Protein kinase C (PKC) \(\beta\)II Induces Cell Invasion through a Ras/Mek-, PKC/ Rac 1-dependent Signaling Pathway*

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Protein kinase C \(\beta\)II (PKC\(\beta\)II) promotes colon carcinogenesis. Expression of PKC\(\beta\)II in the colon of transgenic mice induces hyperproliferation and increased susceptibility to colon cancer. To determine molecular mechanisms by which PKC\(\beta\)II promotes colon cancer, we established rat intestinal epithelial (RIE) cells stably expressing PKC\(\beta\)II. Here we show that RIE/PKC\(\beta\)II cells acquire an invasive phenotype that is blocked by the PKC\(\beta\) inhibitor LY379196. Invasion is not observed in RIE cells expressing a kinase-deficient PKC\(\beta\)II, indicating that PKC\(\beta\)II activity is required for the invasive phenotype. PKC\(\beta\)II induces activation of K-Ras and the Ras effector, Rac1, in RIE/PKC\(\beta\)II cells. PKC\(\beta\)II-mediated invasion is blocked by the Mek inhibitor, U0126, and by expression of either dominant negative Rac1 or kinase-deficient atypical PKC\(\alpha\). Expression of constitutively active Rac1 induces Mek activation and invasion in RIE cells, indicating that Rac1 is the critical downstream effector of PKC\(\beta\)II-mediated invasion. Taken together, our results define a novel PKC\(\beta\) \(\rightarrow\) Ras \(\rightarrow\) PKC/ Rac1 \(\rightarrow\) Mek signaling pathway that induces invasion in intestinal epithelial cells. This pathway provides a plausible mechanism by which PKC\(\beta\)II promotes colon carcinogenesis.

Colorectal cancer is the second leading cause of cancer death in the United States (1). Colon carcinogenesis is a complex multistep process involving progressive disruption of intestinal epithelial cell proliferation, differentiation, and survival mechanisms (2). Colon carcinogenesis is driven by environmental factors that modulate cell signaling pathways, and by genetic mutation of transforming oncoproteins, and deletion or mutation of DNA repair enzymes and tumor suppressor genes (2). An important challenge is to understand how environmental and genetic factors interact to define colon cancer risk.

Protein kinase C (PKC)\(^{3}\) is a family of 12 lipid-dependent serine/threonine kinases involved in proliferation, differentiation, and survival (3, 4). Specific, reproducible changes in PKC isozyme expression patterns occur during carcinogen-induced colon carcinogenesis in rodents. Normal rat colonic epithelium contains multiple PKC isozymes, including PKC \(\alpha\), \(\delta\), and \(\xi\) (5, 6). In azoxymethane-induced rat colonic tumors, the levels of PKC \(\alpha\), \(\delta\), and \(\xi\) are reduced and the level of PKC\(\beta\)II is increased when compared with normal colonic epithelium (5, 6). We recently demonstrated reduced PKC\(\alpha\) expression and increased PKC\(\beta\)II and PKC\(\alpha\) expression in azoxymethane-induced mouse colon tumors (7, 8). Our subsequent studies provided direct evidence that both PKC\(\beta\)II and PKC\(\alpha\) play critical but distinct roles in colon carcinogenesis (7–10).

We have developed and analyzed transgenic mice that express elevated PKC\(\beta\)II in the colonic epithelium (7, 9, 10). Transgenic PKC\(\beta\)II mice exhibit hyperproliferation of the colonic epithelium and are prone to carcinogen-induced colon cancer (9, 10). This cancer-prone phenotype results, at least in part, from the imposition of a PKC\(\beta\)II-mediated hyperproliferative phenotype (9, 10). To assess the molecular mechanisms by which PKC\(\beta\)II promotes colon cancer, we established non-transformed rat intestinal epithelial (RIE) cell lines that over-express PKC\(\beta\)II (RIE/PKC\(\beta\)II) cells (10, 11). Genomic analysis of RIE/PKC\(\beta\)II cells revealed that PKC\(\beta\)II induces the expression of the Cox-2 enzyme and suppresses the expression of the transforming growth factor \(\beta\) receptor type II (TGF\(\beta\)RII) (11). As a result, RIE/PKC\(\beta\)II cells have lost the ability to respond to the growth inhibitory effects of TGF-\(\beta\), and this loss is dependent upon the activity of Cox-2 (11). Therefore, PKC\(\beta\)II establishes a novel, pro-carcinogenic PKC\(\beta\)II \(\rightarrow\) Cox-2 \(\rightarrow\) TGF\(\beta\)RII signaling pathway that confers resistance to TGF-\(\beta\) (11). This pathway is activated in transgenic PKC\(\beta\)II mice and accounts, at least in part, for the hyperproliferative phenotype exhibited by these mice (11). Carcinogens induce this pathway, whereas chemopreventive \(\omega-3\) fatty acids inhibit PKC\(\beta\)II activity, suppress PKC\(\beta\)II-mediated hyperproliferation, and attenuate the cancer-prone phenotype exhibited by transgenic PKC\(\beta\)II mice (10, 11).

Here, we demonstrate that PKC\(\beta\)II induces an invasive phenotype in RIE cells through a signaling pathway that is distinct from the PKC\(\beta\)II \(\rightarrow\) Cox-2 \(\rightarrow\) TGF\(\beta\)RII pathway responsible for TGF-\(\beta\) resistance. PKC\(\beta\)II-mediated invasion is dependent upon Ras, PKC\(\alpha\), Rac1, and Mek. Our findings define a novel proinvasive PKC\(\beta\)II \(\rightarrow\) Ras \(\rightarrow\) PKC/ Rac1 \(\rightarrow\) Mek signaling pathway. Our data indicate that PKC\(\beta\)II promotes colon cancer through activation of at least two distinct signaling pathways, one that confers TGF-\(\beta\) resistance, and a second that induces invasion, in intestinal epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—The PKC\(\beta\)-selective inhibitor LY379196 was a kind gift of Dr. James R. Gillig, Lilly Pharmaceutical Co. Puromycin dihydrochloride was purchased from Calbiochem. Polybrene and crystal violet were from Sigma. Anti-PKC\(\alpha\), anti-actin, and horseradish peroxidase-conjugated donkey anti-goat secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. The PhosphoPlus Mek kit 1/2 antibody was from Cell Signaling Technology. Anti-FLAG epitope antibody was from Sigma. Anti-Rac1 mouse monoclonal antibody was purchased from Calbiochem.

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* The abbreviations used are: PKC, protein kinase C; RIE, rat intestinal epithelial; Mek, mitogen-activated protein kinase/extracellular signal-regulated kinase; TGF-\(\beta\), transforming growth factor \(\beta\); TGF\(\beta\)RII, TGF-\(\beta\) receptor type II; PAK, p21-activated kinase; DME, Dulbecco’s modified Eagle’s; GFP, green fluorescent protein; GST, glutathione S-transferase; RBD, Ras-binding domain; kd, kinase-deficient.

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FIG. 1. PKCβII induces an invasive phenotype in RIE cells. A, overexpression of human PKCβII in RIE cells. RIE cells were stably transfected with either a control pBabe retrovirus expression vector (R) or a pBabe vector containing the full-length human PKCβII cDNA (R/PI). B, expression of kinase-deficient PKCβII (kdPKCβII) does not induce an invasive phenotype in RIE cells. RIE, RIE/PKCβII, and RIE/kdPKCβII cells were assessed for invasion as described in C above. Values represent the mean number of invading cells in triplicate determinations ± S.E. *p < 0.05 versus RIE cells; **p < 0.03 versus RIE/PKCβII cells.

RESULTS
PKCβII Induces an Invasive Phenotype in RIE Cells—We previously characterized transgenic mice expressing elevated levels of PKCβII in the colononic epithelium (7, 9, 10). These mice exhibit colonic hyperproliferation and enhanced colon carcinogenesis (9). To elucidate the molecular mechanisms by which PKCβII promotes colon carcinogenesis, we established a cell model system to dissect PKCβII-mediated signaling pathways (10, 11). RIE cells are immortalized but not transformed and, like most cells within the intestinal epithelium, express little or no PKCβII (Fig. 1A). Therefore, we established RIE cells stably expressing human PKCβII by transgenesis (Fig. 1A). Surprisingly, RIE/PKCβII cells acquire a highly invasive phenotype (Fig. 1B). Treatment of RIE and RIE/PKCβII cells with the selective PKCβ inhibitor LY379196 (16) blocked invasion of RIE/PKCβII cells while having no effect on RIE cells (Fig. 1C).

Unlike RIE/PKCβII cells, RIE cells expressing a kinase-defi-
the upper chamber of the Transwell insert (5 × 10^4 cells/well), and DME medium containing 10% fetal bovine serum was added to the lower chamber. After 22 h at 37°C, 5% CO_2, non-invasive cells in the upper chamber were removed, and invasive cells were fixed in 100% methanol and stained with 0.5% crystal violet in 2% ethanol. Five random microscopic fields at ×400 magnification were counted in each filter using a calibrated ocular grid. Experiments were carried out in triplicate. The data are expressed as the average number of cells/field ± S.E. In some cases, the data are presented as fold change from RIE cells, with the value for RIE cells set as 1. In some cases, cells were treated with LY379196 (50 nM), U0126 (10 μM), or celecoxib (25 μM) for 24 h prior to being subjected to the invasion assay using medium containing the appropriate inhibitor at the concentration indicated above.

RESULTS
PKCβII Induces an Invasive Phenotype in RIE Cells—We previously characterized transgenic mice expressing elevated levels of PKCβII in the colonic epithelium (7, 9, 10). These mice exhibit colonic hyperproliferation and enhanced colon carcinogenesis (9). To elucidate the molecular mechanisms by which PKCβII promotes colon carcinogenesis, we established a cell model system to dissect PKCβII-mediated signaling pathways (10, 11). RIE cells are immortalized but not transformed and, like most cells within the intestinal epithelium, express little or no PKCβII (Fig. 1A). Therefore, we established RIE cells stably expressing human PKCβII by transgenesis (Fig. 1A). Surprisingly, RIE/PKCβII cells acquire a highly invasive phenotype (Fig. 1B). Treatment of RIE and RIE/PKCβII cells with the selective PKCβ inhibitor LY379196 (16) blocked invasion of RIE/PKCβII cells while having no effect on RIE cells (Fig. 1C).

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Fig. 2. PKCβII-mediated invasion requires Mek activity but not Cox-2. A, RIE and RIE/PKCβII cells were subjected to immunoblot analysis for K-Ras, H-Ras, and actin as described under "Experimental Procedures." B, RIE and RIE/PKCβII cells were incubated in the absence of serum for 24 h to induce quiescence and then stimulated with 5% fetal bovine serum (FBS) for the indicated times. C, RIE and RIE/PKCβII cells were incubated in the absence of serum for 24 h as described in A or maintained in serum-containing medium. D, RIE and RIE/PKCβII cells were incubated in the presence of the Mek inhibitor U0126, the Cox-2 inhibitor celecoxib, or diluent control and assayed for invasion as described under "Experimental Procedures." Values represent the mean number of invading cells in triplicate determinations ± S.E. *, p < 0.05 versus RIE cells; **, p < 0.05 versus RIE/PKCβII in the absence of U0126; ***, p < 0.05 versus RIE cells and not significantly different from RIE/PKCβII cells.

PKCβII activates K-Ras in RIE Cells—There is evidence for reciprocal cross-talk between PKC and Ras (17–20). Many cellular effects of the PKC activator phorbol myristate acetate are dependent upon Ras, and PKC activates cellular Ras in response to many extracellular stimuli (19). Therefore, we measured Ras expression and activity in RIE and RIE/PKCβII cells (Fig. 2A). RIE and RIE/PKCβII cells express abundant K-Ras but very little H-Ras (Fig. 2A), consistent with the epithelial nature of RIE cells. Expression of PKCβII in RIE cells has no demonstrable effect on the level of K-Ras expression and does not induce H-Ras expression. We next assessed the level of K-Ras activity in RIE and RIE/PKCβII cells in the presence and absence of serum (Fig. 2B). Although RIE cells exhibit little or no detectable active Ras in the absence of serum, RIE/PKCβII cells show a significant amount of active Ras under serum-free conditions. Treatment of RIE cells with 5% serum leads to transient activation of Ras in both RIE and RIE/PKCβII cells. However, both the duration and the magnitude of serum-stimulated Ras activation are higher in RIE/PKCβII cells than in RIE cells.

Expression of oncogenic Ras in RIE cells induces invasion (21). To assess whether PKCβII-mediated invasion is Ras-dependent, we initially measured, and then inhibited, the activity of the Ras effector, Mek. RIE/PKCβII cells exhibit an elevated level of phosphorylated, active Mek when compared with RIE cells (Fig. 2C). The increase in phospho-Mek in RIE/PKCβII cells reflects increased Mek activity since it is not accompanied by a corresponding increase in total Mek levels. Treatment of RIE and RIE/PKCβII cells with the selective Mek 1 and 2 inhibitor U0126 (22) blocked invasion of RIE/PKCβII cells while having no effect on RIE cells, indicating that PKCβII-mediated invasion is dependent upon Mek activity (Fig. 2D).

We recently demonstrated that PKCβII induces Cox-2 and suppresses TGFβII in RIE/PKCβII cells and that Cox-2 activity is required for PKCβII-mediated resistance to TGF-β and suppression of TGFβRII expression (11). However, treatment of RIE and RIE/PKCβII cells with the selective Cox-2 inhibitor celecoxib had no effect on the invasive behavior of either RIE or RIE/PKCβII cells, demonstrating that PKCβII-mediated invasion does not require Cox-2 activity (Fig. 2D).

PKC βII Activates Rac1, but Not RhoA, in RIE Cells—Oncogenic Ras-mediated invasion requires the GTPase activity of Rac1 (13, 23). Interestingly, RIE/PKCβII cells exhibit elevated levels of GTP-bound, active Rac1 when compared with RIE cells (Fig. 3A). Densitometric analysis of multiple experiments demonstrated that RIE/PKCβII cells contain 5-fold higher Rac1 activity than RIE cells without an increase in the total levels of Rac1 (Fig. 3B). RIE and RIE/PKCβII cells exhibit a similar, low level of RhoA activity in the absence of serum (Fig. 3C). Furthermore, treatment with serum leads to activation of RhoA to a similar extent in RIE and RIE/PKCβII cells. Therefore, PKCβII induces activation of Rac1 but not of the related GTPase RhoA in RIE/PKCβII cells. LY379196 reduces Rac1 activity in RIE/PKCβII cells to levels comparable with those observed in RIE cells while having no effect on Rac1 activity in RIE cells (Fig. 4A). Furthermore, although RIE/PKCβII cells exhibit elevated Rac1 activity, RIE/kdPKCβII cells exhibit Rac1 activity indistinguishable from RIE cells (Fig. 4B). Taken together, these results demonstrate that PKCβII-mediated Rac1 activation, like cell invasion, requires PKCβII activity.

PKCβII-mediated Invasion Requires Rac1 and the Atypical PKC Isozyme, PKCδ—We next assessed whether Rac1 activity is required for PKCβII-mediated invasion. Expression of a dominant negative Rac1 mutant, RacN17, blocks invasion of RIE/PKCβII cells while having no effect on RIE cells (Fig. 4C), indicating that Rac1 activity is required for PKCβII-mediated invasion. The atypical PKC isoyme PKCδ resides in a complex with the cell polarity protein Par6 and Rac1, where its activity may regulate signaling to Rac1 (24–27). We recently demonstrated that oncogenic Ras-mediated invasion requires both Rac1 and PKCδ, indicating the involvement of this complex in Ras-mediated invasion (8). Therefore, we assessed the involvement of PKCδ in PKCβII-mediated invasion. Genomic and immunoblot analysis demonstrated that PKCβII does not induce
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PKCβII plays a critical promotive role in colon carcinogenesis. PKCβII is elevated in both mouse and human colon tumors and in aberrant crypt foci in azoxymethane-treated mice, indicating that induction of PKCβII is an early event in colon carcinogenesis (7). Expression of PKCβII in the colonic epithelium of transgenic mice induces hyperproliferation and increased susceptibility to carcinogen-induced aberrant crypt foci and tumor formation (9), demonstrating that PKCβII promotes colon carcinogenesis.

We have used rat intestinal epithelial cells as a model of the normal colonic epithelium to investigate the molecular mechanisms by which PKCβII promotes colon carcinogenesis (10, 11). Expression of PKCβII in RIE cells induces several aspects of the transformed phenotype, including the acquisition of TGF-β resistance and an invasive phenotype (Fig. 6). RIE cells, like normal colonic epithelial cells, are growth-inhibited by TGF-β (9). However, RIE/PKCβII cells no longer respond to TGF-β (9). We have demonstrated that PKCβII induces a TGF-β-resistant state in RIE cells through activation of a novel PKCβII → Cox-2 → TGFβIII signaling axis (11). We also demonstrated that this pathway operates in the colonic epithelium of transgenic PKCβII mice in vivo and that dietary ω-3 fatty acids exert their chemopreventive effects on colon carcinogenesis, at least in part, through inhibition of this pathway (11). Cox-2 is frequently over-expressed in colon cancers (31) and colon cancer cell lines (32, 33) and has been implicated in multiple functions critical to colon carcinogenesis including angiogenesis (34), tumor cell proliferation (35, 36), invasiveness (37), and metastatic potential (38-41). Our data are consistent with the role of Cox-2 in the loss of TGF-β responsiveness, which is observed in the vast majority of colon cancers and colon cancer cell lines (42, 43). PKCβII-mediated induction of Cox-2 provides a plausible mechanism by which Cox-2 expression and TGF-β resistance is induced in the early stages of colon carcinogenesis. PKCβII may also be responsible for maintaining Cox-2 expression and TGF-β resistance in established colon cancer cell lines and colon cancers.

Here, we demonstrate that PKCβII induces an invasive phenotype in RIE cells. Activated K-Ras is important for PKCβII-mediated invasion since the MEK1-selective inhibitor U0126 blocks invasion of RIE/PKCβII cells. Interestingly, although Cox-2 is critical for PKCβII-mediated suppression of TGFβIII (10), it does not appear to be involved in PKCβII-mediated invasion. Thus, PKCβII induces TGF-β resistance and invasion through two distinct pathways.

PKCβII induces the activity of the Ras effector, Rac1, a critical downstream effector of Ras transformation (13). Rac1 is essential for Ras-mediated changes in the actin cytoskeleton that induce invasion (14) and is required for PKCβII-mediated invasion. Expression of a constitutively active Rac1 mutant, Rac1V12, is sufficient to induce invasion in RIE cells in the absence of PKCβII. Furthermore, invasion in RIE/PKCβII cells transfected with Rac1V12 is no longer blocked by LY379196, demonstrating that active Rac1 is sufficient to induce PKCβII-independent invasion. Interestingly, Rac1V12 induces activation of Mek, the activity of which is required for Rac1V12-induced invasion. Taken together, these data provide compelling evidence that both Rac1 and Mek are critical effectors of PKCβII-induced invasion. The proinvasive activity of significant changes in PKCα mRNA or protein expression (data not shown). However, expression of a kinase-deficient mutant of PKCα (kpPKCα), which acts in dominant negative fashion (28), inhibits invasion in RIE/PKCβII cells while having no effect on RIE cells (Fig. 4D). Thus, both oncogenic Ras- and PKCβII-mediated invasion are dependent upon PKCα. We recently demonstrated that Rac1 is downstream of PKCα in cellular invasion and Ras-mediated transformation (8).

RIE cells transfected with a constitutively active Rac1 mutant (RIE/RacV12 cells) exhibit an invasive phenotype similar to that observed in RIE/PKCβII cells (Fig. 5A). Furthermore, although invasion of RIE/PKCβII cells is blocked by LY379196, RIE/PKCβII cells expressing RacV12 exhibit an invasive phenotype that is not blocked by LY379196 (Fig. 5B). Thus, Rac1 functions downstream of PKCβII and is sufficient to induce invasion. RIE/RacV12 cells exhibit elevated levels of phospho-Mek (Fig. 5C), consistent with reports that Rac1 can activate Mek (29, 30). Furthermore, invasion of RIE/RacV12 cells is blocked by U0126, indicating that active Mek is required for RacV12-mediated invasion (Fig. 5D). Taken together, these results demonstrate that Rac1 is the relevant downstream target for PKCβII-mediated invasion and that active Rac1 is both necessary and sufficient to induce an invasive phenotype in RIE cells through activation of Mek.
PKCβII can be explained through the activation of Ras, which is capable of activating both Rac1 and Mek. The fact that RacV12 can also activate Mek in the absence of PKCβII suggests that Rac1 is the critical effector of invasion. The relative contribution of the canonical Ras/Raf pathway, and of Rac1, in the activation of Mek-dependent invasion will require further experimentation.

Our data are consistent with earlier reports that PKC isoforms are important regulators of cytoskeletal function (24, 44–46). PKCβII has been shown to interact with the actin cytoskeleton (44). Here we demonstrate that PKCβII activates Rac1, and for the first time, demonstrate the functional importance of PKCβII-mediated activation of Rac1. We recently demonstrated that like Rac1, the atypical PKC isoyme, PKCι, is required for Ras-mediated transformation (8). PKCι resides between Ras and Rac1 in a pathway required for Ras-mediated invasion and anchorage-independent growth (8). PKCβII-mediated invasion is also dependent upon PKCι, providing further evidence for the involvement of Ras in PKCβII-induced invasion. PKCι resides in a complex with Rac1 and Par6 that regulates the polarity of epithelial cells (25–27). Our present data indicate that this complex is also involved in the process of cellular invasion through upstream input from PKCβII and activated Ras. Future studies will be aimed at determining the role of PKCι/Par6/Rac1 complexes in transformation and invasion.

Taken together, our data are consistent with a model in which PKCβII induces invasion through activation of a PKCβII → Ras → PKCι/Rac1 → Mek signaling pathway (Fig. 6). Our data have important implications for the role of PKCβII
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Fig. 6. Schematic showing the two PKCβII-mediated signaling pathways responsible for TGF-β resistance and invasion in RIE cells. Lines with arrows denote a signaling pathway. A line with a circled plus sign denotes the transcriptional activation of the gene by PKCβII; a line with a circled minus sign denotes the suppression of gene expression by PKCβII.

in colon carcinogenesis. We previously demonstrated that induction of PKCβII expression occurs very early in colon carcinogenesis, prior to acquisition of oncogenic mutations such as activated Ras. PKCβII activates cellular Ras, suggesting that PKCβII activation represents a phenotype of an activated ras allele. Thus, the pathway delineated in the present report could explain the critical role of PKCβII in early carcinogenesis, prior to acquisition of a Ras mutation. An important question is whether PKCβII is required to maintain the transformed phenotype of colon cancer cells harboring a Ras mutation or whether it becomes dispensable in the presence of oncogenic Ras. An important question is whether PKCβII is required to maintain the transformed phenotype of colon cancer cells. Future studies will investigate the role of PKCβII in transformed colon cancer cells.

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