described previously (8, 9). 12 weeks after AOM exposure, mice were analyzed for formation of aberrant crypt foci (*ACF*) in the colon as described previously (8, 9). Data represent the mean number of ACF/mouse ± S.E. PKCβKO mice exhibit a significant decrease in ACF formation when compared with wild-type or PKCβKO/PKCβII mice (*, $p < 0.02$).

within the colonic epithelium is both necessary and sufficient to confer susceptibility to AOM-induced colon carcinogenesis.

Expression of Human PKCβII in RIE Cells Induces Expression of Endogenous PKCβII—Since PKCβII expression in the colonic epithelium is an important determinant of colon cancer susceptibility, we next assessed the mechanism by which PKCβII expression is regulated. An important clue came from a genomic analysis of RIE and RIE/PKCβII cells for potential PKCβII gene targets. This analysis revealed that the endogenous rat PKCβ gene is induced by the presence of human PKCβII (data not shown). To confirm these results, we generated real time PCR reagents that detect rat PKCβ mRNA species but not the human PKCβII transgene. Alternative splicing of PKCβ mRNAs is complicated, with evidence of tissue-specific differences in splice patterns (18–20). The PKCβ gene can be alternatively spliced to produce at least three mRNA splice variants termed PKCβI, PKCβIIa, and PKCβIIb (20) (Fig. 2*A*). To determine whether PKCβII induces expression of a specific splice variant of the endogenous PKCβ gene, we developed PCR reagents to detect each of these splice variants and determined the level of each variant by quantitative real time PCR in RIE and RIE/PKCβII cells (Fig. 2*B*). Whereas RIE cells contain no detectable PKCβ mRNA species, RIE/PKCβII cells express abundant endogenous PKCβIIa and PKCβIIb mRNA but no detectable PKCβI mRNA. The pattern of expression of the three splice variants of PKCβ is very similar to the pattern of expression of these variants in rat brain, a very abundant source of PKCβI and PKCβII mRNA. In contrast to RIE/PKCβII cells, RIE cells transfected with a kinase-deficient mutant of PKCβII (RIE/kdPKCβII cells) express no detectable rat PKCβ mRNAs. Taken together, these data demonstrate that PKCβII induces expression of PKCβII. The PKCβIIa mRNA species is the major form produced, with lower but detectable amounts of the PKCβIIb mRNA. PKCβII autoinduction requires the kinase activity of PKCβII, since kdPKCβII does not induce endogenous PKCβ expression.

Immunoblot analysis of total cell extracts from RIE and RIE/PKCβII cells with a specific antibody to PKCβII revealed, as expected (12), that RIE cells express no detectable PKCβII protein (Fig. 2*C*). In contrast, two immunoreactive bands corresponding to PKCβII are detected in RIE/PKCβII cells. Immunoblot analysis with an anti-FLAG antibody confirmed the identity of the slower migrating band as transgenic FLAG-tagged human PKCβII. The faster migrating band is not recognized by the anti-FLAG antibody and co-migrates with rat brain PKCβII, indicating that it corresponds to endogenous rat PKCβII. Immunoblot analysis using a PKCβI-specific antibody revealed no detectable PKCβI protein in RIE or RIE/PKCβII cells (data not shown). These results indicate that expression of PKCβII in RIE cells induces endogenous PKCβII mRNA and protein.

PKCβII Activates the Human PKCβ Promoter—PKCβII-mediated induction of PKCβII expression could be caused by multiple mechanisms, including activation of transcription of the PKCβ gene and stabilization of the PKCβII mRNA. To distinguish between these mechanisms, we next determined the half-life of the PKCβII mRNA in RIE/PKCβII cells as described previously (11). We obtained a half-life of >24 h (data not shown), consistent with the reported half-life of PKCβII in other cells (18). However, we were unable to detect PKCβII mRNA in RIE cells, making a comparison of half-life in the presence and absence of PKCβII impossible. Therefore, we cannot eliminate the possibility that stabilization of the PKCβII mRNA contributes to PKCβII-mediated induction of PKCβII mRNA and protein.

We next determined whether PKCβII induces the activity of the human PKCβ promoter. For this purpose, we cloned two promoter constructs consisting of 500 bp and 2.3 kb from the PKCβ gene from a genomic DNA library from K562 human myelocytic leukemia cells. The PKCβ promoter is extremely GC-rich in the immediate 5′ region upstream of the transcriptional start site, making it a prime candidate for methylation-mediated gene silencing. In addition, the promoter does not contain a conventional TATA box. When these PKCβ promoter constructs are placed in front of a luciferase reporter plasmid and transfected into RIE and RIE/PKCβII cells, both constructs exhibit a 3–5-fold induction in the presence of PKCβII (Fig. 3*A*). We recently demonstrated that PKCβII activates cellular K-Ras in RIE/PKCβII cells (12). As a consequence of K-Ras activation, PKCβII induces PKCι-, Rac1-, and MEK-dependent invasion of these cells (12). The human PKCβ promoter was previously shown to be activated by phorbol esters through AP1 and AP2 elements within the promoter (21). Therefore, we assessed whether PKCβII-dependent activation of the PKCβ promoter requires Ras/MEK-dependent signaling (Fig. 3*B*). The two MEK1 and -2 inhibitors, U0126 and PD98059, both cause significant inhibition of PKCβ promoter activity in RIE/PKCβII cells.

We recently demonstrated that PKCβII also induces the expression of the Cox-2 enzyme in RIE/PKCβII cells (11). However, the selective Cox-2 inhibitor celecoxib had no effect on PKCβ promoter activity in RIE/PKCβII cells, indicating that Cox-2 is not involved in PKCβII-mediated induction of the PKCβ promoter. To confirm the involvement of MEK in the regulation of PKCβII expression, we determined the effect of U0126 and PD98059 on expression of endogenous PKCβII mRNA in RIE/PKCβII cells (Fig. 3*C*). Both U0126 and PD98059 significantly repressed PKCβII mRNA expression in RIE/PKCβII cells, whereas 25 μM celecoxib had no effect on PKCβII mRNA despite the fact that this concentration of celecoxib completely blocks PKCβII-mediated repression of TGFβRII (11). These results demonstrate that PKCβII induces its own expression in RIE/PKCβII cells through a MEK-dependent signaling pathway.

PKCβ Promoter Activity Correlates with PKCβII Expression in Human Colon Cancer Cells—We next determined whether activation of the PKCβ promoter could be responsible for regulating the expression of PKCβII in human colon cancer cells.

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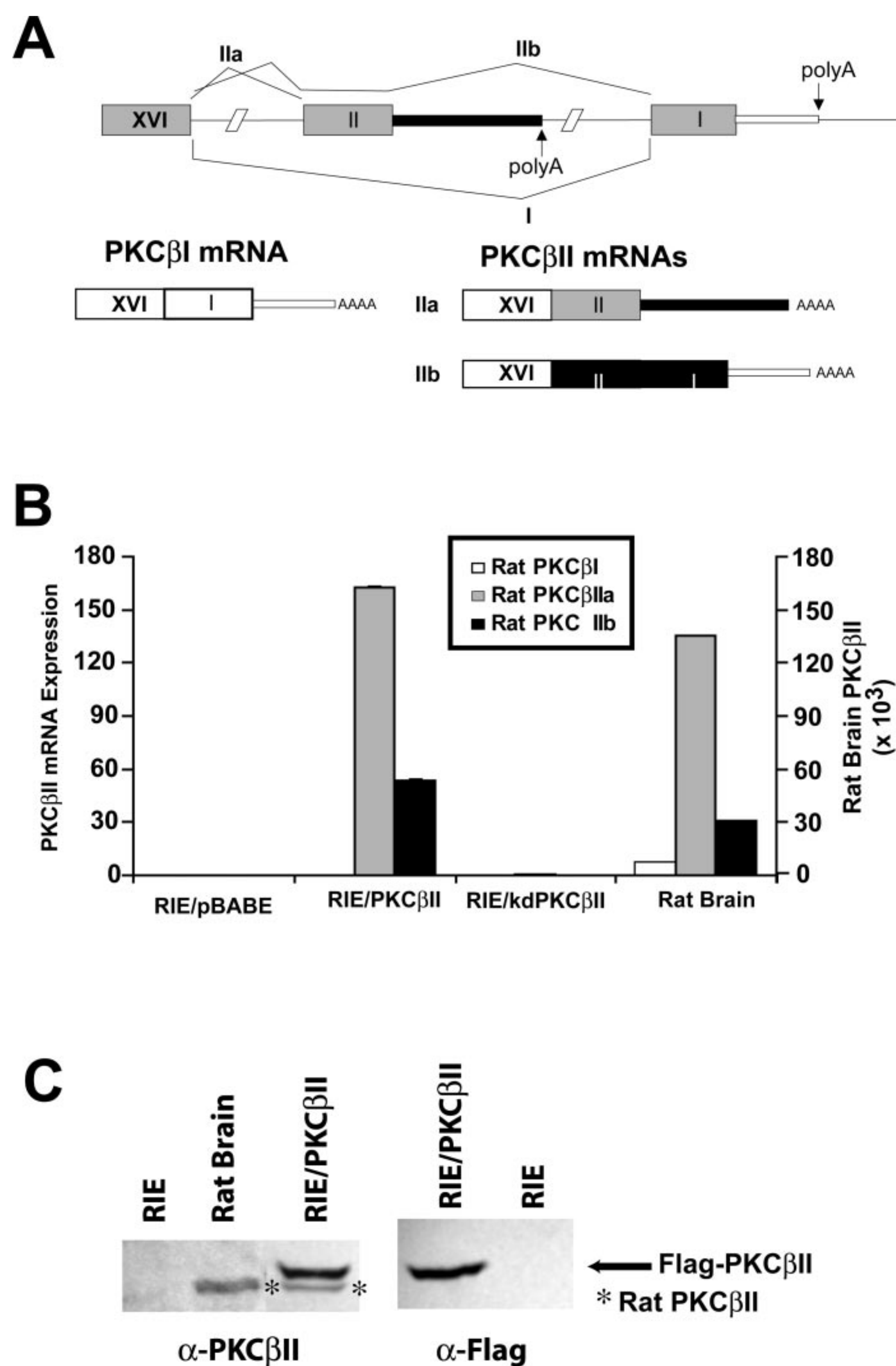

FIG. 2. **PKCβII induces its own expression in RIE cells.** *A*, schematic showing the three major splice variants of the PKCβ gene. A single PKCβI mRNA species is generated by exon exclusion. At least two PKCβII mRNA species (βIIa and βIIb) can be generated by alternative splicing of the final exon and 3′-untranslated region of the PKCβ gene. *B*, PKCβII induces expression of the PKCβIIa and PKCβIIb mRNA species but no detectable PKCβI mRNA. Kinase-deficient PKCβII does not induce endogenous PKCβII expression. Rat brain is an abundant source of the PKCβI, βIIa, and βIIb mRNA species and serves as a positive control. *C*, immunoblot analysis demonstrates that PKCβII induces endogenous PKCβII protein expression. Cell extracts from RIE and RIE/PKCβII cells were subjected to immunoblot analysis using anti-PKCβII and anti-FLAG antibodies. RIE/PKCβII cells express two PKCβII immunoreactive bands corresponding to the FLAG-tagged human PKCβII transgenic protein (*arrow*) and endogenous rat PKCβII (*asterisk*). Note that the lower migrating band comigrates with authentic rat brain PKCβII. Anti-FLAG blotting confirms the identity of the higher migrating band as FLAG-tagged human PKCβII.

For this purpose, we assessed PKCβ promoter activity in human colon cancer cells that express different levels of endogenous PKCβII. Immunoblot analysis of HT29 and HCT116 cells demonstrate that HT29 cells express very little PKCβII, whereas HCT116 cells express much higher levels of PKCβII (Fig. 4*A*). The relative level of PKCβII protein expression correlates well with the steady state levels of PKCβII mRNA in these two cell lines, since HCT116 cells express ~10-fold more PKCβII mRNA than HT29 cells (Fig. 4*B*). Transfection of the human PKCβ promoter into HT29 and HCT116 cells revealed that the activity of both the 500-bp and 2.3-kb PKCβ promoters was 10–15-fold higher in HCT116 cells than in HT29 (Fig. 4*C*), consistent with the difference in endogenous PKCβII mRNA and protein levels in these cells. Thus, PKCβ promoter activity directly correlates with the steady state levels of PKCβII mRNA and protein in two established human colon cancer cell lines. Similar results were obtained in Caco2 and DLD-1 cells that express low and high PKCβII levels, respectively. These data indicate that PKCβII expression is controlled, at least in part, through transcriptional regulation of the PKCβ gene in a variety of human colon cancer cells.

We next determined whether PKCβII regulates the activity of its own promoter and mRNA levels in human colon cancer cells. HT29 and HCT116 cells were transfected with the 500-bp human PKCβ promoter construct and treated with either the PKCβ-selective inhibitor LY317615 or the MEK1 and -2 inhibitor U0126 (Fig. 5*A*). Both LY317615 and U0126 caused significant inhibition of PKCβ promoter activity in HCT116 cells, consistent with our results in RIE/PKCβII cells. In contrast, HT29 cells, which express very low endogenous PKCβII, exhibit very low PKCβ promoter activity that is not inhibited by either LY317615 or U0126. Thus, the ability of LY317615 to inhibit PKCβ promoter activity correlates directly with PKCβII expression in HCT116 and HT29 cells, indicating that

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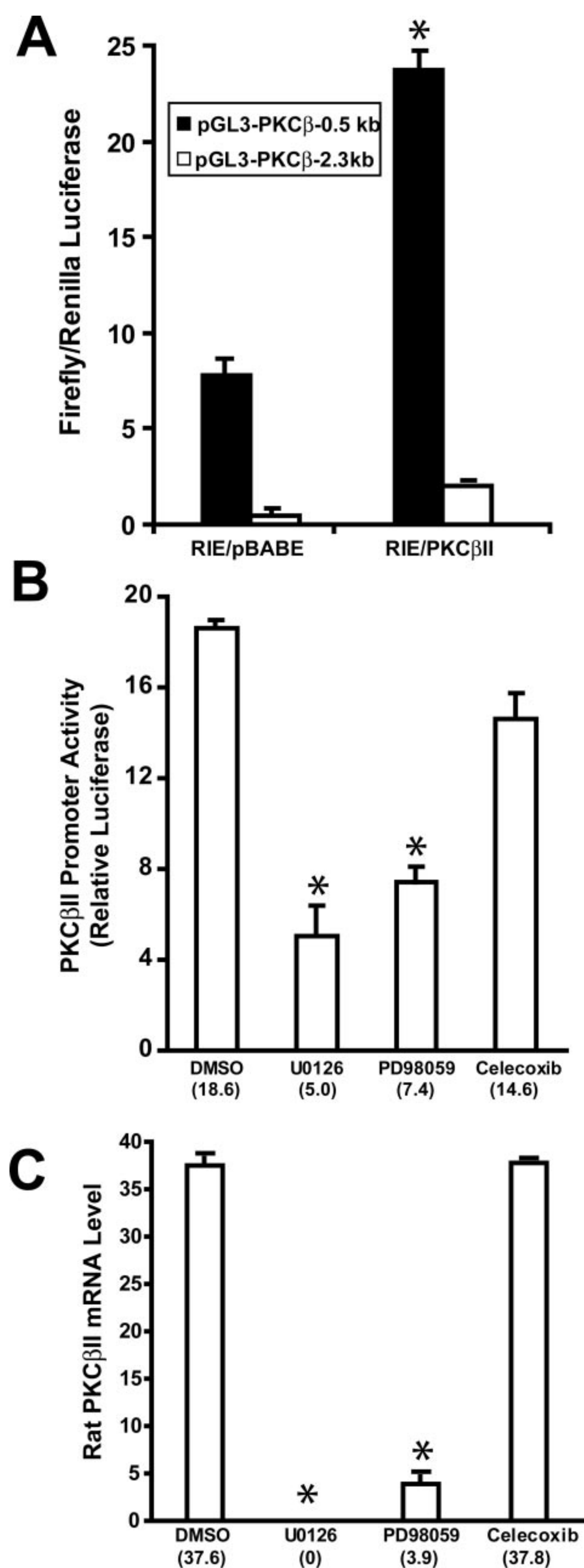

FIG. 3. **PKCβ gene promoter activity in RIE and RIE/PKCβII cells.** *A*, two human PKCβ gene promoter constructs of 0.5 and 2.3 kb were linked to a luciferase reporter plasmid pGL3, transfected into RIE or RIE/PKCβII cells, and assessed for promoter activity. Both the 0.5- and 2.3-kb promoter constructs were more active in RIE/PKCβII cells than in RIE cells. Data are expressed as mean ± S.D. *, $p < 0.05$ *versus* RIE/pBABE cells. *B*, the 0.5-kb PKCβ promoter was transfected into RIE/PKCβII cells and assessed for transcriptional activity in the presence of U0126 (10 μM), PD98059 (30 μM), celecoxib (25 μM), or 0.1% Me_2SO (*DMSO*). Results are expressed as mean ± S.D. of relative promoter activity normalized to *Renilla* luciferase activity. *, $p < 0.05$ *versus* Me_2SO. *C*, endogenous rat PKCβII mRNA levels were determined by real time PCR as described under "Experimental Procedures." Data represent the mean ± S.D. *, $p < 0.05$ *versus* Me_2SO.

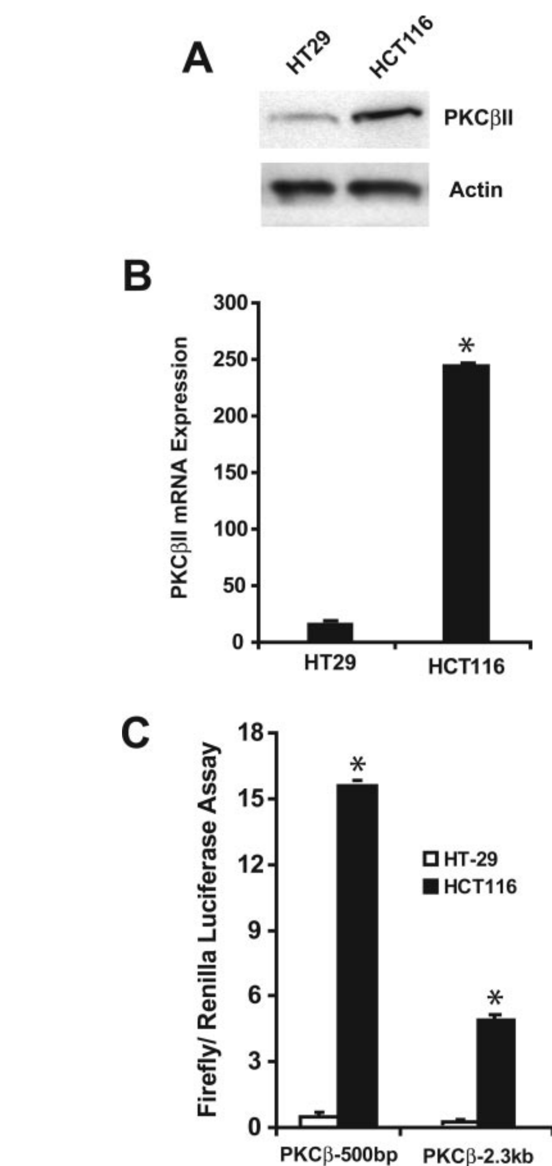

FIG. 4. **PKCβ promoter activity correlates with PKCβII expression in human colon cancer cells.** *A*, immunoblot analysis of HT29 and HCT116 human colon cancer cells for PKCβII and actin. *B*, steady state levels of PKCβII mRNA in HT29 and HCT116 cells were determined by real time PCR. Data are the mean ± S.D. *, $p < 0.05$ *versus* HT29 cells. *C*, human PKCβ promoter activity was determined by luciferase assay as described under "Experimental Procedures." Both the 0.5- and 2.3-kb PKCβ promoter constructs are more active in HCT116 cells than in HT29 cells. Promoter activity was normalized to *Renilla* to control for transfection efficiency. Data represent the mean ± S.D. *, $p < 0.05$ *versus* HT29 cells.

LY317615-mediated inhibition of PKCβ promoter activity in HCT116 cells is due to inhibition of PKCβII. Endogenous PKCβII mRNA levels in HCT116 cells are significantly reduced by both LY317615 and U0126, consistent with the effect of these compounds on PKCβ promoter activity (Fig. 5*B*). Our data demonstrate that PKCβII expression is regulated through PKCβII-mediated transcriptional control of the PKCβ promoter in human colon cancer cell lines that express significant levels of PKCβII. Our data further demonstrate that the PKCβ

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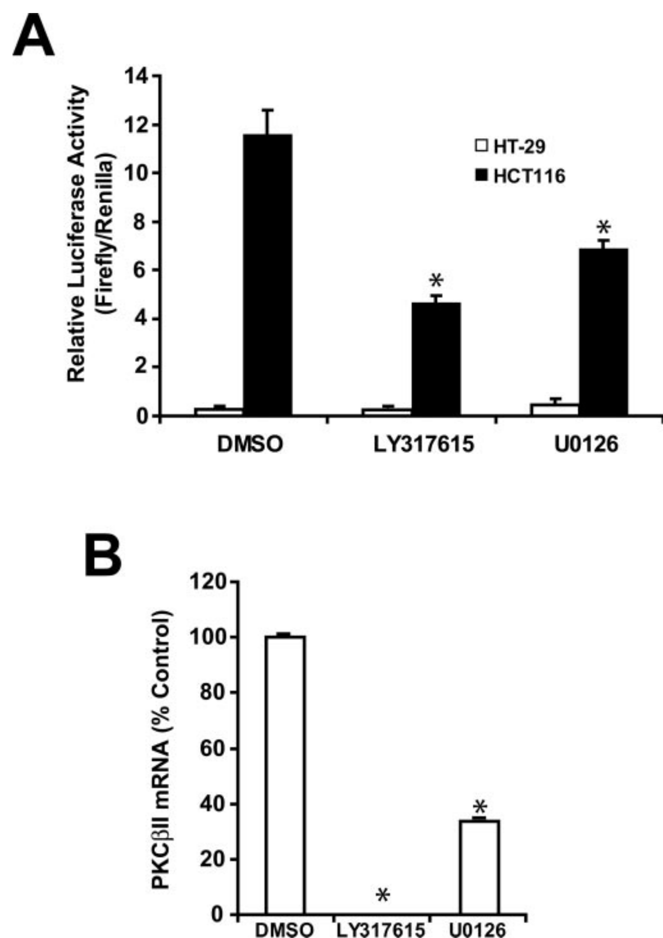

FIG. 5. **PKCβ promoter activity and mRNA expression in HCT116 cells is blocked by LY317615 and U0126.** *A*, HT29 and HCT116 cells were transfected with the human PKCβ promoter construct, and promoter activity was assessed in the presence of the PKCβ inhibitor LY317615 (25 μM), the MEK1 and -2 inhibitor U0126 (10 μM), or 0.01% Me_2SO (*DMSO*). Results represent the mean ± S.D. *, $p < 0.05$ *versus* Me_2SO-treated HCT116 cells. *B*, PKCβII mRNA abundance was determined in HCT116 cells in the presence of LY317615, U0126, or Me_2SO as described under "Experimental Procedures." Results represent the mean ± S.D. and are expressed as percentage of control expression in Me_2SO-treated cells. *, $p < 0.05$ *versus* Me_2SO.

promoter is regulated through a PKCβII-, MEK1-, and MEK2-dependent pathway in these cells.

PKCβII Regulates Its Own Expression in the Colonic Epithelium in Vivo—We next assessed whether PKCβII regulates its own expression in the colonic epithelium *in vivo*. For this purpose, we developed species-specific real time PCR assays to detect human and rodent PKCβII (Fig. 6*A*). We determined that our reagents are species-specific using RNA isolated from human K562 myelocytic leukemia cells and mouse brain as positive controls for human and mouse PKCβII, respectively. The human PKCβII reagents detect PKCβII in K562 RNA but not in mouse brain RNA. Conversely, the mouse PKCβII reagents detect abundant PKCβII in mouse brain RNA but do not detect PKCβII RNA in K562 cells.

Having demonstrated the specificity of our PCR reagents, we next assessed whether expression of human PKCβII in the colonic epithelium of transgenic PKCβII mice induces the expression of endogenous mouse PKCβII in the colon (Fig. 6*B*). In wild-type mice, we detect a low but detectable level of mouse PKCβII mRNA in the colon, consistent with our previous immunohistochemical and immunoblot results demonstrating that PKCβII is expressed at a relatively low level in the mouse colon (7). As expected, no human PKCβII mRNA is detected in

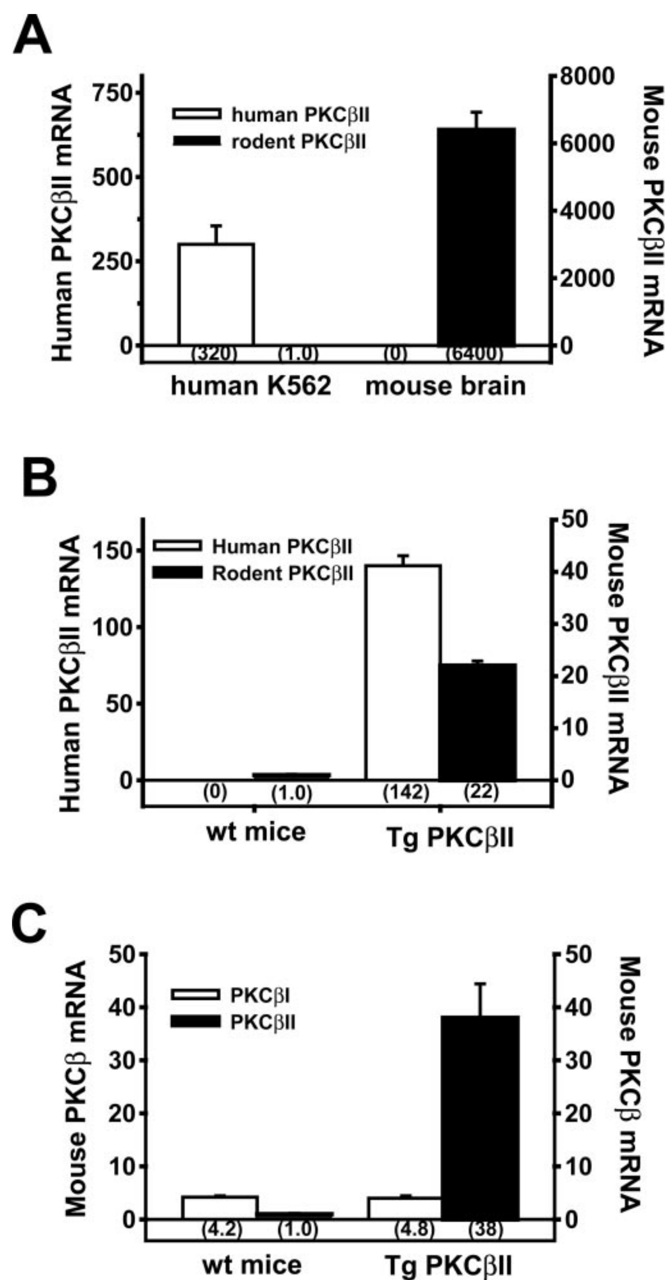

FIG. 6. **PKCβII induces its own expression in the colon *in vivo*.** *A*, characterization of real time PCR reagents that distinguish human and rodent PKC βII mRNAs. *B*, RNA from the colon of wild-type and transgenic PKCβII mice was isolated and subjected to real time PCR analysis for human transgenic and endogenous mouse PKCβII mRNA. *C*, real time PCR assays for mouse PKCβI and PKCβII were used to determine the level of PKCβI and PKCβII mRNA in wild-type and transgenic PKCβII mice.

wild-type mice. However, in transgenic PKCβII mice, we not only detect abundant transgenic human PKCβII mRNA but also a much higher level of mouse PKCβII mRNA when compared with wild-type mice. We next used RT-PCR reagents that distinguish the mouse PKCβI and PKCβII mRNA species of the PKCβ gene to determine whether human PKCβII induces one or the other of these variants preferentially (Fig. 6*C*). In wild-type mice, we detect both PKCβI and PKCβII mRNA species, with PKCβI mRNA being ~4-fold more abundant than PKCβII mRNA. These results are consistent with our immunoblot analysis, which showed that PKCβI is more abundant than PKCβII in normal mouse colon tissue (7). In transgenic PKCβII mice, we find that whereas the level of PKCβI mRNA remains un-

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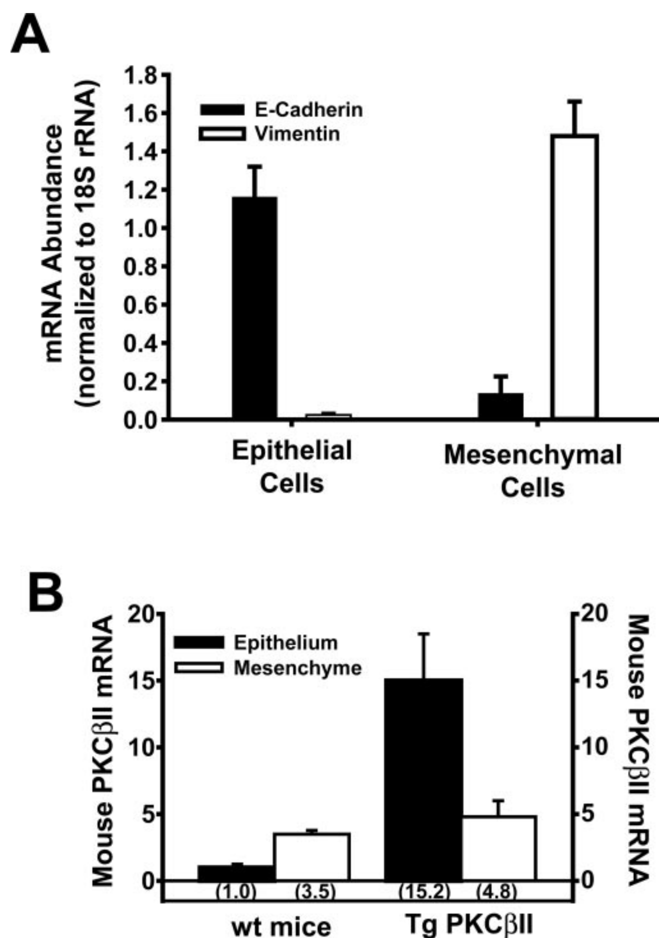

FIG. 7. **PKCβII autoinduction is confined to the colonic epithelium *in vivo*.** *A*, enrichment of colonic epithelial and mesenchymal cells. Extracts enriched in colonic epithelial and mesenchymal cells were isolated from mice and assessed for E-cadherin and vimentin mRNA by QRT-PCR as described under "Experimental Procedures." Results represent the mean ± S.D. *B*, colonic epithelial and mesenchymal cells isolated from wild-type and transgenic PKCβII mice were assessed for mouse PKCβII by QRT-PCR.

changed, the level of PKCβII mRNA is strongly induced. Thus, PKCβII preferentially induces PKCβII mRNA but not PKCβI mRNA, consistent with our results in RIE/PKCβII cells.

Finally, we wished to determine whether the induction of PKCβII occurs in colonic epithelial cells and/or in surrounding mesenchymal cells. For this purpose, RNA isolated from colonic epithelial and mesenchymal cell fractions were subjected to QRT-PCR analysis for E-cadherin and vimentin mRNA, biochemical markers of epithelial and mesenchymal cells, respectively (Fig. 7*A*). Epithelial cell preparations exhibited abundant E-cadherin mRNA but a very low level of vimentin mRNA. Conversely, mesenchymal cell preparations exhibited abundant vimentin but low levels of E-cadherin. These results indicate that these two cell fractions are highly enriched in epithelial and mesenchymal cells, respectively. Using our mouse PKCβII-specific RT-PCR reagents, we observe that wild-type mice express ~3.5-fold higher levels of PKCβII mRNA in the mesenchymal cell fraction than in the colonic epithelial cell fraction (Fig. 7*B*). In transgenic PKCβII mice, we find that whereas the level of PKCβII mRNA in the colonic mesenchyme remains essentially unchanged, that in the colonic epithelium is much higher than in wild-type mice. Taken together, our results demonstrate that PKCβII induces its own expression in the colonic epithelium *in vivo*. Autoinduction is selective for the PKCβII splice variant(s) of the PKCβ gene and is largely confined to the colonic epithelium. These data are consistent with the pattern of expression of the human PKCβII transgene in these mice, which is confined to the colonic epithelium by virtue of the modified fatty acid-binding protein promoter construct driving transgene expression (8, 9, 22). Our *in vivo* data are consistent with our results in RIE/PKCβII and human colon cancer cells *in vitro* and suggest that similar mechanisms are involved in the autoregulation of PKCβII in intestinal epithelial cells *in vitro* and *in vivo*.

DISCUSSION

PKCβII has emerged as a critical gene involved in colon carcinogenesis (3, 7, 9–12). PKCβII expression is elevated in early preneoplastic lesions and in established colon tumors in carcinogen-treated mice (7). Expression of PKCβII in the colon by transgenesis leads to colonic hyperproliferation and increased susceptibility to colon carcinogenesis (9, 11). Here, we demonstrate that PKCβ gene expression is a critical determinant of susceptibility to colon carcinogenesis. PKCβKO mice exhibit resistance to AOM-induced ACF formation, preneoplastic lesions in the colon that are highly predictive of colon tumor formation (16, 17). Significantly, reintroduction of PKCβII expression to the colonic epithelium by transgenesis is sufficient to restore sensitivity to AOM in PKCβKO mice. This observation provides strong genetic evidence that PKCβII expression within the colonic epithelium is both necessary and sufficient to confer susceptibility to AOM-induced colon carcinogenesis in mice. These data are particularly interesting in light of the observation that PKCβ may be important for tumor-mediated angiogenesis in other tumor systems (14), a process that could be mediated by PKCβ expressed in either epithelium-derived tumor cells, tumor-associated mesenchymal elements, or both. Although we cannot rule out a role for mesenchymal cell PKCβII in the support of tumorigenesis, our data clearly demonstrate that expression of PKCβII in colonic epithelial cells is critical for colon carcinogenesis. Our compound transgenic PKCβKO/PKCβII mice represent an important genetic model in which to assess the relative contribution of epithelial and stromal expression of PKCβII in AOM-induced colon carcinogenesis. In addition, these mice will be useful in assessing the role of PKCβII in other colon cancer models such as the APC^{min} mouse.

Given the importance of epithelial PKCβII in AOM-induced colon carcinogenesis, we assessed the mechanisms by which PKCβII expression is regulated in RIE cells, human colon cancer cells, and the colonic epithelium *in vivo*. Microarray analysis identified the PKCβ gene as a potential transcriptional target of PKCβII in RIE/PKCβII cells. Real time PCR analysis confirmed PKCβ as a gene target for PKCβII and demonstrated that autoregulation requires PKCβII kinase activity. The PKCβ gene is subject to regulation at both transcriptional and post-transcriptional levels (18, 20, 21, 23). The PKCβ gene encodes two distinct isoforms, PKCβI and PKCβII, which differ in their cellular function (3). Abundant evidence indicates that the PKCβ gene can be regulated through alternative splicing to yield multiple mRNA species (18–20). For example, insulin has been shown to induce a splicing switch from PKCβI and PKCβII isoforms that is mediated by alternative splicing mechanisms (19, 20). However, our real time PCR analysis revealed no evidence for alternative splicing as a major mechanism controlling PKCβ gene expression in RIE/PKCβII cells, human colon cancer cells, or the colonic epithelium. Indeed, PKCβII appears to preferentially induce PKCβII mRNAs in these tissues, with the three major PKCβ mRNA species expressed to levels consistent with those expressed in rat brain. These results suggest that alternative splicing is not a major aspect of PKCβII-mediated autoinduction.

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Analysis of two human PKCβ promoter constructs demonstrates that transcriptional activation of the PKCβ promoter is a major mechanism by which PKCβII expression is regulated by PKCβII in RIE and human colon cancer cells. The human PKCβ promoter is induced by the presence of PKCβII in RIE/PKCβII cells. We previously demonstrated that expression of PKCβII in RIE cells activates cellular K-Ras and induces an invasive phenotype through activation of the small molecular weight GTPase and Ras effector, Rac1 (12). PKCβII-mediated Rac1 activation and cellular invasion are dependent upon MEK1 and -2 activity, demonstrating that PKCβII induces invasion through a Ras → Rac → MEK signaling axis (12). We also demonstrated that PKCβII expression leads to loss of TGFβ responsiveness in RIE/PKCβII cells (10, 11). PKCβII mediates TGFβ resistance through induction of *Cox-2* gene expression (11). Interestingly, PKCβII autoinduction is blocked by inhibition of MEK1 and -2 activity but not by inhibition of Cox-2. These results indicate that the pathway by which PKCβII induces TGFβ resistance is distinct from that by which it induces cellular invasion and its own expression.

PKCβII autoinduction is an important mechanism regulating PKCβII expression in human colon cancer cells. HT29 cells express extremely low levels of PKCβII mRNA and protein, whereas HCT116 cells express higher levels of PKCβII. Consistent with the level of endogenous PKCβII in these cell lines, HT29 cells support low PKCβ promoter activity, whereas HCT116 cells support much higher PKCβ promoter activity. PKCβ promoter activity in HCT116 cells is blocked by the selective PKCβ inhibitor LY317615 and the MEK1 and -2 inhibitor U0126, whereas that in HT29 cells is not significantly affected by PKCβ or MEK1 and -2 inhibition.

Autoinduction of PKCβII also occurs in the colonic epithelium *in vivo*. Expression of transgenic PKCβII in the colonic epithelium of transgenic mice leads to induction of PKCβII, but not PKCβI, in the colonic epithelium. Our results reveal an important, novel mechanism by which PKCβII expression may be regulated during AOM-induced colon carcinogenesis. It is interesting to note that dietary ω-6 fatty acids and secondary bile acids promote colon carcinogenesis and are potent activators of conventional PKC isozymes, including PKCβII (24, 25). Taken together with our data, one could envision a mechanism by which dietary risk factors can promote colon carcinogenesis by activating PKCβII, which in turn induces its own expression through the autoregulatory mechanism described in the present study.

Numerous PKC isozymes have been implicated in various aspects of transformation. Recently, we demonstrated that atypical PKCι is required for oncogenic Ras-mediated transformation both *in vitro* and *in vivo* (8). PKCι, like PKCβII, is induced during colon carcinogenesis, and elevated expression of PKCι leads to enhanced susceptibility to colon carcinogenesis, whereas disruption of PKCι signaling blocks carcinogenesis (8). Interestingly, at least one procarcinogenic pathway elucidated in our recent studies involves both PKCβII and PKCι. PKCι is required downstream of Ras for PKCβII-dependent invasion in RIE/PKCβII cells (12). In this regard, we recently demonstrated that both PKCβII and PKCι expression is induced in human chronic myelogenous leukemia cells (26). Induction of PKCβII and PKCι expression in CML cells is dependent upon Bcr-Abl and the Ras/MEK pathway (26). In CML cells, PKCι induction is due to transcriptional activation of an ELK1-like element in the proximal PKCι promoter (26). Similar AP1 and AP2 sites have been implicated in the regulation of the PKCβ promoter (21). It will be of interest to determine whether PKCβII autoregulation is dependent upon PKCι and Rac1 activity, as is the case for PKCβII-mediated invasion (12).

Acquisition of an oncogenic K-Ras mutation, which occurs frequently in AOM-induced colon tumors and sporadic human colon cancers, could potentially activate PKCβII autoinduction through activation of MEK1 and -2 activity. Consistent with this possibility, the human colon cancer cell lines examined in this study that harbor oncogenic Ras mutations, HCT116 and DLD-1 cells, both express relatively high levels of PKCβII, whereas those that do not harbor oncogenic Ras mutations, HT29 and Caco2 cells, express very low levels of PKCβII. It will be of interest to determine the prevalence of elevated PKCβII expression in human colon cancers as a function of oncogenic Ras status and clinical outcome. Such a study is currently under way in our laboratory.

Finally, our results have important implications for the use of PKCβ inhibitors as antineoplastic agents. Currently, LY317615 (also known as enzastaurin) is in phase 2 clinical trials for refractory large B-cell lymphoma and recurrent high grade glioma. Based on our cell-based and preclinical animal model studies, enzaspaurin is an attractive candidate for treatment and particularly prevention of colon cancer.

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