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Elevated Protein Kinase C βII Is an Early Promotive Event in Colon Carcinogenesis[1]

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ABSTRACT

Protein kinase C (PKC) has been implicated in colon carcinogenesis in humans and in rodent models. However, little is known about the specific role of individual PKC isozymes in this process. We recently demonstrated that elevated expression of PKC βII in the colonic epithelium induces hyperproliferation *in vivo* (N. R. Murray *et al.*, J. Cell Biol., *145:* 699–711, 1999). Because hyperproliferation is a major risk factor for colon cancer, we assessed whether specific alterations in PKC βII expression occur during azoxymethane-induced colon carcinogenesis in mice. An increase in PKC βII expression was observed in preneoplastic lesions (aberrant crypt foci, 3.7-fold) compared with saline-treated animals, and in colon tumors (7.8-fold; $P = 0.011$) compared with uninvolved colonic epithelium. In contrast, PKC α and PKC βI (a splicing variant of PKC βII) expression was slightly decreased in aberrant crypt foci and dramatically reduced in colon tumors. Quantitative reverse transcription-PCR analysis revealed that PKC mRNA levels do not directly correlate with PKC protein levels, indicating that PKC isozyme expression is likely regulated at the posttranscriptional/translational level. Finally, transgenic mice expressing elevated PKC βII in the colonic epithelium exhibit a trend toward increased colon tumor formation after exposure to azoxymethane. Taken together, our results demonstrate that elevated expression of PKC βII is an important early, promotive event that plays a role in colon cancer development.

INTRODUCTION

Colon carcinogenesis is a multistep process involving both epigenetic changes in signal transduction pathways and genetic alterations in oncogenes and tumor suppressor genes, resulting in progressive disregulation of cell proliferation and survival mechanisms (1, 2). PKC[3] is a family of 12 distinct serine/threonine kinases that participate in signaling pathways involved in cellular proliferation, differentiation, and apoptosis in diverse cell systems (3). Differences in tissue expression, subcellular localization, and activator/substrate specificity predict that individual PKC isozymes have distinct cellular functions (4–7). Alterations in PKC isozyme levels have been observed in human and rodent colon cancers, suggesting a role for PKC in colon carcinogenesis (2, 8–10). However, little is known about the specific role of individual PKC isozymes in the carcinogenic process *in vivo*.

Several studies have investigated expression of the PKC β isozyme in rat and human colon tumors, with varied results (10–14). PKC β protein expression was found to increase (8, 11), remain the same (10), or decrease (12) in colon tumors as compared with normal epithelium. When the level of PKC β mRNA was analyzed, it was found to be decreased in colon tumors when compared with normal epithelium (15, 16). However, the *PKC β* gene actually codes for two distinct proteins generated by alternative splicing, PKC βI and PKC βII, which differ in the last 50 amino acids (17). Because the expression of these two PKC β isozymes may be differentially regulated (18), it is important to analyze the expression of each individual PKC β isozyme to assess its potential role in colon cancer.

We recently demonstrated that overexpression of PKC βII in the colonic epithelium results in hyperproliferation and increased susceptibility to carcinogen-induced preneoplastic lesions (19), demonstrating a direct role for the PKC βII isozyme in proliferation in the colonic epithelium. Based on these data, we hypothesized that an increase in expression of PKC βII is an early event in colon carcinogenesis that occurs in preneoplastic tissue to provide a proliferative advantage to precancerous cells. To test this hypothesis, we analyzed the expression of PKC βII, its splice variant, PKC βI, and PKC α in normal colonic epithelium, in AOM-induced early preneoplastic ACF, and in colon tumors.

Our results demonstrate that expression of PKC βII increases dramatically early in the carcinogenic process. In contrast, expression of the related PKC α and PKC βI isozymes decreases later in tumor development. Furthermore, transgenic mice overexpressing PKC βII in the colon exhibit increased susceptibility to carcinogen-induced colon tumors. Taken together, our results demonstrate that a specific increase in PKC βII expression is an early event in colon carcinogenesis that has a direct, promotive role in the development of ACF and colon tumors. Our data also suggest that loss of PKC α and PKC βI expression may be involved in later stages of tumorigenesis. The present study is the first to directly investigate PKC isozyme expression in both ACF and colon tumors in the mouse. Our results provide a better understanding of the potential role of specific PKC isozymes in the multistep process of colon carcinogenesis.

MATERIALS AND METHODS

Carcinogen Treatment. Female 6–7-week-old C57BL/6J mice (The Jackson Laboratory) were injected i.p. with AOM at a dose of 10 mg/kg body weight or with an equal volume of saline once a week for 2 weeks, as described previously (20). All animals were housed five mice/cage in microisolator cages in a pathogen-free barrier facility maintained at a constant temperature and humidity on a 12-h light/12-h dark cycle. Mice were provided with a standard autoclavable chow (Purina 7012; 5% fat) and autoclaved water *ad libitum* until the termination of the experiment. Animals were sacrificed at either 20 or 36 weeks after the second AOM injection for analysis.

Isolation of ACF. At 20 weeks after the second AOM injection, animals (AOM- or saline-treated animals) were sacrificed by cervical dislocation. The colons were removed, flushed with ice-cold PBS, and cut longitudinally. For protein analysis, colons were fixed flat between sheets of filter paper in 70% ethanol for 30 min at 4°C (21). After staining with 1% methylene blue in PBS for 5 min, the fixed colons were assessed for the presence of ACF at low magnification (×40) on a dissecting microscope using criteria described previously (22, 23). ACF from eight to nine AOM-treated mice (range, 42–88 ACF/group; average, 2 ± 1 crypts/ACF; range, 1–7 crypts/ACF) were isolated using fine-tipped forceps as described previously (21), pooled in ice-cold radioimmunoprecipitation assay buffer [50 mM Tris (pH 7.2), 150 mM NaCl, 2 mM EDTA, 0.4 mM EGTA, 20 μM sodium fluoride, 0.5% deoxycholate, 1% NP40, 0.1% SDS, 0.1 mM sodium orthovanadate, 25 μg/ml aprotinin, 20

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[3] The abbreviations used are: PKC, protein kinase C; AOM, azoxymethane; ACF, aberrant crypt foci; QRT-PCR, quantitative reverse transcription-PCR.

μg/ml leupeptin, 2.5 μg/ml pepstatin, 1 μg/ml soybean trypsin inhibitor, and 34.5 μg/ml 4-(2-aminoethyl)benzene sulfonyl fluoride], and boiled immediately in SDS Laemmli sample buffer. The protein concentration of these extracts was determined using a Coomassie Filter Protein Assay (24). Normal colonic crypts were isolated from the colon of saline-treated animals in a pattern representative of the distribution of ACF in colons from AOM-treated animals and processed as described above.

For RNA analysis, ACF and normal crypts were isolated as described above, except that the colon was flushed with diethyl pyrocarbonate-treated PBS, and crypt isolation was performed on unfixed tissue. All steps were carried out under RNase-free conditions. The isolated ACF and normal crypts were put into Trizol reagent (Life Technologies, Inc.) and immediately frozen at −80°C until analysis.

Isolation of AOM-induced Mouse Colon Tumors and Normal Colonic Epithelial Tissue. At 36 weeks after the second AOM injection, animals were sacrificed by CO_2 asphyxiation; the colons were isolated, cut longitudinally, and rinsed well with ice-cold PBS; and tumors were identified visually. Tumors were removed from the colon, divided, and processed for histopathological analysis and protein and RNA isolation. For protein isolation, normal colonic epithelium and tumor tissues were processed as described above. The protein concentration of each sample was determined by the BCA assay (Pierce), and samples were subjected to immunoblot analysis as described previously (19).

For RNA isolation, tumors and scraped normal colonic epithelial tissues were snap frozen in liquid nitrogen and stored at −80°C until extraction. Total cellular RNA was isolated from frozen colonic mucosa and tumor tissue using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. The integrity of 18S and 28S rRNA was determined by ethidium bromide staining after electrophoretic separation in agarose gels.

For histopathological examination, tumors were fixed in 4% paraformaldehyde for 4 h at 4°C, washed several times with ethanol, paraffin embedded as described previously (25), and stained with H&E. Complete histopathological analyses were carried on all tumor samples by a board-certified pathologist (Z. G.).

Immunoblot Detection of PKC Isozymes. Protein extracts from normal colonic crypts, ACF, and colon tumors were subjected to SDS-PAGE on 10% gels. After transfer to nitrocellulose (Schleicher & Schuell), samples were subjected to immunoblot analysis using isozyme-specific antibodies to PKC α (26), PKC βI, PKC βII, and actin (Santa Cruz Biotechnology). Immune complexes were detected by binding of affinity-purified peroxidase-labeled secondary antibodies (Kirkegaard & Perry Laboratories) and reaction with the SuperSignal Pico (PKC α, PKC βI, and β-actin) or SuperSignal Femto (PKC βII; Pierce) chemiluminescence system. Rat brain lysate was used as a positive control for each antibody. Band intensity was quantified by densitometric scanning (Molecular Dynamics). The level of expression of individual PKC isozymes was normalized to the β-actin levels in each sample.

Immunohistochemical Analysis of PKC βII Expression in Mouse Colon Tumors and Normal Colonic Epithelium. Immunohistochemical analysis was performed on 5-μm sections of normal colonic epithelium and colon tumors that were paraformaldehyde fixed and paraffin embedded. After deparaffinization and rehydration, sections were processed for antigen retrieval as described by the manufacturer (DAKO) and treated with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidases. PKC βII expression was detected using the ABC staining system and an isotype-specific antibody to PKC βII (Santa Cruz Biotechnology).

QRT-PCR Analysis of PKC Isozyme mRNA Expression. Total cellular RNA from ACF, normal colonic epithelium, and colon tumors was isolated using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Reverse transcription was performed using 2 μg of total RNA, 1 μg of oligo(dT) primer (Promega), 10 mM DTT, 0.5 mM deoxynucleotide triphosphates, and 200 units of Superscript II reverse transcriptase (Life Technologies, Inc.) in a 50-μl reaction as described previously (11). Samples without reverse transcriptase served as negative controls. Amplification of the cDNAs was carried out using EasyStart PCR mix-in-a-tube (Molecular Bioproducts), 2.5 units of Taq polymerase (Promega), and the appropriate primer pairs for each PKC isozyme. Primers used for PKC isozyme-specific PCR and the expected sizes of their products are as follows: (*a*) PKC α, 5′-TGAATCCTCAGTGGAATGAGT-3′ (forward primer) and 5′-GGTTGCTTTCTGTCTTCTGAA-3′ (reverse primer), 325 bp; (*b*) PKC βI, 5′-TGTGATGGAGTATGTGAACGGGGG-3′ (forward primer) and 5′-TCGAAGTTGGAGGTGTCTCGCTTG-3′ (reverse primer), 640 bp; (*c*) PKC βII, 5′-CATCTGGGATGGGGTGACAACC-3′ (forward primer) and 5′-CGGTCGAAGTTTTCAGCGTTTC-3′ (reverse primer), 420 bp; and (*d*) β-actin, 5′-GTGGGCCGCTCTAGGCACCAA-3′ (forward primer) and 5′-CTCTTGATCTCACGCACGATTTC-3′ (reverse primer), 540 bp. The optimized linear range for each PKC isozyme was determined as 30 cycles for PKC α, 35 cycles for PKC βI and βII, and 25 cycles for β-actin at 95°C for 45 s, 60°C for 45 s, and 72°C for 2 min, followed by a 10-min incubation at 72°C. An initial denaturation step was performed at 95°C for 2 min. PCR products were separated in 1.8-% agarose gel (FMC Bioproducts), and the intensity of ethidium bromide fluorescence was quantitated using an Eagle Eye (Stratagene) densitometer. Quantitation of PKC mRNA expression was normalized to β-actin mRNA levels, which have been shown to be unchanged during colon carcinogenesis (27).

Quantitation of Colon Tumor Parameters in AOM-treated Transgenic PKC βII and Nontransgenic Mice. PKC βII transgenic mice and nontransgenic littermates (19) were injected with AOM as described above. At 36 weeks after the second AOM injection, the mice were sacrificed, and the colons were removed, cut open longitudinally, and rinsed well with cold PBS. Colon tumors were identified visually, and the location and size (mm^2) of each tumor were recorded.

Statistical Analysis. Values are expressed as fold of control expression ± SE. Statistical significance was calculated by using a one-way ANOVA; $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Elevated PKC βII Expression in ACF. We recently demonstrated that increased expression of PKC βII leads to hyperproliferation of the colonic epithelium (19). Because hyperproliferation is an early promotive event in colon carcinogenesis, we assessed the level of expression of PKC βII in early preneoplastic lesions in AOM-treated mice. ACF are the earliest identifiable preneoplastic lesions in

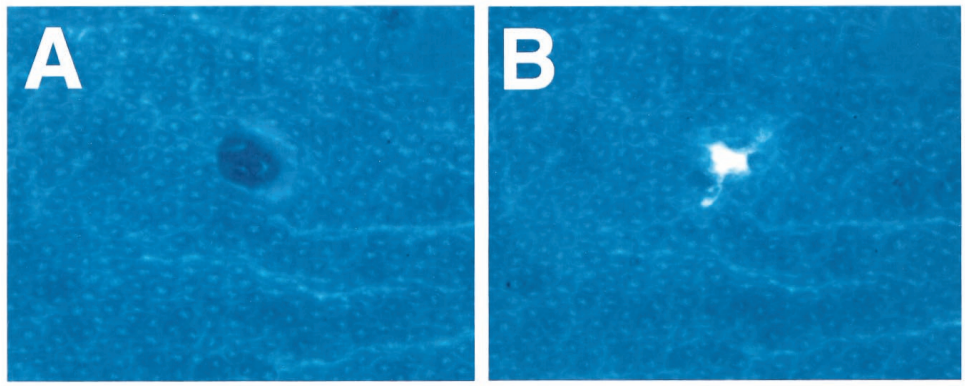

Fig. 1. ACF dissection from an AOM-treated mouse colon. C57BL/6J mice received injection of AOM or saline once a week for 2 weeks (19). At 20 weeks after the second AOM injection, mice were sacrificed, and the colons were isolated. The colons were cut longitudinally, fixed flat in 70% ethanol, and stained with 1% methylene blue for 5 min. ACF were identified under low magnification (×40) using previously defined criteria for ACF (22, 23). A representative colonic epithelium containing an ACF from an AOM-treated mouse is shown before (*A*) and after (*B*) microdissection of the ACF.

the progression of normal colonic epithelium to colon carcinoma in both carcinogen-induced rodent models and humans (22, 23, 28). AOM reproducibly induces ACF and colon tumors in rodents, which exhibit many of the same genetic and signal transduction defects identified in human colon carcinomas (29–31). C57BL/6J mice were injected with AOM or saline as described. At the preneoplastic stage of colon carcinogenesis (20 weeks after injection), mice were sacrificed, and their colons were removed and analyzed visually for the presence of ACF. Individual ACF were microdissected from stained colons for biochemical analysis as described in "Materials and Methods." A representative colonic epithelium containing an ACF from an AOM-treated mouse is shown before (Fig. 1*A*) and after (Fig. 1*B*) microdissection of the ACF. No ACF were observed in saline-treated control animals, consistent with our previous report (19). ACF from AOM-treated mice and normal colonic epithelial tissue from saline-treated mice were isolated as described previously (21), pooled, and subjected to immunoblot and RNA analysis as described in "Materials and Methods." A representative immunoblot for PKC α, βI, and βII in ACF and normal crypts is shown in Fig. 2*A*. A single band with an apparent molecular mass of 85 kDa for PKC α, βI, and βII was detected, consistent with the predicted size for each intact protein. The changes in PKC isozyme levels from two independent experiments were quantitated by densitometric analysis, and the average relative levels of expression were presented as fold of control (Fig. 2*B*). PKC βII protein was increased 3.7-fold in ACF relative to normal epithelium (range, 2.0–5.3-fold). In contrast, PKC α and βI were modestly decreased in ACF. These findings demonstrate that multiple changes in PKC isotype expression occur in the early, preneoplastic stage of colon tumor formation.

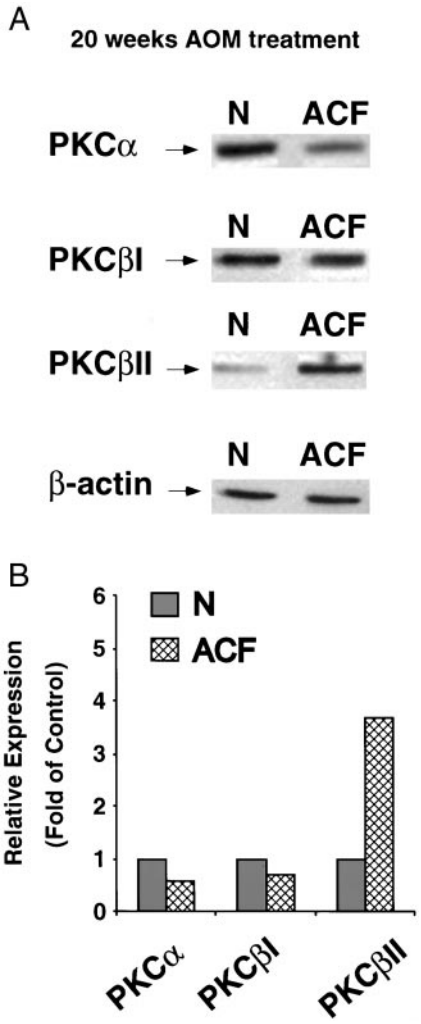

Fig. 2. Immunoblot analysis for PKC isozyme expression in ACF and normal colonic crypts. Carcinogen treatment and colon isolation were performed as described in the Fig. 1 legend. ACF from AOM-treated mice and normal crypts from saline-treated mice were isolated from fixed, stained colons as described in "Materials and Methods." Total cellular protein (5 μg) was subjected to immunoblot analysis using isozyme-specific PKC antibodies. *A*, a representative immunoblot analysis for the indicated PKC isoform is shown. *N*, normal crypts from saline-treated animals; *ACF*, ACF from AOM-treated animals. *B*, immunoblots were quantitated by densitometric scanning of the film. Results are presented as the average of two independent experiments.

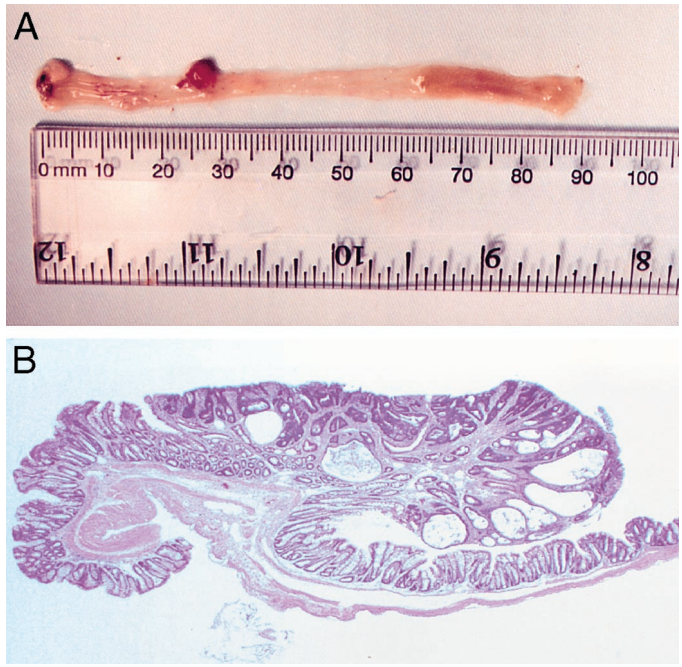

Fig. 3. Colon tumor from an AOM-treated mouse. *A*, gross pathology of a tumor-containing colon from an AOM-treated mouse. *B*, histopathology of typical severely dysplastic, tubular adenoma from the colon of an AOM-treated mouse. Sample was stained with H&E (×10).

PKC Isozyme Expression Is Altered in Colon Tumors. We next determined the level of PKC α, βI, and βII protein in mouse colon tumors at 36 weeks after AOM treatment. Tumors were isolated and stained with H&E as described in "Materials and Methods." Histopathological analysis determined that all tumors were tubular adenomas, a majority of which were severely dysplastic/carcinoma *in situ*. No invasion of the lamina propria was observed in any lesions. The gross pathology and histopathology of a representative colon tumor are shown in Fig. 3, *A* and *B*, respectively. Immunoblot analysis of representative mouse colon tumors demonstrated a dramatic increase in PKC βII expression, whereas PKC βI and α were strikingly reduced or lost in colon tumors compared with normal epithelium (Fig. 4*A*). Quantitative analysis of samples from multiple tumors demonstrated a statistically significant increase in PKC βII expression in all AOM-induced mouse colon tumors analyzed (7.7 ± 2.1; $P = 0.011$), whereas PKC α and βI were both significantly decreased in tumors [0.16 ± 0.05 ($P = 4 \times 10^{-7}$) and 0.08 ± 0.05 ($P = 2 \times 10^{-7}$), respectively] compared with uninvolved colonic mucosa. Similar alterations in PKC isozyme expression were observed in human colon tumors, confirming the similarity of the mouse carcinogen model to human colon tumors.[4] Taken together, our results indicate that an increase in PKC βII expression occurs early in colon carcinogenesis and is maintained in the later stages of this process.

[4] Y. Gökmen-Polar and A. P. Fields, unpublished results.

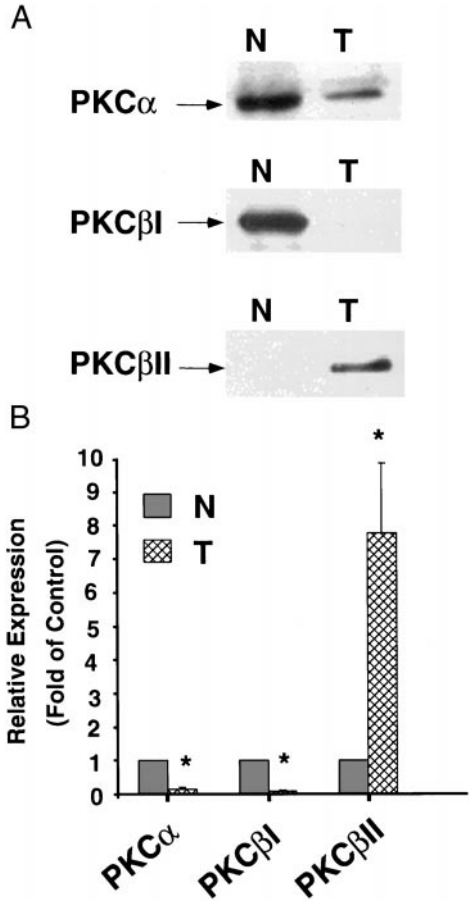

Fig. 4. Expression of PKC isozymes in AOM-induced mouse colon tumors and uninvolved colonic epithelium. C57BL/6J mice were injected with AOM to induce colon tumors as described in "Materials and Methods." Thirty-six weeks after AOM treatment, the mice were sacrificed, and the colons were excised. Uninvolved colonic epithelium and colon tumors were isolated from the same animal as described in "Materials and Methods." *A*, total cellular protein (10 μg) was subjected to immunoblot analysis using isozyme-specific PKC antibodies. Each sample was normalized according to the β-actin levels. Representative results of immunoblot analysis with a specific antibody to the indicated PKC isozyme are shown. *N*, uninvolved mouse colonic epithelium; *T*, mouse colonic tumors. *B*, immunoblots from five pairs of tumor and uninvolved tissue were quantitated, and the mean $\pm$ SE value is presented for each PKC isozyme. $*$, $P < 0.05$.

Our data also demonstrate that the elevation of PKC βII is isozyme specific because a progressive decrease in PKC α and βI is observed during tumor development.

Immunohistochemical Detection of PKC βII Expression. To examine the distribution of PKC βII in colon tumors, immunohistochemical detection of PKC βII was performed on normal epithelium from the distal colon and colon tumors isolated from AOM-injected mice (Fig. 5). Low level PKC βII staining was detected in both the base of the crypts and at the luminal surface of the normal epithelium (Fig. 5*A*). The PKC βII staining at the base of the colonic crypts is concordant with the location of the stem cell population in the distal colon, consistent with a role for PKC βII in proliferation. However, the presence of immunoreactive PKC βII in postmitotic colonocytes suggests that PKC βII may serve multiple roles in this tissue. In colon tumors (Fig. 5*B*), a high level of PKC βII staining was detected throughout the tumor, with the highest levels seen at the surface of the tumor. Interestingly, little or no staining was detected in the stromal elements of the tumor, indicating that the observed increase in PKC βII in colon tumors is due to an increased expression in the epithelial cells comprising the tumor rather than tumor-associated stromal or immune cells. These results confirm the immunoblot data showing overexpression of PKC βII in colon tumors and demonstrate the colocalization of PKC βII with the proliferative cell fraction in both normal colonic epithelium and colon tumors.

PKC βII mRNA Is Only Slightly Increased in AOM-induced ACF. To investigate the regulatory mechanisms responsible for PKC isozyme expression during the early and late stages of colon tumorigenesis, we analyzed the relative mRNA levels for PKC α, βI, and βII in AOM-induced ACF and normal crypts. Total RNA was isolated from ACF and normal crypts and subjected to QRT-PCR using primers specific for mouse PKC α, βI, and βII isozymes, as described in "Materials and Methods." Representative QRT-PCR analyses are shown in Fig. 6*A*. For each RNA sample, no amplified products were detected in the absence of reverse transcriptase (data not shown). Relative band intensities were quantitated by densitometric scanning and are presented as the fold change in expression in Fig. 6*B*. The level of PKC βII mRNA in ACF was only slightly increased relative to that in normal colonic epithelium, as compared with the 3.7-fold increase in PKC βII protein detected in ACF. In contrast, PKC βI and α mRNA levels in ACF were decreased to a level commensurate with the decrease in protein expression of these isozymes in ACF.

PKC βII mRNA Levels Do Not Reflect the Dramatic Alterations in PKC βII Protein Levels in Colon Tumors. Total RNA was isolated from both colon tumors and normal colonic epithelium at 36 weeks after AOM injection and subjected to QRT-PCR using primers specific for mouse PKC α, βI, and βII isozymes as described in "Materials and Methods" (Fig. 7). Relative mRNA levels were quantitated by densitometric scanning, and the fold increase in expression was calculated for each PKC isozyme (Fig. 7*B*). PKC βII mRNA levels were slightly elevated in tumors, whereas those for PKC α were slightly reduced compared with normal epithelium. The level of PKC βI mRNA was also increased slightly in tumors. Therefore, the small alterations in mRNA expression for these PKC isozymes do not correlate with the dramatic changes in expression of the corresponding protein.

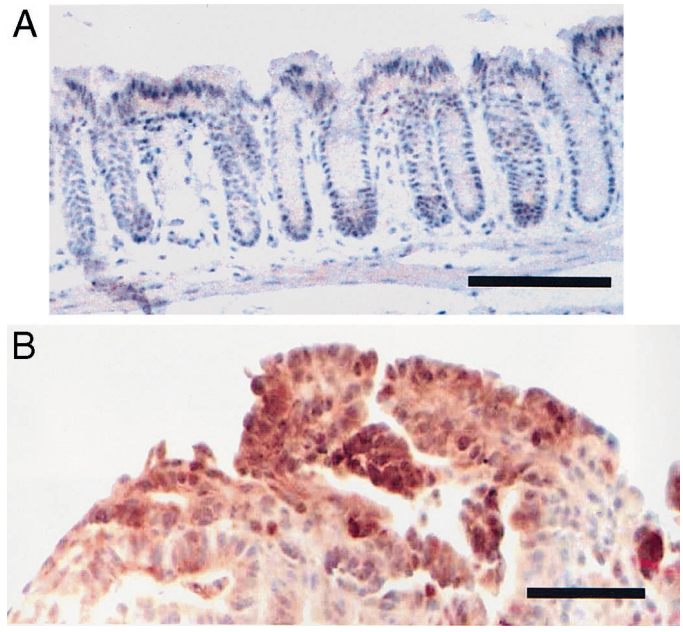

Fig. 5. Immunohistochemical analysis of PKC βII in mouse colon tumor and normal colonic epithelium. Immunohistochemical detection of PKC βII expression was performed as described in "Materials and Methods." PKC βII expression was detected using the ABC staining system and an isozyme-specific antibody to PKC βII. Antigen was visualized by 3,3′-diaminobenzidine staining. *A*, normal mouse colonic epithelium, *bar* = 100 μM; *B*, colon tumor from AOM-treated mouse, *bar* = 50 μM.

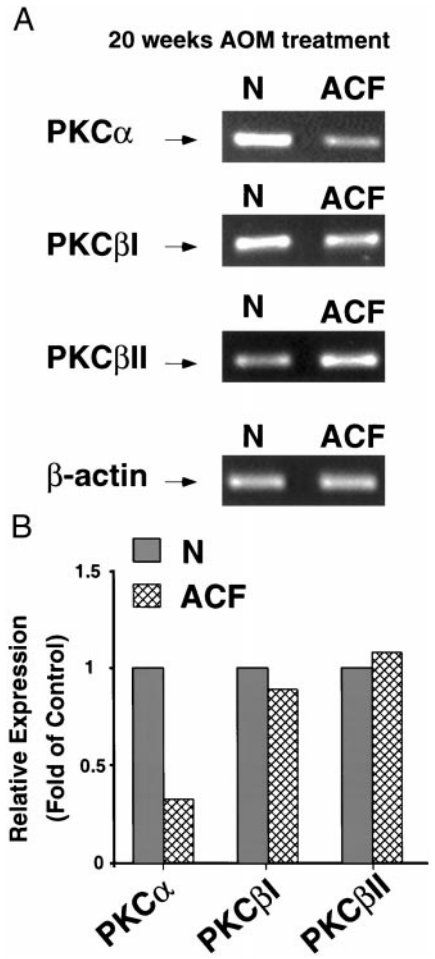

Fig. 6. QRT-PCR analysis for PKC α, βI, and βII mRNA in ACF and normal colonic epithelium. Total RNA was isolated from pooled ACF from AOM-treated mice or from normal colonic epithelium from saline-treated mice, reverse-transcribed, and amplified by PCR using PKC isozyme-specific primers as described in "Materials and Methods." The PCR products were electrophoresed on a 1.8% agarose gel and stained with ethidium bromide. *A*, representative reverse transcription-PCR results are shown for each PKC isozyme. Each sample was normalized to the β-actin levels. *N*, normal crypts from saline-treated mice; *ACF*, ACF from AOM-treated mice. *B*, quantitation of relative expression of PKC isozyme mRNA by densitometric scanning of the stained gel. Results are presented as the average of two independent experiments.

Transgenic PKC βII Mice Are More Susceptible to Development of Carcinogen-induced Colon Tumors. We have demonstrated that transgenic mice that overexpress PKC βII in the colonic epithelium are more susceptible to AOM-induced colon carcinogenesis, as assessed by an increase in the total number of ACF and in the number of ACF of higher multiplicity than nontransgenic mice (19). The number and multiplicity of ACF have been demonstrated to be highly predictive of subsequent tumor formation in carcinogen-induced rodent models (32, 33). To further evaluate the effect of transgene expression on susceptibility to AOM-induced colon tumor formation, transgenic PKC βII mice and nontransgenic littermates were treated with AOM and analyzed for tumor formation as described in "Materials and Methods." Transgenic PKC βII mice exhibit a higher incidence (9 of 15 mice; 60%) of colon tumors than nontransgenic mice (4 of 12 mice; 33%) in response to AOM treatment. In addition to tumor incidence, other tumor parameters also showed an upward trend in transgenic PKC βII mice. Thus, tumor multiplicity (average number of tumors:tumor-bearing mouse) increased from 1.3 ± 0.3 (5 tumors in 4 nontransgenic tumor-bearing mice) to 1.5 ± 0.3 (14 tumors in 9 transgenic PKC βII, tumor-bearing mice). Tumor burden (average tumor area per tumor-bearing mouse) was also elevated from 12.5 ± 5.5 mm^2/mouse in nontransgenic mice to 19.3 ± 5.2 mm^2/mouse in transgenic PKC βII mice. Finally, the average tumor size was greater in transgenic PKC βII mice (12.4 ± 1.6 mm^2/tumor) than in nontransgenic mice (10.0 ± 2.6 mm^2/tumor). Because of the small number of mice used in this study, the increases observed in transgenic PKC βII did not reach statistical significance. However, the increases in all of these colon tumor parameters suggest that elevated expression of PKC βII in the colonic epithelium results in increased susceptibility to colon carcinogenesis and tumor formation.

DISCUSSION

Colon carcinogenesis is a multistep process that results from multiple genetic and nongenetic alterations (1). Identification of the alterations that occur in the earliest detectable preneoplastic lesions may allow development of early colon cancer screening and prevention methods. Elevated expression of PKC βII has been associated with neoplastic transformation in both rat and human colonic epithelium (8, 11). In the present study, we assessed the timing and importance of changes in PKC βII expression during colon carcinogenesis.

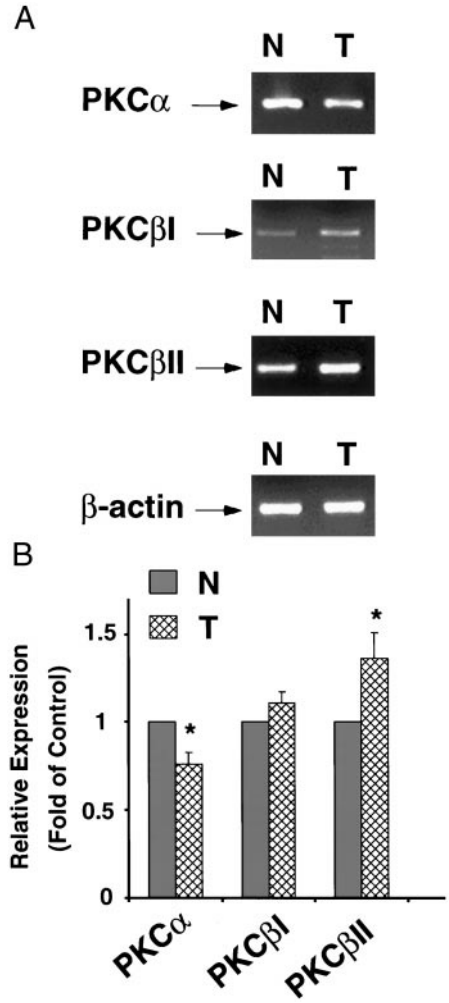

Fig. 7. Relative mRNA levels of PKC isozymes in AOM-induced mouse colon tumors and normal colonic epithelium. Total RNA (2 μg) was extracted from uninvolved colonic epithelium and tumor tissues from AOM-treated mice, reverse-transcribed, and amplified by PCR as described in "Materials and Methods." *A*, representative reverse transcription-PCR results for each PKC isozyme are shown. *N*, normal, uninvolved mouse epithelium; *T*, tumor tissue. *B*, relative expression of PKC isozyme mRNA was quantitated from five pairs of tumor and uninvolved tissue, and the mean ± SE value is presented for each PKC isozyme. *, $P < 0.05$.

Using the mouse carcinogen (AOM) colon carcinogenesis model, we determined the levels of PKC α, βI, and βII expression in colon tumors and in ACF, the earliest preneoplastic lesions found in both AOM-treated mice and humans.

Our findings suggest that PKC βII plays a role at multiple stages of colon carcinogenesis and that changes in more than one PKC isozyme may be involved in the multistep process of colon tumorigenesis. In ACF, the level of PKC βII protein expression was strikingly increased compared with a small reduction in PKC α and βI protein expression, implicating PKC βII in early preneoplastic changes. In colon tumors, we observed a significant elevation in PKC βII protein expression compared with that seen in normal colonic epithelium, whereas the expression of PKC α and βI was significantly reduced or lost. Therefore, increased PKC βII expression occurs in the early, preneoplastic stages of colon carcinogenesis and remains high in colon tumors. On the other hand, the dramatic decrease in PKC α and βI protein expression in tumors but not ACF suggests that decreases in PKC α and βI expression may be important for later stages of tumor development. Similar changes in PKC isozyme expression have been observed in human colon tumors,[4] emphasizing the relevance of the AOM-induced colon carcinogenesis model to sporadic human colon cancer. These results are in accordance with previous reports from our laboratory, and others have demonstrated that PKC βII promotes cellular proliferation in human leukemia cells and colon cancer cell lines (7, 34). A direct role for PKC βII in colon carcinogenesis has also been established by our recent finding that transgenic PKC βII mice exhibit colonic hyperproliferation and increased susceptibility to colon carcinogenesis, as determined by an increase in the total number of ACF and in the number of ACF of higher multiplicity as compared with nontransgenic mice (19). In the present study, we extend these findings and report a trend toward higher tumor incidence in transgenic PKC βII mice as compared with nontransgenic mice. Average tumor burden, tumor size, and tumor multiplicity all showed an upward trend in transgenic PKC βII mice compared with nontransgenic mice. Our recent data also demonstrate that elevated PKC βII leads to inhibition of glycogen synthase kinase 3β (GSK-3β) activity and an accumulation of β-catenin (19), suggesting that PKC βII causes colonic hyperproliferation and promotes colon carcinogenesis, at least in part, by activating the adenomatous polyposis coli (APC)/β-catenin signaling pathway (19).

Based on epidemiological studies in man (reviewed in Refs. 35 and 36) and biochemical studies in rodent models (37, 38), it has been well established that diets high in certain fatty acids play a promotive role in colon carcinogenesis. Furthermore, high-fat diets result in the production of fatty acid metabolites and secondary bile acids that stimulate lumenal bacterial phospholipases and activate colonic epithelial cell PKC by production of diacylglycerol (39, 40). Based on these data, we have proposed a model for PKC βII in colon carcinogenesis in which stimulation of PKC βII by various dietary lipid components results in the activation of the APC/β-catenin signaling pathway, resulting in hyperproliferation and an increase in susceptibility to colon carcinogenesis (19). Based on our present findings, we propose that an increase in PKC βII expression early in colon carcinogenesis results in hyperproliferation and ACF formation. Our previous studies demonstrated that transgenic PKC βII mice exhibit increased numbers of ACF and also increased multiplicity of ACF, suggesting a role for PKC βII in both initiation and progression (19). Future studies will address the issue of whether the expression level of PKC βII in individual ACF correlates with genetic and morphological changes associated with progression to carcinoma.

Interestingly, the expression of PKC βI protein, a splice variant of PKC βII, is dramatically reduced or lost in AOM-induced mouse colon tumors. These data suggest that PKC βI and PKC βII play distinct and possibly opposing roles in colon tumorigenesis and that the differences in expression may be regulated at the level of alternative splicing. A switch mechanism from PKC βI to βII mRNA has been reported in response to insulin in which alternative splicing results in increased protein levels of PKC βII (18). In L6 skeletal muscle cells, insulin enhances the inclusion of a PKC βII-specific exon and thereby specifically increases the expression of PKC βII protein (41). Experiments are underway to determine whether a similar switch mechanism is involved in the dramatic increase of PKC βII and loss of PKC βI protein expression during colon carcinogenesis.

Expression of PKC α is dramatically reduced or lost in AOM-induced colon tumors, suggesting a negative relationship between PKC α and cell growth. Consistent with these findings, a compelling body of evidence indicates that PKC α is associated with negative growth regulation and cell cycle arrest in various cell lines (42–46). Our earlier data also demonstrated that overexpression of PKC α is involved in cytostasis and phorbol 12-myristate 13-acetate (PMA)-induced differentiation in human K562 erythroleukemia cells (7), whereas inhibition of PKC α expression blocks phorbol 12-myristate 13-acetate-induced differentiation (3). In the nontransformed intestinal epithelial IEC-18 cell line, activation of PKC α results in agonist-induced cell cycle arrest and induction of Cip/Kip family of cyclin-dependent kinase inhibitors, concomitant with the hypophosphorylation of the retinoblastoma protein (Rb) (47). Furthermore, overexpression of PKC α causes inhibition of cellular proliferation and enhanced differentiation in CaCo-2 human adenocarcinoma cells, whereas antisense PKC α-transfected CaCo-2 cells exhibit enhanced cellular proliferation and transformed phenotype (45, 46). Nude mice injected with antisense PKC α-expressing CaCo-2 cells exhibited an increased number of tumors and an increased tumor burden compared with mice injected with sense PKC α-expressing CaCo-2 cells (46). Additionally, a role for PKC α in cell adhesion has been implicated in the poorly differentiated colorectal carcinoma VOM cell line (48). PMA-induced activation of PKC α restores normal epithelial morphology in VOM cells (48). These data, as well as those presented here, are consistent with the hypothesis that the loss of expression of PKC α during colon carcinogenesis allows premalignant cells to overcome normal growth-inhibitory processes. It remains to be determined whether PKC α plays a negative growth-regulatory role in the colonic epithelium *in vivo*.

To investigate the regulatory mechanisms responsible for the alterations of PKC isozyme expression, we analyzed mRNA expression of PKC α, βI, and βII in AOM-induced ACF and colon tumors. In both ACF and tumors, the level of PKC βII mRNA was increased, whereas the level of PKC α mRNA was decreased. However, although the changes in mRNA levels were significant, the dramatic changes in PKC isozyme protein expression could not be explained by transcriptional control alone. This is particularly true for PKC βI, where protein expression is almost undetectable in tumors, whereas the level of mRNA in tumors is slightly increased relative to normal epithelium. Other regulatory mechanisms that could contribute to the observed alterations in PKC isozyme expression include changes in message stability or translatability, protein phosphorylation, and protein stability. Future studies will explore the contribution of these mechanisms to PKC isozyme expression in colonic epithelial cells.

In summary, the expression of PKC isozymes changes dramatically during colon carcinogenesis. Expression of PKC βII increases early in preneoplastic lesions (ACF) and is maintained in colon tumors. PKC α expression declines progressively throughout the carcinogenic process. A dramatic change in expression of PKC βI also occurs later in the carcinogenic process, with essentially no detectable PKC βI remaining in colon tumors. Our data demonstrate that changes in mRNA levels for individual PKC isozymes do not coincide with alterations in protein expression. Therefore, caution should be used in

interpreting quantitative analysis of changes in PKC isozyme mRNA in the absence of information on protein expression. Future studies will focus on the delineation of the molecular mechanisms responsible for the alterations of individual PKC isozyme expression. Finally, development of transgenic mouse models for individual PKC isozymes will provide valuable tools to analyze the role of individual PKC isozymes in colonic epithelial cell homeostasis and colon carcinogenesis.

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