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Overexpression of Protein Kinase C β_{II} Induces Colonic Hyperproliferation and Increased Sensitivity to Colon Carcinogenesis

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Abstract. Protein kinase C β_{II} (PKC β_{II}) has been implicated in proliferation of the intestinal epithelium. To investigate PKC β_{II} function in vivo, we generated transgenic mice that overexpress PKC β_{II} in the intestinal epithelium. Transgenic PKC β_{II} mice exhibit hyperproliferation of the colonic epithelium and an increased susceptibility to azoxymethane-induced aberrant crypt foci, preneoplastic lesions in the colon. Furthermore, transgenic PKC β_{II} mice exhibit elevated colonic β-catenin levels and decreased glycogen synthase kinase 3β activity, indicating that PKC β_{II} stimulates the Wnt/adenomatous polyposis coli (APC)/β-catenin proliferative signaling pathway in vivo. These data demonstrate a direct role for PKC β_{II} in colonic epithelial cell proliferation and colon carcinogenesis, possibly through activation of the APC/β-catenin signaling pathway.

Key words: protein kinase C • colon carcinogenesis • signal transduction • proliferation • transgenic mice

Colon carcinogenesis is a complex multistep process involving progressive disruption of homeostatic mechanisms controlling intestinal epithelial cell proliferation, differentiation, and programmed cell death. This disruption appears to be mediated by dietary and environmental factors that modulate intestinal epithelial cell signaling pathways, as well as genetic mutation of transforming oncogenes and deletion or mutation of DNA repair enzymes and tumor suppressor genes (Bertagnolli et al., 1997). Recent studies have demonstrated the primary importance of the Wnt/APC/β-catenin signaling pathway in colon carcinogenesis (Pennisi, 1998). Mutations in either APC or β-catenin that lead to activation of this pathway are present in the vast majority of colon cancers and colonic carcinoma cell lines (Pennisi, 1998).

Accumulating evidence implicates protein kinase C (PKC)[1] in intestinal epithelial cell proliferation and colon carcinogenesis both in rodents and humans (Weinstein, 1990; Chapkin et al., 1993). PKC activity is higher in actively proliferating colonic epithelial cells than in their quiescent counterparts (Craven and DeRubertis, 1987), suggesting a role for PKC activation in epithelial cell proliferation. A link between PKC and colon carcinogenesis comes from the observation that components of cancer-promoting high fat diets lead to an increase in both colonic epithelial cell PKC activity and cellular proliferation (Craven and DeRubertis, 1988; Reddy et al., 1996). High fat diet-induced hyperproliferation is thought to predispose the colonic epithelium to further genetic and biochemical changes associated with progression along the carcinogenic pathway. PKC has also been shown to play a requisite role in the Wnt/APC/β-catenin proliferative signaling pathway, suggesting a plausible molecular mechanism by which PKC could stimulate colonic epithelial cell proliferation and colon carcinogenesis (Cook et al., 1996).

Several lines of evidence indicate that the PKC β_{II} isozyme (PKC β_{II}) is selectively involved in colonic epithelial cell proliferation and colon carcinogenesis. First, PKC β_{II} is the most responsive of the PKC isozymes expressed in the colonic epithelium to activation by secondary bile acids (Pongracz et al., 1995). Secondary bile acid levels are elevated in rodents fed a cancer-promotive high fat diet and this increase has been implicated in early carcinogenic events (for review see Reddy, 1975). Second, expression of most colonic PKC isozymes (e.g., PKC α, δ, and ζ) is reduced in the presence of chronically elevated diacylglyc-

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1. *Abbreviations used in this paper:* ACF, aberrant crypt foci; AOM, azoxymethane; APC, adenomatous polyposis coli; DAG, diacylglycerol; DBA, dolichos biflorus agglutinin; FABP, fatty acid binding protein; GSK-3β, glycogen synthase kinase 3β; PAS, periodic acid Schiff; PKC, protein kinase C; PKC β_{II}, protein kinase C β_{II} isozyme; PNA, peanut agglutinin; RT-PCR, reverse transcriptase PCR; TUNEL, TdT-mediated dUTP-biotin nick end labeling; UEAI, Ulex europaeus-I.

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erol (DAG), such as is present in preneoplastic colonic epithelial cells (Wali et al., 1991; Jiang et al., 1996; Chapkin et al., 1997; Jiang et al., 1997). However, intestinal PKC β_{II} is largely resistant to such activator-mediated downregulation (Saxon et al., 1994, Sauma et al., 1996). Third, the levels of PKC β_{II} are dramatically elevated both during the initial stages of tumorigenesis and in colonic carcinomas when compared with normal colonic tissue (Craven and DeRubertis, 1992; Davidson et al., 1994, 1998). Finally, PKC β_{II} is directly involved in colon carcinoma cell proliferation in vitro (Lee et al., 1993; Sauma et al., 1996).

These studies provide compelling but indirect evidence that PKC β_{II} plays an important role in intestinal epithelial cell proliferation and colon carcinogenesis, and are consistent with our studies demonstrating that PKC β_{II} is required for leukemia cell proliferation (Murray et al., 1993). Therefore, we hypothesized that PKC β_{II} is directly involved in intestinal epithelial cell proliferation in vivo and that elevated colonic PKC β_{II} expression and activity would enhance colon carcinogenesis. To directly test this hypothesis, we generated transgenic mice that express elevated levels of PKC β_{II} in the intestinal epithelium. These animals exhibit both hyperproliferation of the colonic epithelium and an increased susceptibility to colon carcinogenesis. Furthermore, our data indicate that the β-catenin/APC proliferative signaling pathway is stimulated by PKC β_{II} in these animals.

Materials and Methods

Production and Maintenance of Transgenic Mice

A transgene construct consisting of the rat liver fatty acid binding protein (FABP) promoter (−596 to +21; kindly provided by Dr. Jeffrey Gordon, Washington University, St. Louis, MO), the full-length human PKC β_{II} cDNA, and the SV40 large T antigen polyadenylation signal sequence was produced by conventional cloning methods. The resulting PKC β_{II} transgene construct was confirmed by direct microsequencing before microinjection. The pFABP/PKC β_{II} transgene construct was propagated in the mammalian expression vector pREP4 and the transgene insert was excised using NheI (5′) and XbaI (3′), purified, and microinjected into C57BL/6J × C3H/HeJ F_2 mouse oocytes as previously described (Hogan et al., 1994). The microinjections and generation of transgenic founder mice were conducted at the University of Texas Medical Branch Transgenic Mouse Facility. Transgenic founder mice were identified by Southern blot analysis. In brief, genomic tail DNA (5 μg) was digested to completion with Taq I (Roche), resolved by agarose gel electrophoresis, transferred to nylon membrane (Amersham), and transgenic DNA detected with a radiolabeled probe corresponding to the SV40 polyadenylation sequence. Three transgenic founder animals were identified from a screen of 120 live births. Transgene copy number was determined for each transgenic line by quantitative Southern blot analysis as previously described (Hogan et al., 1994). Genotype was confirmed by slot blot analysis using a radiolabeled probe corresponding to the polyadenylation sequences within the transgene (Sambrook et al., 1989).

Founder mice were mated with C57BL/6J mice (The Jackson Laboratory) to establish the transgene on a stable genetic background. Transgenic PKC β_{II} mice and progeny were bred and housed in microisolator cages maintained at constant temperature and humidity on a 12-h on/12-h off light cycle in a pathogen-free barrier facility. Mice were provided a standard autoclavable chow (Purina 7012, 5% fat) and autoclaved water ad libitum.

Detection of Transgenic PKC β_{II} RNA

Total RNA was extracted from tissue samples using a Totally RNA kit (Ambion). Reverse transcription was carried out using 6 μg RNA, 1 μg oligo(dT) primer, 10 mM dithiothreitol, 0.5 mM dNTPs, and 200 U SuperScript II reverse transcriptase (GIBCO BRL). Amplification of the transgenic RNA was carried out using 20 ng of the following primers, which amplify human PKC β_{II} but not endogenous mouse PKC β_{II}: forward, 5′ CGTCCTCATTGTCCTC 3′; reverse 5′ GACCTTGGTTCCCTGACTG 3′. An optimized amplification program of denaturation (94°C, 15 s), annealing (56°C, 15 s), and extension (74°C, 45 s) for 40 cycles using PCR Supermix (GIBCO BRL) was used. Human brain RNA was used as a positive control; mouse brain RNA and samples incubated without reverse transcriptase served as negative controls.

PKC Immunoblot and Immunohistochemical Analysis

Immunoblot analysis for PKC β_{II} expression in mouse colonic epithelium was performed essentially as previously described (Davidson et al., 1994). In brief, mice were killed by CO_2 asphyxiation, the colons were isolated and slit open longitudinally and rinsed well with PBS, and the colonic epithelium was scraped using a plastic coverslip. Total cell extracts were prepared in RIPA buffer [50 mM Tris, pH 7.2, 150 mM NaCl, 2 mM EDTA, 0.4 mM EGTA, 20 μM NaF, 0.5% deoxycholate, 1% NP-40, 0.1% SDS, 0.1 mM Na_3VO_4, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 25 μg/ml pepstatin, 1 μg/ml soybean trypsin inhibitor, and 34.5 μg/ml 4-(2-aminoethyl) benzene sulfonyl fluoride]. Equal amounts (30 μg) of protein were subjected to immunoblot analysis using an isotype-specific antibody for PKC β_{II} (Santa Cruz Biotechnology).

Immunohistochemistry was performed using an enhanced biotinyl tyramide system (New England Nuclear) on sections from the proximal and distal colon fixed in ethanol, embedded in paraffin, and sectioned (5 μm) as previously described (Jiang et al., 1995), with the following modifications. After deparaffinization and rehydration of tissues, sections were treated with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidase, blocked with TNB reagent (Dupont New England Nuclear), and incubated with polyclonal antibody to PKC β_{II} (Hocevar and Fields, 1991). Specificity was confirmed using antibody preincubated with excess antigen peptide as previously described (Jiang et al., 1995). Sections were incubated with biotinylated secondary antibody followed by addition of streptavidin-conjugated peroxidase. Biotinyl tyramide amplification reagent was then added followed by a second streptavidin-peroxidase incubation. Visualization was with DAB chromagen.

Measurement of Colonic Epithelial Cell Cytokinetics

12-wk-old mice were killed and their colons were dissected and measured for overall length. The distal colon (1 cm from rectal end) was fixed in 4% paraformaldehyde and processed for histology as described previously (Jiang et al., 1995). Tissues were embedded in paraffin, sectioned (5-μm thickness) and stained with hematoxylin and eosin. 25 full-length, longitudinally cut crypts from each animal were analyzed for crypt height (micrometer) and number of cells per crypt height. 25 crypts cut on the cross-section at random height were counted to determine the average crypt circumference (in number of cells). These data were used to calculate cell size (crypt height in micrometer/crypt height in cell number) and estimate the total cells per crypt (mean cells per crypt column × mean crypt circumference).

Proliferation. Cell proliferation was determined by immunohistochemical detection for proliferating cell nuclear antigen (PCNA) in distal colon sections. Primary antibody against PCNA (PC10 clone; DAKO) was diluted 1:50 in PBS and preincubated with 1:200 biotinylated anti–mouse IgG (Santa Cruz Biotechnology) overnight at 4°C. After deparaffinization, sections were processed for antigen retrieval as described by the manufacturer (DAKO), treated with 1% hydrogen peroxide for 10 min to inactivate endogenous peroxidases, and blocked with normal goat serum. The slides were then incubated with the PCNA/anti–mouse IgG antibody conjugate for 60 min at room temperature. Antigen–antibody complexes were detected with avidin and peroxidase–labeled biotin (ABC staining system; Santa Cruz Biotechnology) and visualized with DAB. Slides were counterstained with hematoxylin to provide contrast. 20 full-length, longitudinally cut crypts were divided into thirds and scored visually for cells staining darkly for PCNA (Lin et al., 1996). The labeling index (percent of labeled cells) and proliferative zone (highest cell from the bottom of the crypt staining for PCNA divided by the total cells per crypt height) were calculated for each set of animals.

Differentiation. The differentiation status of colonic epithelial cells was measured by detection of the specific binding of three different lectins. After deparaffinization, sections were incubated for 60 min at room temperature in normal goat serum. Three different biotinylated lectins (dolichos biflorus agglutinin [DBA], peanut agglutinin [PNA], and Ulex europaeus-I [UEAI]; Vector Labs.) were diluted to 10 μg/ml in PBS. Sections

were incubated with one of the three lectin solutions for 60 min at room temperature. Sections were then washed in three changes of PBS and incubated with 5 μg/ml of rhodamine red-X–conjugated Streptavidin (Jackson Immunoresearch Labs.) in PBS for 30 min at room temperature. After three 5-min washes in PBS, sections were mounted in aqueous media containing 95% glycerol in PBS and analyzed by fluorescence microscopy. Sections were also analyzed histologically by Alcian blue/periodic acid Schiff (PAS) staining for detection of mature, mucin-producing goblet cells.

Apoptosis. The percentage of cells undergoing apoptosis (apoptotic index) was determined in paraformaldehyde-fixed distal colon tissue by the TdT-mediated dUTP-biotin nick end labeling of fragmented DNA (TUNEL) assay (Gavrieli et al., 1992) using the apoTACS kit from Trevigen. The tissue sections were counterstained with methyl green. 100 longitudinally cut, full-length crypts were scored for apoptotic cells based on a combination of positive staining and morphological criteria as previously described (Kerr et al., 1995).

Carcinogen Treatment and Aberrant Crypt Foci Analysis

40 (20 transgenic PKC β_{II} mice, 20 nontransgenic littermates) 6–7-wk-old female mice were injected intraperitoneally with azoxymethane (10 mg/kg body wt) or saline weekly for 2 wk as previously described (Chang et al., 1997). At 5 and 20 wk after the second injection, five animals per group were killed by CO_2 asphyxiation and the colons were removed. The colons were flushed with PBS to remove fecal pellets, slit open longitudinally, and fixed flat between two pieces of filter paper under a glass plate in 70% ethanol for 24 h. Fixed colons were stained with 0.2% methylene blue in PBS for 5 min before being mounted on a glass slide for observation at low magnification (×40) on a light microscope. Aberrant crypt foci (ACF) were scored blindly by a single observer (A.P. Fields) for total number and multiplicity (number of crypts/focus) using previously defined criteria (McLellan et al., 1991).

β-Catenin Immunoblot Analysis and Glycogen Synthase Kinase 3β Kinase Assay

Colonic epithelia from transgenic and nontransgenic mice were scraped and equal amounts of protein from total tissue lysates were subjected to immunoblot analysis using a specific β-catenin polyclonal antibody (Santa Cruz Biotechnology Inc.) or a specific GSK-3β monoclonal antibody (Transduction Laboratories). For glycogen synthase kinase (GSK)-3β kinase assay, colonic epithelium scrapings were solubilized in lysis buffer [10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 1% Triton X-100, 150 mM NaCl, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 25 μg/ml pepstatin, 1 μg/ml soybean trypsin inhibitor, 34.5 μg/ml 4-(2-aminoethyl) benzene sulfonyl fluoride, 20 μM NaF, and 0.1 mM Na_3VO_4]. Lysates containing 300 μg of protein were precleared with 75 μl of protein A agarose and then added to 75 μl of protein A agarose beads that had been preincubated with 5 μg of anti–GSK-3β monoclonal antibody (Transduction Labs.). Samples were incubated for 1 h at 4°C, and beads were pelleted and washed once with lysis buffer and once with kinase assay buffer (8 mM MOPS, pH 7.4, 0.2 mM EDTA, 10 mM Mg acetate, and 0.1 mM ATP). The washed and pelleted beads were then resuspended in 40 μl of kinase assay buffer containing 10 μCi [γ^{32}P]ATP and 250 μmol of GSK-3β–specific substrate peptide (Upstate Biotechnology, Inc.). Reactions were incubated for 20 min at 25°C and stopped by pelleting the beads and adding the supernatant to 20 μl of 40% trichloroacetic acid. Reactions were spotted on P-81 filters and washed three times in 0.75% phosphoric acid and once with acetone. Incorporated radioactive phosphate was quantitated by Cerenkov counting. Nonspecific and background counts were calculated by performing parallel assays with a nonphosphorylatable GSK-3β substrate peptide.

Results

Generation and Identification of PKC β_{II} Transgenic Mice

To investigate the role of PKC β_{II} in colonic epithelial cell biology, we generated transgenic mice overexpressing PKC β_{II} in the intestinal epithelium. For this purpose, we used the rat liver FABP promoter, which has been well characterized to target transgene expression to the intestinal epithelium (Cohn et al., 1991; Simon et al., 1993). A schematic diagram of the transgene construct is presented in Fig. 1 A. The FABP promoter (−596 to +21) was fused

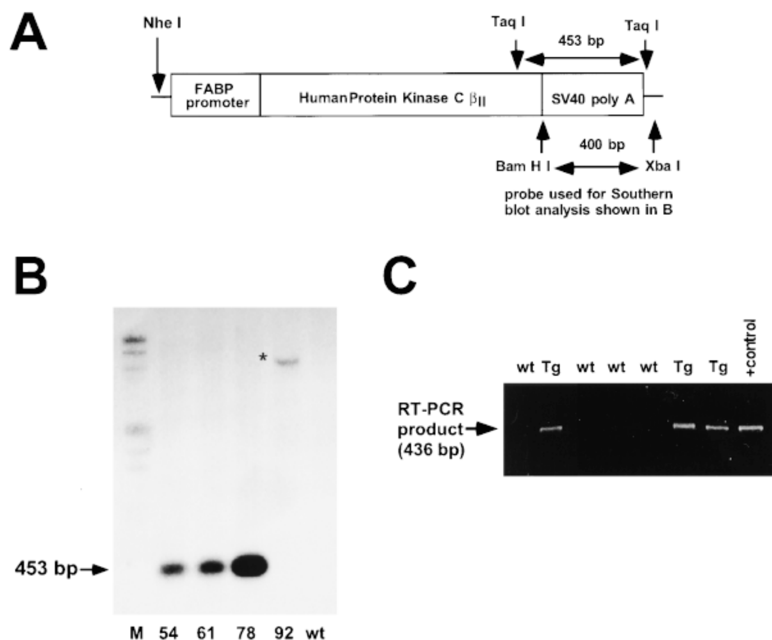


Figure 1. (A) Generation and characterization of transgenic PKC β_{II} mice. Schematic diagram of the PKC β_{II} transgene construct. A transgene construct consisting of the rat liver FABP promoter (−596 to +21), full-length human PKC β_{II} cDNA, and the SV40 large T antigen polyadenylation signal sequence was generated. Restriction sites for excision of the construct from the cloning vector (NheI/XbaI), for generation of radiolabeled probe (BamHI/XbaI), and for digestion of genomic DNA for Southern blot analysis (Taq I) are indicated with arrows. (B) Southern blot analysis to identify founder transgenic mice. Tail DNA was digested with Taq I to generate a 453-bp fragment containing the SV40 polyadenylation sequence from the transgene (see A). 5 μg of digested genomic DNA was resolved by gel electrophoresis and transferred to nitrocellulose, and the membrane was incubated with ^{32}P-labeled probe to the SV40 polyadenylation sequence. M, radiolabeled DNA mol wt markers; 54, 61, 78, and 92 indicate genomic DNA from four founder animals carrying the transgene construct; wt, genomic DNA from a nontransgenic mouse; * indicates an anomalous size band reacting with the transgene probe. (C) Transgenic PKC β_{II} RNA is expressed in the colon of transgenic mice. Total RNA was isolated from scraped colonic epithelium from a litter of mice in the 54 transgenic line. Reverse transcription and amplification was carried out using primers specific to regions of sequence divergence between human and mouse PKC β_{II}. Amplification products were separated on agarose gels and the product was visualized by ethidium bromide staining. Human brain RNA was used as a positive control (+ control); tg, RNA from transgenic animals; *wt*, RNA from nontransgenic animals. Genotype was confirmed by slot blot analysis.

to the cDNA for human PKC β_{II} and the SV40 poly A signal sequence by conventional cloning. Southern blot analysis of tail DNA identified four potential transgenic founders (designated Nos. 54, 61, 78, and 92) from 120 live births (Fig. 1 B). Animal 92 gave a reactive band of higher mol wt than the expected 453 bp Taq 1 fragment generated from the intact transgene (Fig. 1, asterisk). Further analysis using overlapping PCR primer sets demonstrated that this animal contained a truncated transgene, whereas animals 54, 61, and 78 contained multiple copies of the intact transgene construct. All three of the founder animals were fertile and subsequent analysis of progeny by quantitative Southern blot analysis demonstrated that they carried 6, 15, and 31 copies of the transgene, respectively. Furthermore, all three transgenic lines exhibit germline transmission of the transgene to subsequent progeny (data not shown).

Characterization of PKC β_{II} Transgene Expression

PKC β_{II} transgenic RNA expression was detected by reverse transcriptase (RT)-PCR using primers specific for the human PKC β_{II} transgene. A representative RT-PCR analysis of a litter of mice from the 54 transgenic line is shown in Fig. 1 C. As can be seen, transgenic PKC β_{II} mRNA is detected in the colonic epithelium of all three transgenic mice, but not in nontransgenic littermates. Further analysis demonstrated that PKC β_{II} transgene expression is fully penetrant, being detected in the colonic epithelium of all transgenic mice tested. Furthermore, no false positive RT-PCR products have been detected in nontransgenic animals, demonstrating the specificity of the RT-PCR primers for the human PKC β_{II} transgene construct. Similar results were obtained in the 61 and 78 transgenic lines (data not shown).

We next determined the level of PKC β_{II} protein expression in the colonic epithelium of transgenic mice. Colonic epithelial cell lysates from transgenic and nontransgenic animals from the 54 transgenic line were prepared and subjected to immunoblot analysis using a PKC β_{II} isozyme-specific antibody (Hocevar and Fields, 1991). Con-

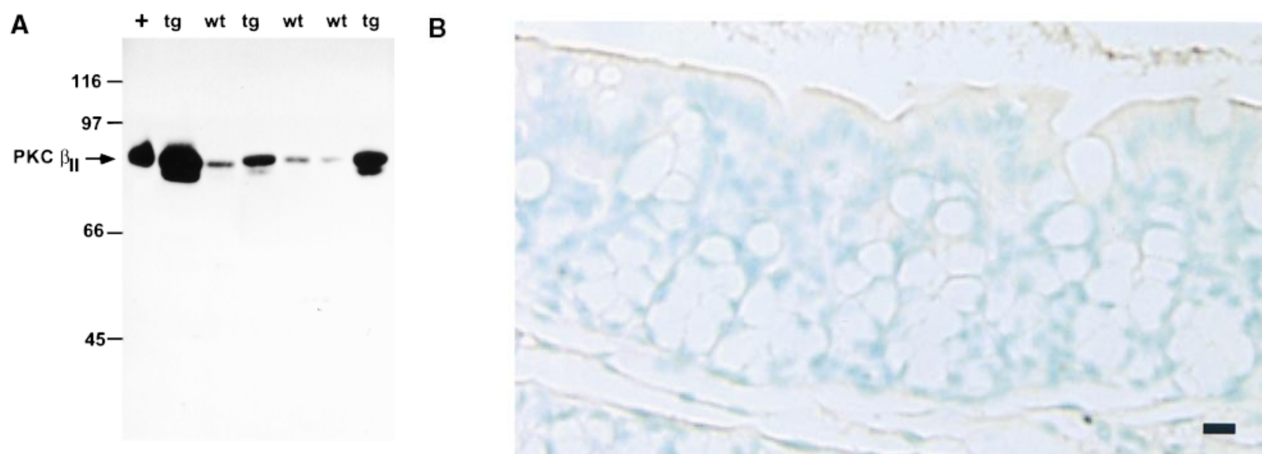


Figure 2. PKC β_{II} protein is overexpressed in the colons of transgenic mice. (A) Immunoblot analysis for PKC β_{II}. A litter of mice from the 54 transgenic line was killed, the colons were isolated and scraped, and total cell lysates (30 μg) were subjected to immunoblot analysis using a PKC β_{II}–specific antibody as previously described (Murray et al., 1993). Densitometric analysis indicates that transgenic animals express an average of fivefold higher level of PKC β_{II} than do nontransgenic littermates. tg, lysates from transgenic animals; wt, lysates from nontransgenic animals; +, rat brain extract used as positive control. (B and C) Immunohistochemical localization of PKC β_{II} in proximal colon of transgenic mice. Proximal colon from transgenic mice and nontransgenic littermates in the 54 transgenic line were fixed in 70% ethanol, embedded in paraffin, sectioned, and immunostained for PKC β_{II} as described in Materials and Methods. In nontransgenic colon (B), endogenous PKC β_{II} localizes to the mid-crypt areas and to the luminal surface of the crypts. In transgenic colon (C), PKC β_{II} staining is greater than in the nontransgenic animal and is observed along the entire crypt axis. Original magnification: ×400. Bars, 10 μm.

sistent with the presence of transgenic PKC β_{II} mRNA, PKC β_{II} protein levels in the colonic epithelium of transgenic mice are elevated relative to their nontransgenic littermates (Fig. 2 A). Transgenic PKC β_{II} exhibits a relative molecular mass of ~85 kD, comigrating with mouse brain PKC β_{II} used as a positive control. Similar results were obtained in the small intestine of these animals and from animals in the 61 and 78 transgenic lines (data not shown). Quantitation of PKC β_{II} expression by densitometric analysis of the immunoblots indicated that 54 line transgenic mice express an average of fivefold more PKC β_{II} protein than do nontransgenic littermates. Immunoprecipitation kinase assays showed an approximately fivefold increase in calcium- and phospholipid-dependent PKC β_{II} activity in the colonic epithelium of transgenic mice, demonstrating that transgenic PKC β_{II} is catalytically active and exhibits the same cofactor dependence of endogenous PKC/β_{II} (data not shown). Animals in the 54 transgenic line gave a consistently high level of transgene expression and therefore this line was selected for further analysis.

We next assessed the pattern of transgenic PKC β_{II} protein expression within the colonic epithelium by immunohistochemistry (Fig. 2, B and C). For this purpose, tissue from the proximal colon of transgenic and nontransgenic mice was immunostained for PKC β_{II}. Consistent with our RT-PCR and immunoblot results, the colonic epithelium from transgenic animals (Fig. 2 C) exhibits increased immunostaining for PKC β_{II} when compared with nontransgenic littermates (Fig. 2 B). In nontransgenic mice, PKC β_{II} staining is observed in the mid-crypt regions and on the luminal surface of the epithelium. In transgenic animals, PKC β_{II} staining is greatest in the mid-crypt region but is detectable throughout the entire crypt axis. Previous characterization of the transgene promoter demonstrated that the rat liver FABP promoter is active in both proliferating and postmitotic cells in the colonic epithelium of transgenic mice (Hansbrough et al., 1991). The distribution of endogenous PKC β_{II} overlaps that of the stem cell population, which is located in the mid-crypt region in the proximal colon (Sato and Ahnen, 1992). In the proximal colon, maturing colonic epithelial cells migrate from the proliferative mid-crypt region toward the base and the luminal surface of the crypt (Sato and Ahnen, 1992). The fact that endogenous PKC β_{II} expression colocalizes with the stem cell population is consistent with the hypothesis that PKC β_{II} plays a functional role in colonic epithelial cell proliferation. Transgenic PKC β_{II} expression was detected in both the proximal and distal colon, indicating transgene expression throughout the colonic epithelium.

Effect of Transgene Expression on Colonic Crypt Morphometry

To investigate the biological effects of overexpression of PKC β_{II} in the colonic epithelium, we analyzed the following colonic morphometric parameters: colon length, colonic crypt height (in micrometer and cell number), crypt circumference (in cell number), and cell size (crypt height in micrometer/crypt height in cell number) (Table I). This analysis revealed no statistical difference in the length of the colon, cell size, crypt height in micrometers, or crypt circumference between transgenic and nontransgenic littermates. However, colonic crypts from transgenic mice tended to be longer and have a larger circumference than those from nontransgenic mice. In addition, a highly significant increase in the number of cells per crypt height, and in the total number of cells per crypt, was observed in transgenic mice (Table I). Similar results were obtained in a second transgenic mouse line (line 78; 22.2 cells per crypt height in transgenic versus 20.7 in nontransgenic mice, $P = 0.009$; and 356.4 total cells per crypt in transgenic versus 321.1 in nontransgenic mice, $P = 0.007$), indicating that this effect is due to the presence of the PKC β_{II} transgene rather than an insertional mutagenic event. Both of these cytokinetic parameters are highly regulated and are determined by the balance among cell proliferation, differentiation, and apoptosis. These results demonstrate that increased expression of PKC β_{II} disrupts one or more of the homeostatic mechanisms regulating cell number in the colonic epithelium.

Table I. Effect of PKC β_{II} Transgene Expression on Morphometric Parameters in the Colon

	Control	Transgenic	*P* value
Colon length (cm)	8.5 ± 0.2	8.6 ± 0.1	NS
Crypt height (μm)	102.6 ± 3.9	108.3 ± 2.3	NS
Crypt height (No. cells)	20.1 ± 0.4	22.3 ± 0.2	0.0002
Cell size (crypt height [μm]/ crypt height [No. cells])	5.1 ± 0.1	4.9 ± 0.1	NS
Crypt circumference (No. cells)	16.3 ± 0.3	16.8 ± 0.2	NS
Total No. cells per crypt	327.7 ± 7.2	372.8 ± 2.5	0.0002

Results are shown as means ± SEM on a sample size of 10 mice/group. NS, not significantly different.

Increased PKC β_{II} Expression Induces Hyperproliferation of the Colonic Epithelium

Elevated PKC β_{II} could increase the number of colonic epithelial cells by increasing the level of proliferation, or by decreasing differentiation and/or apoptosis, in the colonic crypt. To distinguish between these possibilities, each of these cytokinetic parameters was measured. Immunohistochemical staining for PCNA revealed that the colonic epithelium from transgenic mice contain significantly more PCNA-positive cells than those from nontransgenic mice (Fig. 3). Quantitation of PCNA-positive nuclear staining (indicative of cells in S-phase; Lin et al., 1996; Shpitz et al., 1997) gave a labeling index of 28.3 ± 0.2% for transgenic mice compared with 21.4 ± 0.9% for nontransgenic mice (Table II). This difference is highly significant ($P = 0.0001$) and clearly contributes to the increase in crypt cell number observed in transgenic mice. The difference in labeling index was most pronounced in the bottom third of the crypts, the region containing the stem cell population in the distal colon. The size of the proliferative zone (calculated as the highest labeled cell in the crypt column) was also larger in transgenic colons; however, this difference was not statistically significant (Table II). Taken together, these data demonstrate that elevated PKC β_{II} expression stimulates hyperproliferation of the stem cell population residing within the base of the crypt, rather than stimulating postmitotic cells higher in the crypt to reenter the cell cycle.

The differentiation state of the colonic epithelium was

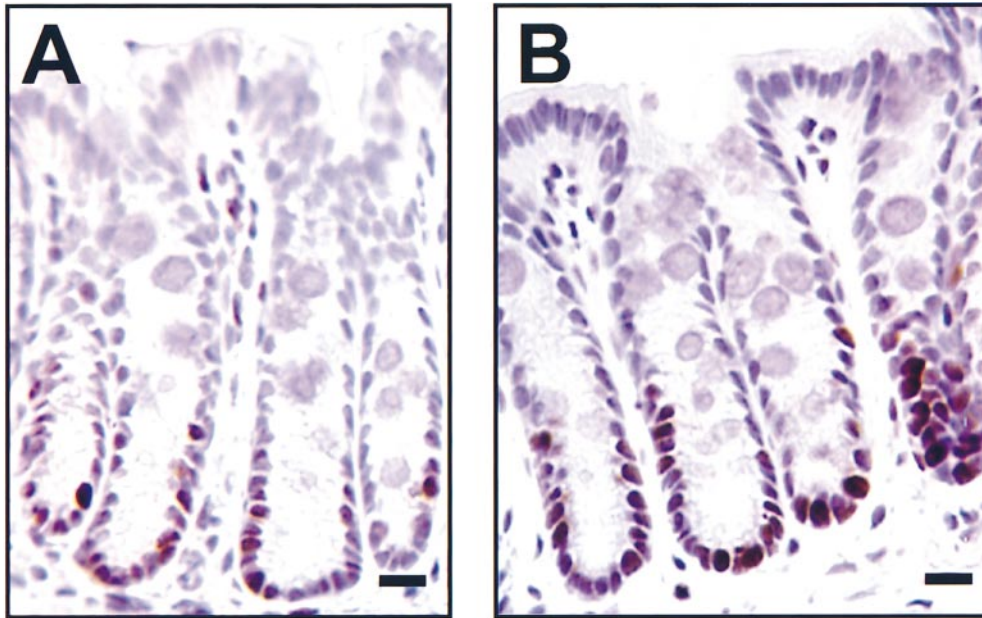


Figure 3. Transgenic PKC β_{II} mice exhibit increased proliferation of the colonic epithelium. 12-wk-old transgenic and nontransgenic mice were killed and their colons were isolated and fixed in paraformaldehyde as previously described (Chang et al., 1997). Sections were stained for PCNA with DAB (brown) using the ABC staining system (Santa Cruz Biotechnology). Sections were counterstained with hematoxylin (blue). A shows the nontransgenic mouse colon, and B shows the transgenic mouse colon. Original magnification: ×400. Bars, 10 μm.

examined by staining with a panel of lectins and histochemical markers to identify the major differentiated colonic epithelial cell lineages. Fig. 4, A and B, shows distal colonic epithelium from transgenic and nontransgenic mice stained with the two histochemical stains, Alcian blue and PAS, that detect goblet cells. The staining pattern seen in transgenic and nontransgenic animals is indistinguishable. Mucin production was detected by staining with several fluorescently labeled lectins (Fig. 4, C–H). DBA binds fairly uniformly to mucin-producing cells in normal distal colonic epithelium (Fig. 4, C and D; Campo et al., 1988; Caldero et al., 1989; Chang et al., 1997; Hong et al., 1997). PNA gives a golgi (supranuclear) staining pattern on a subset of mucin-producing enterocytes (Fig. 4, E and F; Freeman, 1983; Campo et al., 1988; Caldero et al., 1989; Boland and Ahnen, 1995) and UEAI gives low level staining in normal mucosa of the distal colon (Fig. 4, G and H; Caldero et al., 1989). Analysis of the number and location of cells staining with the various lectins revealed no significant changes in the number of goblet cells or in the intensity or pattern of lectin labeling in transgenic PKC β_{II} versus nontransgenic mice. These data indicate that increased expression of PKC β_{II} has no demonstrable effect on the differentiation status of the major colonic enterocytic cell lineages.

Table II. Effect of PKC β_{II} Transgene Expression on Proliferative Parameters in the Colon

	Control	Transgenic	*P* value
Labeling index			
Entire crypt	21.4 ± 0.9	28.3 ± 0.2	0.0001
Top 1/3 crypt	0.2 ± 0.2	0 ± 0	NS
Middle 1/3 crypt	13.8 ± 2.5	17.8 ± 1.0	NS
Bottom 1/3 crypt	50.0 ± 2.7	67.5 ± 1.4	0.0005
Proliferative zone			
Entire crypt column	37.1 ± 2.5	41.4 ± 1.1	NS

Results are shown as means ± SEM on a sample size of four to five mice/group. NS, not significantly different, ($P > 0.05$).

The level of apoptosis in the colonic epithelium was measured using an in situ TUNEL assay (Fig. 5, A and B). An example of TUNEL staining of an apoptotic cell, which typically occurs near the top of the crypt, is shown in Fig. 5 A. As expected, we detected a very low level of apoptosis in the colon of transgenic PKC β_{II} and nontransgenic mice. The apoptotic index in the distal colon of nontransgenic mice was not significantly different from that in transgenic PKC β_{II} mice (Fig. 5 B). Apoptosis is thought to contribute to the loss of cells required to maintain a balance with cell proliferation within the colonic epithelium (Chang et al., 1997; Potten et al., 1997). However, apoptotic cells are quickly eliminated in the colonic crypt, so that apoptosis is detected at a very low level (Hall et al., 1994; Merritt et al., 1994; Risio et al., 1996). Our results are similar to the level of apoptosis in mouse colon reported by others (Merritt et al., 1994; Risio et al., 1996), and demonstrate that increased expression of PKC β_{II} has no significant effect on the level of apoptosis in the colonic epithelium.

Transgenic PKC β_{II} Mice Are More Susceptible to Formation of Carcinogen-induced Aberrant Crypt Foci

Increased cellular proliferation is a significant risk factor for development of colon cancer (Chang et al., 1997; Einspahr et al., 1997). Therefore, we assessed whether transgenic PKC β_{II} mice exhibit an increased susceptibility to colon carcinogenesis. 1,2-dimethylhydrazine and its metabolite, azoxymethane (AOM), are organ-specific carcinogens that have been extensively characterized for their ability to induce colon cancer in rodents (Deschner and Long, 1977; Deschner et al., 1979). AOM reproducibly induces colon tumors that exhibit many of the same genetic and signal transduction defects identified in human colon carcinomas (Deschner et al., 1977; Deschner et al., 1979;

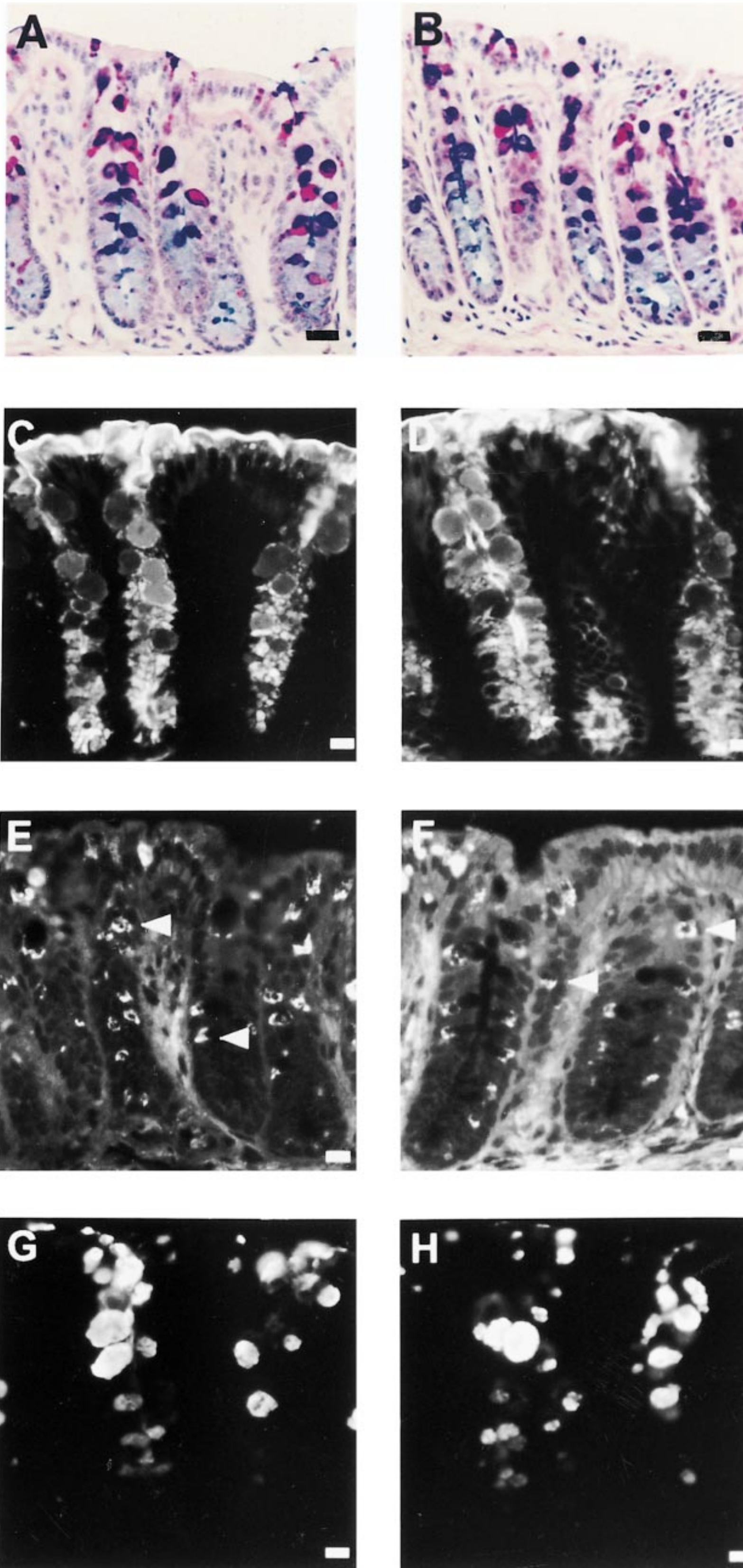
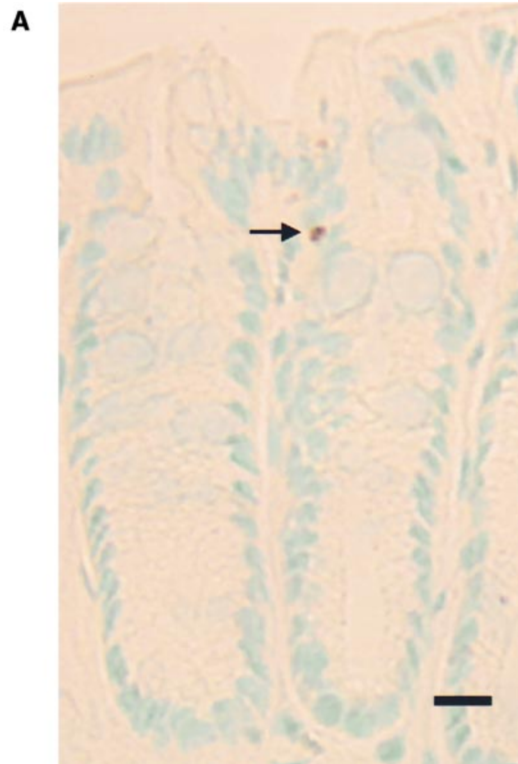


Figure 4. Transgenic PKC β_{II} mice show no change in colonic epithelial cell differentiation. (A and B) Alcian blue/PAS staining. Mucin-containing goblet cells in colonic epithelium of nontransgenic (A) and transgenic PKC β_{II} (B) mice were stained with Alcian blue/PAS. (C–H) Lectin staining. Sections from nontransgenic (C, E, and G) and transgenic (D, F, and H) mouse colonic epithelium were incubated with three different biotinylated lectins and detected with avidin-conjugated rhodamine red-X. C and D, DBA; E and F, PNA; G and H, UEAI. Arrowheads indicate Golgi staining in PNA-stained sections. Bars, 10 μm.

Erdman et al., 1997; Maltzman et al., 1997; DeFilippo et al., 1998; Sheng et al., 1998). AOM also induces ACF, which represent well-established preneoplastic colonic lesions in both rodents and humans (McLellan and Bird, 1988; McLellan et al., 1991; Takayama et al., 1998). Both the number and multiplicity (i.e., number of crypts per focus) of ACF are highly predictive of subsequent tumor development (Magnuson et al., 1993; Bird, 1995; Shivapurkar



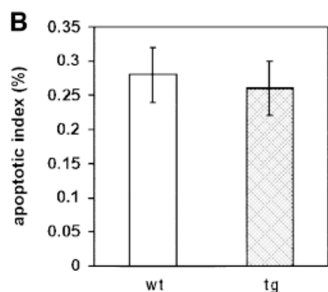


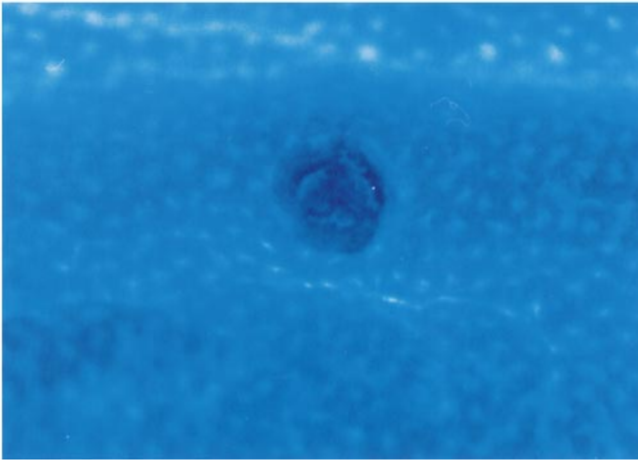
Figure 5. Transgenic PKC β_{II} mice exhibit no change in apoptosis. (A) Detection of apoptosis in the colonic epithelium by in situ TUNEL. Distal colonic epithelium was isolated and fixed in paraformaldehyde as previously described (Chang et al., 1997) and cells undergoing apoptosis were detected by an in situ TUNEL method. A representative apoptotic cell is indicated by the arrow. (B) Quantitative analysis of in situ TUNEL staining. 100 crypts from transgenic and nontransgenic mice were scored for apoptotic cells and the apoptotic index was calculated (percentage of apoptotic cells). Results are expressed as the mean ± SEM. Bar, 10 μm.

et al., 1997). Therefore, AOM-induced colon carcinogenesis is a highly relevant model for human colon cancer.

To determine whether transgenic PKC β_{II} mice differ from nontransgenic mice in their sensitivity to AOM-induced colon carcinogenesis, 6–7-wk-old transgenic PKC β_{II} mice and nontransgenic littermates (five mice/group) received either AOM (10 mg/kg body wt) or saline by intraperitoneal injection once a week for 2 wk. At 5 and 20 wk after the second AOM injection, mice were killed and their colons were analyzed for the presence of ACF. In agreement with the literature (Bird, 1987; McLellan and Bird, 1988; McLellan et al., 1991), we observed no ACF in saline-injected animals, confirming that ACF arise as a result of AOM exposure. Colons from both transgenic and nontransgenic animals treated with AOM contained ACF exhibiting the distinguishing characteristics described by Bird and colleagues (Bird, 1987; McLellan and Bird, 1988; McLellan et al., 1991). Specifically, ACF appeared as enlarged crypts, often three or four times the size of adjacent crypts, that were raised above the surface of the surrounding mucosa. ACF characteristically stained darker than surrounding crypts, had thicker than normal intercryptal spaces, and exhibited thickening of the crypt wall, suggestive of epithelial stratification. The crypt lumens in ACF were elongated and often serrated, in contrast to the round, smooth lumens of normal crypts. ACF contained either a single aberrant crypt or involved two or more adjacent crypts. Fig. 6 A shows the morphology of a typical ACF consisting of three crypts from an AOM-treated animal. The total number of ACF/colon and the multiplicity of ACF was determined at 5 and 20 wk after the last AOM injection (Fig. 6, B–D). AOM-treated transgenic mice had a statistically significant increase in the total number of ACF/colon and in the number of ACF of higher multiplicity at both 5 and 20 wk (Fig. 6, B and C). At 20 wk, the total number of ACF did not increase significantly from that measured at 5 wk; however, the number of ACF of higher multiplicity did increase in transgenic PKC β_{II} mice (Fig. 6 D). Interestingly, at 5 wk, although the total number of ACF and the number of ACF of higher multiplicity were greater in transgenic mice, the average multiplicity of ACF in these two groups did not differ (Fig. 6 D). However, by 20 wk, transgenic mice exhibited an increase not only in the number of ACF but also in the average crypt multiplicity (Fig. 6 D). Since the number of ACF, particularly those of higher multiplicity, are highly predictive of subsequent colon tumor incidence, these data demonstrate that transgenic PKC β_{II} mice are more susceptible to AOM-induced colon carcinogenesis than nontransgenic littermates. Furthermore, these data suggest that elevated PKC β_{II} is involved not only in the early promotive phase of ACF development but also in their progression to lesions of higher multiplicity and malignant potential.

The APC/β-Catenin Signaling Pathway Is Activated in the Colonic Epithelium of Transgenic PKC β_{II} Mice

Colonic epithelial cell proliferation is under the control of the Wnt/APC/β-catenin proliferative signaling pathway (Pennisi, 1998). PKC has recently been demonstrated to play a key role in Wnt/wingless signaling in tissue culture cells (Cook et al., 1996). Selective PKC inhibitors can block Wnt-mediated inhibition of GSK-3β activity, whereas activation of PKC with PMA leads to inactivation of GSK-3β kinase activity in the absence of Wnt (Cook et al., 1996). GSK-3β is a constitutively active serine/threonine kinase that is a critical downstream target in the Wnt signaling pathway. GSK-3β–mediated phosphorylation of APC facilitates binding of β-catenin to APC, which targets β-catenin for degradation. The ability of PKC to inhibit GSK-3β activity is probably due to its direct phosphorylation of GSK-3β, since PKC has been shown to directly phosphorylate GSK-3β and inhibit its activity in vitro (Goode et al., 1992). To determine whether PKC β_{II} activates the Wnt/APC/β-catenin pathway in vivo, we assessed GSK-3β levels and activity in the colonic epithelium of transgenic PKC β_{II} mice (Fig. 7, A and B). Immunoblot analysis reveals that GSK-3β protein levels



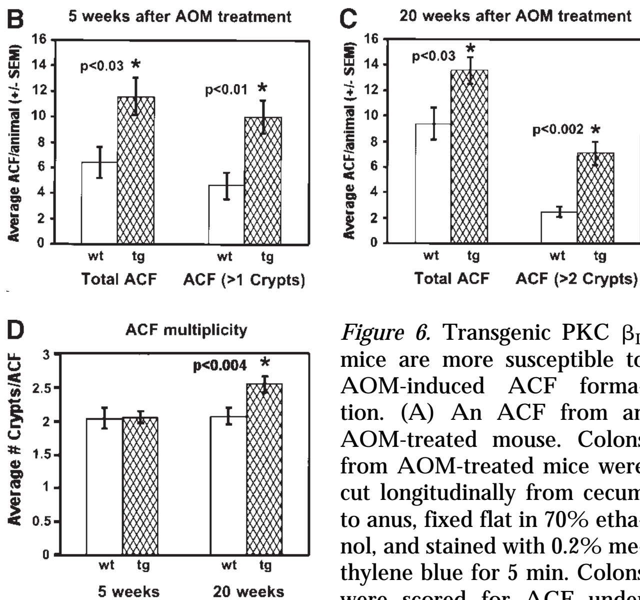


Figure 6. Transgenic PKC β_{II} mice are more susceptible to AOM-induced ACF formation. (A) An ACF from an AOM-treated mouse. Colons from AOM-treated mice were cut longitudinally from cecum to anus, fixed flat in 70% ethanol, and stained with 0.2% methylene blue for 5 min. Colons were scored for ACF under low magnification (×40) using previously defined criteria for ACF (Bird, 1987; McLellan and Bird, 1988). A representative ACF from an AOM-treated animal is shown. This ACF involves three adjacent crypts. Bar, 100 μm. (B–D) Transgenic PKC β_{II} mice have increased levels of AOM-induced ACF. 6-wk-old female mice from the 54 line (transgenic and nontransgenic littermates) were injected with AOM (10 mg/kg body wt) weekly for 2 wk. At 5 and 20 wk after the second injection the mice were killed and the colons were analyzed for the presence of ACF as described in panel A. wt, nontransgenic; tg, transgenic. (B) ACF analysis at 5 wk after treatment. Total ACF per animal and ACF involving >1 crypt were plotted for both transgenic and nontransgenic animals. For each group of animals, $n = 5$. (C) ACF analysis at 20 wk after treatment. Total ACF per animal and ACF involving >2 crypt were plotted for both transgenic and nontransgenic animals. For each group of animals, $n = 5$. (D) Crypt multiplicity of ACF at 5 and 20 wk. The average crypt multiplicity for ACF in nontransgenic and transgenic mice was calculated at 5 and 20 wk. For each group of animals, $n = 5$. Error bars represent the SEM.

are similar in transgenic and nontransgenic mice (Fig. 7 A). However, immunoprecipitation kinase assays demonstrate that GSK-3β activity in transgenic mice is 50% of that observed in nontransgenic littermates (Fig. 7 B). The observed decrease in GSK-3β activity is due to a decrease in the specific kinase activity of the enzyme since GSK-3β expression was unchanged in transgenic PKC β_{II} mice (Fig. 7 A). The extent of GSK-3β inhibition is similar to that observed in response to optimal concentrations of either soluble Wnt or PMA in fibroblasts in vitro (Cook et al., 1996). As another measure of Wnt pathway activation, β-catenin protein levels were assessed by immunoblot analysis (Fig. 7, C and D). β-catenin levels are elevated in transgenic PKC β_{II} mice when compared with nontransgenic littermates (Fig. 7 C). Densitometric analysis of the immunoblot data indicate that on average β-catenin levels are ~40% higher in transgenic PKC β_{II} mice. These data indicate that the Wnt/APC/β-catenin signaling pathway can be stimulated by β_{II} and provide a plausible molecular mechanism by which PKC β_{II} causes hyperproliferation and increased susceptibility to colon carcinogenesis in these animals.

Discussion

Colonic Crypt Homeostasis/Role of (Hyper)proliferation in Susceptibility to Colon Carcinogenesis

Colon carcinogenesis is a multistep process involving the progressive loss of growth control mechanisms and accumulation of genetic mutations that result in an increasing level of neoplasia (Bertagnolli et al., 1997). The process of multistage carcinogenesis has been described as "a progressive disorder in signal transduction" (Weinstein, 1990). According to this model, nongenetic changes in normal signal transduction pathways which increase the susceptibility to further genetic "hits" and therefore play a critical role in the pathogenesis of colon cancer occur early in the carcinogenic process. However, the nature of these early cancer-promotive changes is not well understood. Members of the PKC family of enzymes have been implicated in the regulation of colonic cell proliferation, differentiation, and apoptosis. PKC β_{II} plays a direct role in cellular proliferation in both human leukemia cells and colon cancer cell lines (Murray et al., 1993; Sauma et al., 1996), and increases in PKC β_{II} expression are early events in colon carcinogenesis in vivo (Davidson et al., 1998). Our present data demonstrate that this increase in PKC β_{II} expression plays a promotive role in colon carcinogenesis.

To directly assess the role of PKC β_{II} in colonic epithelial cell proliferation and colon carcinogenesis, we developed a transgenic mouse model in which PKC β_{II} is overexpressed in the intestinal epithelium. Transgenic PKC β_{II} mice exhibit hyperproliferation of the colonic epithelium characterized by an increase in the labeling index and an increase in the number of cells per colonic crypt. Interestingly, no significant changes were observed in colonocyte differentiation status or apoptotic index, indicating a selective effect of PKC β_{II} on the proliferative program of the colonic epithelium. Although we cannot eliminate the possibility that subtle changes have occurred in the regulation of differentiation or susceptibility to apoptosis, our data clearly demonstrate that the change in proliferation is a major contributing factor to the increased colonic crypt cell number observed in transgenic PKC β_{II} mice.

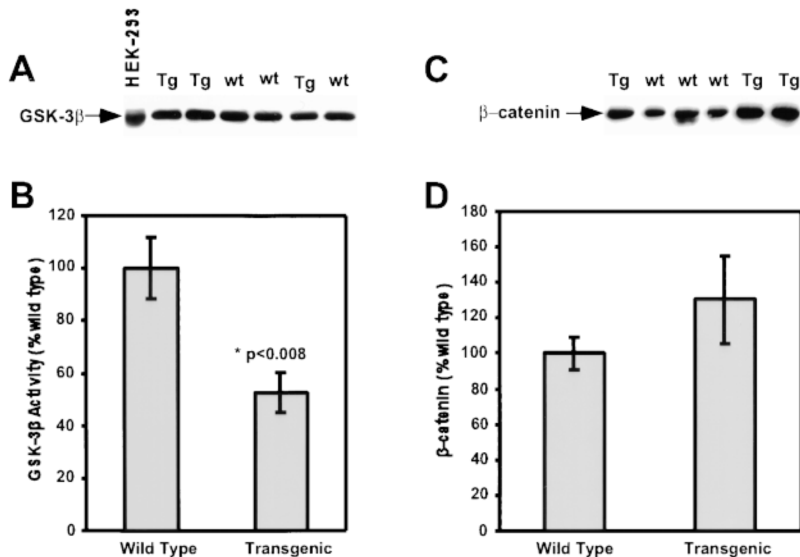


Figure 7. Transgenic PKC β_{II} mice exhibit decreased GSK-3β activity and increased β-catenin levels. (A) Immunoblot analysis for GSK-3β in the colonic epithelium of transgenic (tg) and nontransgenic (wt) mice. (B) Immunoprecipitation kinase assays for GSK-3β were performed on scraped colon extracts from transgenic and nontransgenic mice. Results were normalized to GSK-3β activity in nontransgenic animals. Error bars represent the SEM. (C) Representative immunoblot analysis for β-catenin in the colonic epithelium of transgenic and nontransgenic mice. (D) Densitometric analysis of β-catenin expression. Results were normalized to β-catenin in wild-type mice. $n = 6$ for each group. Error bars represent SEM. Equal amounts of total protein were analyzed in each immunoblot.

Increased proliferation is an important risk factor for induction of colon cancer and is a key biomarker of preneoplastic events (Chang et al., 1997; Einspahr et al., 1997). Our data indicate that PKC β_{II} acts early in the carcinogenic pathway to increase the proliferation of the colonic epithelium, perhaps making it more susceptible to further genetic mutations and formation of preneoplastic lesions, including ACF. The effect of increased PKC β_{II} expression on the susceptibility to induction of colon cancer was tested using a well-characterized rodent carcinogenesis model (Deschner and Long, 1977; Deschner et al., 1979). AOM-induced colon tumors are a good model for sporadic human colon cancer because they exhibit many of the same properties as human colon tumors, including increased proliferation, development of tumors predominantly in the distal colon, and the presence of many of the same genetic mutations found in human tumors. In addition, ACF, the earliest preneoplastic lesions observed in this model, are also thought to be preneoplastic lesions in humans (Pretlow et al., 1992; Takayama et al., 1998). ACF exhibit many of the early phenotypic markers of colon cancer including increased proliferation and frequent mutations in the APC and ras genes (Pretlow et al., 1993; Smith et al., 1994; Shivapurkar et al., 1997). We demonstrate that increased PKC β_{II} expression makes transgenic mice more susceptible to AOM-induced colon carcinogenesis as measured by an increase in the total number of ACF and in the number of ACF of higher multiplicity than nontransgenic mice. ACF are highly predictive of subsequent tumor formation and multiplicity in the rodent carcinogenesis model and of adenoma formation and colon cancer risk in humans (Magnuson et al., 1993; Bird, 1995; Roncucci et al., 1991). Our data indicate that elevated PKC β_{II} expression not only promotes ACF formation, but also stimulates progression of these lesions. These results suggest that PKC β_{II} plays a critical role at multiple stages in the colon carcinogenic pathway.

A Model for the Role of PKC β_{II} in Sporadic Colon Cancer

Accumulating evidence suggests that PKC β_{II} plays a direct role in intestinal epithelial cell proliferation and colon carcinogenesis in both rodents and humans. PKC β_{II} levels and activity are elevated in preneoplastic and neoplastic colons, demonstrating that these changes precede colon carcinoma development (Craven and DeRubertis, 1992; Wali et al., 1995; Davidson et al., 1998). Here, we demonstrate that overexpression of PKC β_{II} in the colonic epithelium leads to hyperproliferation and increased susceptibility to colon carcinogenesis. Furthermore, we demonstrate that elevated PKC β_{II} leads to inhibition of GSK-3β activity and an increase in β-catenin levels. These observations are consistent with in vitro data demonstrating a requisite role for PKC in the Wnt proliferative signaling pathway (Cook et al., 1995), and suggest that PKC β_{II} may play such a role in vivo. Further studies will be required to determine whether PKC β_{II}–mediated activation of this pathway is required for its ability to stimulate proliferation and cancer susceptibility in the transgenic mouse setting.

Taken together, the data lead us to propose a molecular mechanism by which PKC β_{II} stimulates colonic epithelial cell hyperproliferation and increased colon carcinogenesis in transgenic mice (Fig. 8). In this model, PKC β_{II} either directly or indirectly leads to GSK-3β inactivation. PKC has been shown to phosphorylate GSK-3β and inactivate the enzyme in vitro (Goode et al., 1992), suggesting that PKC β_{II} can inhibit GSK-3β by direct phosphorylation and inactivation. Inhibition of GSK-3β leads to an accumulation of β-catenin by decreasing the interaction of β-catenin with APC, which targets β-catenin for degradation. Accumulation of β-catenin causes Tcf-dependent transcriptional activation of growth-related genes to stimulate colonocyte proliferation (Pennisi, 1998). The APC/β-catenin pathway is a major site for mutation during colon car-

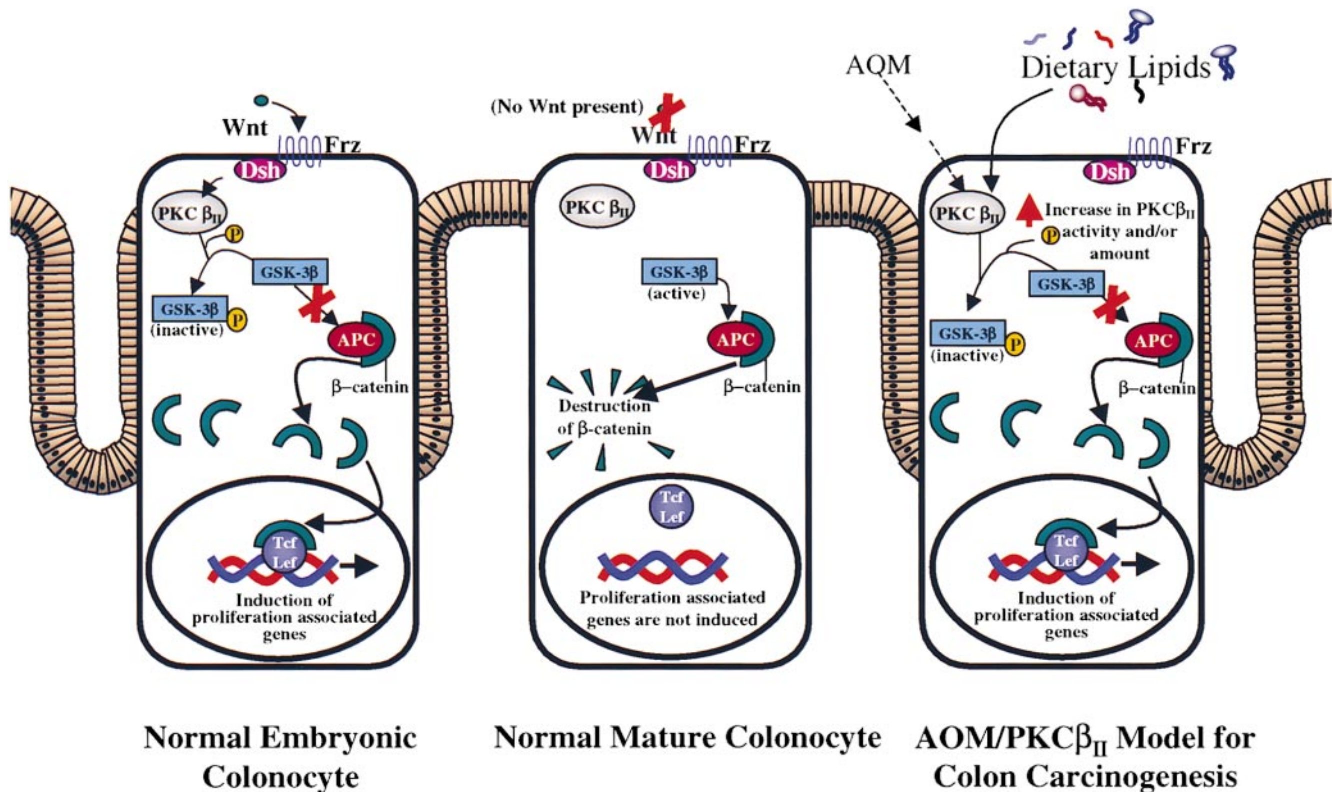


Figure 8. Model of a proposed functional role for PKC β_{II} in colon carcinogenesis. PKC β_{II} is proposed to function in the Wnt signaling pathway in the colonic epithelium. (A) During embryogenesis, Wnt signals through Dsh to PKC β_{II}, which phosphorylates GSK-3β to inhibit its activity. β-catenin levels rise, leading to Tcf-dependent activation of transcription of growth-related genes. (B) In the adult colonocyte, Wnt is not present, GSK-3β activity is constitutively high, and APC binds β-catenin and targets it for degradation. (C) In transgenic PKC β_{II} mice, PKC β_{II} levels and activity are elevated. PKC β_{II} can induce phosphorylation and inactivation of GSK-3β. β-catenin levels rise and Tcf-dependent transcription of growth-related genes is induced, leading to increased proliferation. Similarly, AOM treatment leads to increased expression of PKC β_{II} stimulating this proliferative pathway during colon carcinogenesis. Biochemical evidence suggests that PKC β_{II} can be activated by various lipid components of a cancer-promotive diet, including free fatty acids, secondary bile acids, and bacterially-derived DAG. Direct stimulation of PKC β_{II} by these factors leads to Wnt-independent activation of the APC/β-catenin signaling pathway, hyperproliferation, and enhanced susceptibility to colon carcinogenesis.

cinogenesis (Pennisi, 1998). Mutations in either APC or β-catenin that disrupt β-catenin degradation are present in the vast majority of colon cancers, providing strong evidence that elevated β-catenin levels are important in colon carcinogenesis (Ilyas et al., 1997; Pennisi, 1998). Furthermore, overexpression of a proteolytically-stable NH_2-terminal truncated β-catenin in the intestinal epithelium of transgenic mice leads to hyperproliferation (Wong et al., 1998). Our data suggest that accumulation of β-catenin through PKC β_{II}–mediated inhibition of GSK-3β may play an important promotive role in colon carcinogenesis before the acquisition of mutations in members of this critical signaling pathway.

A major question is how PKC β_{II} activity is modulated during the early stages of colon carcinogenesis. One attractive hypothesis arises from the finding that colonocyte PKC activity can be stimulated by cancer-promotive components of a high fat diet. Diets high in certain fatty acids have been shown to increase the proliferative activity of the colonic epithelium, stimulate colonocyte PKC activity, and increase susceptibility to carcinogen-induced ACF (Craven and DeRubertis, 1988; Risio et al., 1996, Morotomi et al., 1997). This finding is of significance since increased colonic proliferation is a well-established risk factor and biomarker for colon cancer in individuals with familial adenomatous polyposis and ulcerative colitis, as well as in carcinogen-treated rodents (Einspahr et al., 1997).

Cancer-promotive dietary fats function to increase the level of secondary bile acid and fatty acids in the intestinal lumen. Secondary bile acids can in turn activate colonic PKC by a number of mechanisms. First, secondary bile acids and fatty acids can directly activate PKC β_{II} activity and stimulate cellular proliferation in the colonic epithelium (DeRubertis et al., 1984; Fitzer et al., 1987; Ward and O'Brian, 1988; Pongracz et al., 1995). Second, bile acids can promote DAG production by intestinal bacteria, which in turn stimulate colonocyte PKC activity (Morotomi et al., 1990; Morotomi et al., 1991). Third, bile acids can stimulate phospholipid breakdown and DAG generation in colonic epithelial cells (DeRubertis and Craven, 1987), leading to PKC activation. Therefore, we hypothesize that these dietary risk factors increase PKC β_{II} activity in intestinal epithelial cells by multiple mechanisms, re-

sulting in increased epithelial cell proliferation through activation of the APC/β-catenin signaling pathway in a Wnt-independent fashion (Fig. 8 C). This model provides a plausible link between a critical intracellular signaling pathway that is known to be important in colon cancer, and known dietary risk factors for colon carcinogenesis. Our transgenic PKC β_{II} mice will provide a valuable model to test the hypothesis that PKC β_{II} is a relevant target for these cancer-promotive dietary risk factors, and to explore the mechanism by which these factors may impinge on the APC/β-catenin signaling pathway.

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