Protein Kinase-ζ Inhibits Collagen I–Dependent and Anchorage-Independent Growth and Enhances Apoptosis of Human Caco-2 Cells

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Abstract
Colonic carcinogenesis is accompanied by abnormalities in multiple signal transduction components, including alterations in protein kinase C (PKC). The expression level of PKC-ζ, an atypical PKC isoform, increases from the crypt base to the luminal surface and parallels crypt cell differentiation in normal colon. In prior studies in the azoxymethane model of colon cancer, we showed that PKC-ζ was down-regulated in rat colonic tumors. In this study, we showed that PKC-ζ is expressed predominantly in colonic epithelial and not stromal cells, and loss of PKC-ζ occurs as early as the adenoma stage in human colonic carcinogenesis. To assess the regulation of growth and differentiation by PKC-ζ, we altered this isoform in human Caco-2 colon cancer cells using stable constitutive or inducible expression vectors, specific peptide inhibitors or small interfering RNA. In ecdysone-regulated transfectants grown on collagen I, ponasterone A significantly induced PKC-ζ expression to 135% of empty vector cells, but did not alter nontargeted PKC isoforms. This up-regulation was accompanied by a 2-fold increase in basal and 4-fold increase in insulin-stimulated PKC-ζ biochemical activity. Furthermore, PKC-ζ up-regulation caused >50% inhibition of cell proliferation on collagen I (P < 0.05). Increased PKC-ζ also significantly enhanced Caco-2 cell differentiation, nearly doubling alkaline phosphatase activity, while inducing a 3-fold increase in the rate of apoptosis (P < 0.05). In contrast, knockdown of this isoform by small interfering RNA or kinase inhibition by myristoylated pseudosubstrate significantly and dose-dependently increased Caco-2 cell growth on collagen I. In transformation assays, constitutively up-regulated wild-type PKC-ζ significantly inhibited Caco-2 cell growth in soft agar, whereas a kinase-dead mutant caused a 3-fold increase in soft agar growth (P < 0.05). Taken together, these studies indicate that PKC-ζ inhibits colon cancer cell growth and enhances differentiation and apoptosis, while inhibiting the transformed phenotype of these cells. The observed down-regulation of this growth-suppressing PKC isoform in colonic carcinogenesis would be predicted to contribute to tumorigenesis.

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Introduction
Colonic carcinogenesis involves a multistep process of genetic mutations that derange signaling pathways controlling cell proliferation and cell death (1). Members of the protein kinase C (PKC) family are important regulators of colonocyte growth and differentiation that are altered during neoplastic transformation. Experimental models of chemical carcinogenesis have been widely used to elucidate signaling abnormalities that contribute to colon malignancy transformation (2). The clinical, histologic, and molecular features of the azoxymethane model mimic many features of sporadic human colon cancer (3). Previous studies from our laboratory in azoxymethane-induced rat colonic carcinogenesis have identified changes in the expression levels of several PKC isoforms, including down-regulation of PKC-ζ, an atypical PKC isoform (4).

The PKC family includes at least 11 isoforms of closely related serine/threonine protein kinases that regulate important cellular processes, including proliferation, differentiation, and apoptosis (5–8). Members of this family are classified into three subfamilies according to their sequence homology and activating cofactor requirements (9). The conventional PKCs (α, β1, βII, and γ) are activated by calcium (Ca²⁺) and 1,2-diacyl-sn-glycerol, whereas members of the novel class of PKC (δ, ε, θ, and η) are activated by 1,2-diacyl-sn-glycerol, but are Ca²⁺ independent. The atypical PKC isoforms (λ, ξ, and ζ) are both Ca²⁺- and 1,2-diacyl-sn-glycerol independent. Although PKC isoforms have overlapping substrate specificities in vitro, these kinases exhibit distinct patterns of tissue expression and intracellular localizations that likely reflect unique isoform-specific functions.

PKC-ζ has been shown to regulate diverse process, including epidermal growth factor receptor and nuclear factor-κB signaling, cytoskeletal interactions, and metabolic processes in agonist- and cell-specific manners (10–14). In normal colonic epithelium, PKC-ζ expression and membrane association increase with crypt cell migration from the proliferative compartment in the crypt base to the colonic luminal surface, containing growth-arrested differentiated cells undergoing...
apoptosis (15, 16). We previously showed that PKC-ζ is down-regulated in azoxymethane-induced colonic carcinogenesis. We also showed three structurally unrelated agents inhibited azoxymethane tumorigenesis and concomitantly prevented PKC-ζ down-regulation in these tumors (4, 17, 18). A diet enriched in chemopreventive fish oil also preserved PKC-ζ in azoxymethane premalignant mucosa (19). Taken together, increased PKC-ζ expression during normal colonocyte maturation, and loss of expression in colonic tumorigenesis, as well as PKC-ζ preservation by chemopreventive agents, suggested to us that PKC-ζ might regulate colonocyte growth and differentiation. In this regard, this atypical isoform has been shown to regulate proliferation, differentiation, and/or apoptosis in other cell types (20-30).

In human colonic cancers, PKC-ζ expression was also reported to be down-regulated, but not all reports have confirmed this observation (31-33). To address this question, we first confirmed PKC-ζ down-regulation in human colonic tumors. To elucidate the potential consequences of PKC-ζ alterations in colonic carcinogenesis, we then used a number of molecular techniques to modulate PKC-ζ expression and activity and assessed the biologically relevant correlates of growth, differentiation, and apoptosis in Caco-2 cells. These cells were derived from a human colon cancer and have been widely used as a model of intestinal epithelial cell growth and differentiation (34, 35). When grown to confluence, these cells develop a differentiated phenotype with a microvillus brush border and increased alkaline phosphatase expression (36). With confluence, Caco-2 cells also develop domes, indicative of epithelial tight junctions and vectorial ion transport (37). We established stable transfections in these cells with an edcsyne-regulated expression vector encoding full-length cDNA for human PKC-ζ. Because PKC-ζ-transfected cells did not show significant alterations in nontargeted PKC isoforms, the biological consequences of up-regulating PKC-ζ on Caco-2 cellular proliferation, differentiation, and apoptosis could be assessed in an unambiguous manner. To test whether the growth-inhibitory changes that we observed were direct effects of PKC-ζ up-regulation, we also asked if PKC-ζ knockdown or enzymatic inhibition would stimulate cell proliferation. Our findings, and a discussion of their implications for colonic carcinogenesis, serve as the basis for this report.

Results

PKC-ζ Is Down-Regulated in Human Colonic Polyps and Colon Cancers

We have previously shown that PKC-ζ expression was decreased in azoxymethane-induced rat colonic tumors (4), but results in human colonic tumors have been conflicting (31-33). In the current study, we examined four human colonic adenomas and 18 human colon cancers for PKC-ζ expression. The tumors included right- and left-sided colonic adenomas, as well as moderate or well-differentiated colonic cancers. We found that PKC-ζ protein levels were significantly decreased in 3 of 4 premalignant lesions and in 14 of 18 malignant lesions. Representative tumors are shown in Fig. 1. There was no significant difference in PKC-ζ down-regulation in polyps, compared with cancers. To identify the localization of the cell expressing PKC-ζ, we immunostained colonic tumors and adjacent normal mucosa (Fig. 1C). In normal colon, PKC-ζ was expressed only in the colonic crypts with an increasing gradient of expression from the crypt base to the colonic lumen, in agreement with a previous report (15). In five of six colon cancers examined by immunostaining, PKC-ζ was also down-regulated (Fig. 1C). In the remaining tumor, there was persistent PKC-ζ expression in ~20% of malignant colonocytes in a patchy and heterogeneous distribution. These results confirm the findings of others that PKC-ζ is frequently down-regulated in human colonic neoplasms (33). Furthermore, PKC-ζ loss can occur as early as the adenomatous polypl stage in colonic malignant transformation.

Inducible Caco-2 Cell Transfectants Exhibit Collagen I–Regulated PKC-ζ Expression

To assess the potential biological significance of PKC-ζ loss in colonic carcinogenesis, we examined the effects of altering the level of this isoform on human colon cancer cell growth, differentiation, and cell death. We initially up-regulated PKC-ζ levels in Caco-2 cells expressing this isoform under a ponasterone A–inducible, edcsyne-regulated expression vector system (38). Transfectants were treated with 5 μmol/L ponasterone A or vehicle (DMSO) in serum-free medium for up to 8 days. Compared with endogenous PKC-ζ, the V5 epitope tag of the transfected PKC-ζ increased the apparent molecular weight ~3 kDa, allowing SDS-PAGE separation of endogenous and transfected PKC-ζ. Both endogenous and transfected PKC-ζ were detected by PKC-ζ antibodies (Fig. 2). Ponasterone A up-regulated PKC-ζ expression in transfectants plated on collagen I, but not on plastic (Fig. 2; data not shown). The induced isoform was detectable by day 2 and persisted for at least 8 days. Total PKC-ζ expression was significantly increased to 135% of empty vector cells by day 4 in ponasterone A–treated transfectants grown on collagen I (P < 0.05, Fig. 2). In contrast, expression levels of nontargeted endogenous PKC isoforms α, βI, βII, and δ were unchanged in empty vector and PKC-ζ–transfected Caco-2 cells grown on collagen I (Fig. 3).

PKC-ζ Transfectants Possess Increased Basal and Insulin-Stimulated Kinase Activity

We measured PKC-ζ enzymatic activity in immunoprecipitation kinase assays to assess the biochemical significance of increased protein expression. Empty vector and PKC-ζ–transfected cells were treated with 5 μmol/L ponasterone A or DMSO for 2 days. The V5 epitope was detectable in ponasterone A, but not DMSO-treated PKC-ζ transfectants, or in empty vector cells (Fig. 4, inset). We treated Caco-2 transfectants with insulin because this hormone was shown to stimulate PKC-ζ kinase activity in rat adipocytes (39). Ponasterone A–induced or uninduced transfectants were stimulated with 10 nmol/L insulin or PBS (vehicle) for 5 minutes and cell lysates prepared. PKC-ζ was immunoprecipitated and kinase activity was measured as described in Materials and Methods. As shown in Fig. 4, the basal kinase activities of PKC-ζ transfectants and empty vector cells were comparable. Ponasterone A alone significantly increased kinase activity in PKC-ζ transfectants, while insulin further increased phosphorylation in both empty vector and PKC-ζ–transfected cells (Fig. 4).
activity in PKC-ζ transfectants 80% above similarly treated empty vector cells ($P < 0.05$). The addition of insulin to ponasterone A–induced PKC-ζ transfectants further increased PKC-ζ kinase activity that was 4-fold compared with similarly treated empty vector cells ($P < 0.05$). Thus, ponasterone A significantly increased PKC-ζ protein expression and kinase activity, without altering the expression of nontargeted PKC isoforms in these transfectants.

**PKC-ζ Up-Regulation Inhibits Caco-2 Cell Growth**

To ascertain the effect of PKC-ζ up-regulation on Caco-2 cellular proliferation, we compared growth rates of ζ-transfectants and empty vector cells. As assessed by the WST-1 colorimetric cell proliferation assay, PKC-ζ and empty vector transfectants treated with DMSO had similar rates of proliferation (Fig. 5). In contrast, up-regulation of PKC-ζ by ponasterone A significantly inhibited proliferation by >50% in PKC-ζ transfectants, compared with similarly treated empty vector cells grown on collagen I ($P < 0.01$). These results were confirmed by Coulter counting (data not shown). Thus, increased expression of PKC-ζ inhibited Caco-2 cell proliferation on collagen I extracellular matrix.

**PKC-ζ Kinase Inhibition or Knockdown with Small Interfering RNA Increases Caco-2 Cell Growth**

As an independent approach to directly confirm that PKC-ζ regulates Caco-2 cell growth, we examined the effect of inhibiting PKC-ζ activity using the myristoylated PKC-ζ pseudosubstrate (40). As shown in Fig. 6A, the pseudosubstrate peptide dose dependently increased Caco-2 cell proliferation, with 20 μmol/L inhibitor peptide causing nearly a 50% increase in proliferation, compared with vehicle-treated cells ($P < 0.05$). As an alternative approach to confirm that PKC-ζ regulates Caco-2 cell proliferation, we inhibited PKC-ζ expression by RNA interference using PKC-ζ–specific sequences as previously described (41). PKC-ζ was down-regulated by 50%
Polyclonal antibodies. Representative blot from three independent experiments plated in duplicate. Ponasterone A induces an additional PKC-ζ (20 μmol/L) transfectants, but not empty vector (EV) cells that migrate at a slightly higher molecular weight than endogenous PKC-ζ (M, 72 kDa). Blot was reprobed for β-actin as a loading control.

**FIGURE 2.** Ponasterone A induces PKC-ζ up-regulation. Transfectants were seeded overnight on collagen I–coated plates. Cells were treated with 5 μmol/L ponasterone (+ Ponasterone A) or DMSO vehicle (− Ponasterone A) for the indicated days, and whole-cell lysates were prepared. Proteins (20 μg/lane) were resolved by SDS-PAGE on 10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with anti-human PKC-ζ polyclonal antibodies. Representative blot from three independent experiments plated in duplicate. Ponasterone A induces an additional PKC-ζ reactive band in -transfectants, but not empty vector (EV) cells that migrate at a slightly higher molecular weight than endogenous PKC-ζ (M, 72 kDa). Blot was reprobed for β-actin as a loading control.

PKC-ζ Up-Regulation Enhances Caco-2 Cell Differentiation

Cell growth and differentiation are highly coordinated and often inversely related (43). For example, colonocytes migrating up the crypt axis cease dividing and differentiate. Furthermore, PKC-ζ inhibited Caco-2 cell growth and this isoform increases with colonocyte maturation (15). We asked, therefore, whether Caco-2 cell differentiation was regulated by PKC-ζ. Differentiation of these cells is associated with increased activity of alkaline phosphatase, an enzyme in the brush border membrane. As shown in Fig. 7, empty vector cells showed a time-dependent increase in alkaline phosphatase activity as they progressed from preconfluent to confluent cells. Compared with empty vector cells, ponasterone A significantly enhanced the alkaline phosphatase activity of PKC-ζ transfectants 2-fold within 3 days. This increased alkaline phosphatase activity persisted for up to 12 days (P < 0.03). These changes were accompanied by an earlier appearance of dome formation in -transfectants, compared with empty vector cells.

PKC-ζ Up-Regulation Increases the Rate of Caco-2 Cell Apoptosis

In the normal colon, PKC-ζ expression increases as colonocytes mature and eventually undergo apoptosis at the luminal surface (15, 16). We also found increased proliferation in Caco-2 cells with up-regulated PKC-ζ. We asked, therefore, whether up-regulated PKC-ζ might drive increased apoptosis that could contribute to slower cell growth. PKC-ζ has been shown to regulate apoptosis in a number of other cell types (23, 27-30). As assessed by 4',6-diamidino-2-phenylindole (DAPI) staining shown in Fig. 8 and summarized in Table 1, PKC-ζ up-regulation increased the apoptotic rate of Caco-2 cells nearly 3-fold compared with PKC-ζ transfectants treated with DMSO (P < 0.01). The apoptotic rates of empty vector–transfected cells, treated with DMSO or ponasterone A, were similar to parental Caco-2 cells (data not shown). We also confirmed that PKC-ζ enhances the rate of apoptosis by terminal deoxyribonucleotide transferase–mediated nick-end labeling assay (TUNEL; Table 1). Thus, based on our findings of enhanced differentiation and accelerated apoptosis in Caco-2 cells with up-regulated PKC-ζ, along with the observed expression changes of this isoform during colonocyte maturation, PKC-ζ seems to regulate differentiation and apoptosis of colon cancer cells, and perhaps normal colonocytes.

PKC-ζ Inhibition Increases Caco-2 Cell Anchorage–Independent Growth

To assess the influence of PKC-ζ on the transformation phenotype, ecdysone-regulated Caco-2 transfectants were analyzed for their ability to form colonies in soft agar. As shown in Fig. 9A, DMSO-treated empty vector and PKC-ζ transfectants grew at comparable rates in soft agar. Ponasterone A potently inhibited anchorage-independent growth of PKC-ζ transfectants, but not empty vector cells in soft agar (P < 0.01).
In separate experiments, we examined the effects of constitutive expression of wild-type or kinase-dead PKC-ζ on cell growth in soft agar. In agreement with our findings in the inducible expression system, constitutive expression of wild-type PKC-ζ inhibited growth in soft agar (Fig. 9B). In marked contrast, PKC-ζ kinase-dead transfectants grew significantly faster in soft agar compared with cells transfected with wild-type PKC-ζ (Fig. 9B). Thus, down-regulation of PKC-ζ, as we observed in colonic carcinogenesis, might contribute to the transforming phenotype that occurs during colonic malignant progression.

Discussion

Numerous studies have shown multiple signaling abnormalities in regulators of growth and differentiation during colonic malignant transformation (1). These changes include dysregulations in PKC signaling (44). We have previously shown that PKC-ζ was down-regulated in azoxymethane colonic carcinogenesis (4). In this study, we extended these observations to confirm that this isoform is expressed predominantly in epithelial cells of normal human colon. Furthermore, PKC-ζ expression is reduced in >70% of human colonic premalignant and malignant neoplasms in agreement with a prior report (33). These observations, taken together with high levels of expression of PKC-ζ in more mature postmitotic colonocytes, provoked us to test the hypothesis that alterations in PKC-ζ could regulate the transformed phenotype of colon cancer cells. The results of this study show that PKC-ζ inhibits Caco-2 cell growth, enhances differentiation, and increases apoptosis on collagen I extracellular matrix, whereas down-regulation or specific inhibition of PKC-ζ promotes cell growth. Thus, the loss of this isoform seems to favor the development of the malignant phenotype.

We used several different approaches to alter PKC-ζ expression or activity in colon cancer cells: (a) stable constitutive or inducible transfection systems; (b) kinase-dead, dominant-negative constructs; (c) RNA interference strategies; and (d) isoform-specific peptide inhibitors. For up-regulation, we developed Caco-2 transfectants that overexpressed full-length human PKC-ζ under a ponasterone-inducible ecdysone expression system. In these cells, we confirmed the expression of transfected PKC-ζ as shown by the coexpression of the V5 epitope tag. We also showed that these transfected cells, when induced by ponasterone A, possessed enhanced basal and insulin-stimulated PKC-ζ kinase activity. The magnitude of increased PKC-ζ expression in this study was comparable with that achieved in rat thyroid cells that significantly altered (enhanced) cell growth (45). Importantly, there were no changes in the expression levels of nontargeted PKC isoforms in our Caco-2 transfectants. Using these transfected Caco-2 cells, we showed that PKC-ζ up-regulation induced important biological effects on growth, including inhibition of anchorage-dependent and anchorage-independent proliferation. PKC-ζ up-regulation also enhanced differentiation and accelerated apoptosis. Our observation that PKC-ζ inhibited Caco-2 cell growth on collagen I is consistent with a prior report that a collagen I–containing gel, but not plastic, up-regulated dermal fibroblast PKC-ζ activity (46). PKC-ζ, in turn, increased α5 integrin in growth, enhances differentiation, and increases apoptosis on collagen I extracellular matrix, whereas down-regulation or specific inhibition of PKC-ζ promotes cell growth. Thus, the loss of this isoform seems to favor the development of the malignant phenotype.
these fibroblasts. This collagen I dependence suggests that PKC-ζ signaling is activated by outside-in signaling from the extracellular matrix, perhaps via collagen-interacting integrins, such as α2β1 (47). Our strategy to control PKC-ζ up-regulation by an inducible promoter was also important because constitutive up-regulation of this growth-suppressing isoform might have inhibited the expansion of stable Caco-2 transfectants.

As an alternative approach to confirm that PKC-ζ regulates growth, we inhibited the expression or activity of this isoform. In contrast to the antiproliferative effects caused by up-regulating PKC-ζ, specific PKC-ζ siRNA sequences decreased PKC-ζ expression by >50%, concomitantly increasing Caco-2 cell proliferation to 150% of control values by 48 hours. In agreement with these findings, inhibition of PKC-ζ functional activity with myristoylated PKC-ζ pseudosubstrate dose dependently and significantly increased Caco-2 cell proliferation on collagen. Kinase-dead, but not wild-type PKC-ζ, also significantly increased Caco-2 cell proliferation in soft agar, a measure of cellular transformation. Taken together, using multiple different approaches to alter PKC-ζ levels or activity, we have shown for the first time a negative growth regulatory role for PKC-ζ in human colon cancer cells. Using Caco-2 transfectants, we have also provided a direct demonstration that PKC-ζ activates a differentiation program and increases the rate of apoptosis in these cells.

The gradient of PKC-ζ expression in colonic crypts increases as epithelial cells migrate up the crypt axis and parallels growth cessation and differentiation (15). In this regard, PKC-α and PKC-δ also increase from the colonic crypt base to the luminal surface (15). Using similar strategies of stable transfections that selectively target specific PKC isoforms, we previously showed in vitro that PKC-α and PKC-δ also mediate growth-suppressor functions in these colon cancer cells (34, 35, 48). PKC isoforms, however, are not universally growth inhibiting. For example, PKC-pII increases cell proliferation and promotes colonic neoplastic transformation (49). Interestingly, we and others have also shown that PKC-pII is up-regulated in azoxymethane colonic carcinogenesis (4, 49). Taken together, our prior studies showing that several unrelated chemopreventive agents inhibited azoxymethane tumorigenesis and preserved PKC-ζ expression, along with our current in vitro results in Caco-2 cells, suggest that PKC-ζ might regulate a more generalized mechanism to inhibit colonic carcinogenesis. Our demonstration that changes in PKC-ζ can induce these growth effects argues strongly, moreover, that PKC-ζ directly regulates these changes.

Studies in a number of noncolon cancer cell types have shown that PKC-ζ may stimulate or inhibit proliferation (20, 23). PKC-ζ has also been shown to regulate differentiation and apoptosis (24, 26, 28-30). With regard to apoptosis, PKC-ζ was shown to interact with proapoptotic Par-4 and inhibit AKT, a survival kinase (28, 29). These effects of PKC-ζ might also contribute to the tumor suppressor–like functions we have shown in the current report. In contrast to our findings in colon cancer cells, in other cell types, PKC-ζ participates in cell transformation (50). In the colon, moreover, both infectious and neoplastic processes can lead to colonocyte hyperproliferation, but induce opposite effects on PKC-ζ expression and activation (4, 16, 51). These cell- and disease-specific growth responses to PKC-ζ emphasize the importance of examining transformed colonocytes to elucidate the potential growth-regulatory roles of this PKC isoform in colonic carcinogenesis.

Although studies in vivo, and our results in vitro, suggest that PKC-ζ plays an important role in normal colonocyte growth, we inhibited the expression or activity of this isoform. In contrast to the antiproliferative effects caused by up-regulating PKC-ζ, specific PKC-ζ siRNA sequences decreased PKC-ζ expression by >50%, concomitantly increasing Caco-2 cell proliferation to 150% of control values by 48 hours. In agreement with these findings, inhibition of PKC-ζ functional activity with myristoylated PKC-ζ pseudosubstrate dose dependently and significantly increased Caco-2 cell proliferation on collagen. Kinase-dead, but not wild-type PKC-ζ, also significantly increased Caco-2 cell proliferation in soft agar, a measure of cellular transformation. Taken together, using multiple different approaches to alter PKC-ζ levels or activity, we have shown for the first time a negative growth regulatory role for PKC-ζ in human colon cancer cells. Using Caco-2 transfectants, we have also provided a direct demonstration that PKC-ζ activates a differentiation program and increases the rate of apoptosis in these cells.

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Although studies in vivo, and our results in vitro, suggest that PKC-ζ plays an important role in normal colonocyte...
cytokinetics, PKC-ζ knockout mice exhibit grossly normal colonic architecture (10). We speculate that under nonstressed conditions, other growth-inhibiting PKC isoforms, such as PKC-α and PKC-δ, compensate for PKC-ζ loss. Based on prior studies and our findings in this report, we also believe losses of PKC-α and PKC-δ, concomitant with down-regulation of PKC-ζ, would collectively promote colonic tumorigenesis. It will be of interest to examine PKC-ζ-deleted mice under pathologic states, including colonic malignant transformation. We anticipate that these mice might develop a more aggressive tumor phenotype and/or manifest less sensitivity to a number of chemopreventive agents. Based on our results in Caco-2 colon cancer cells, moreover, we believe the observed down-regulation of PKC-ζ in colonic carcinogenesis contributes to tumor progression. Further studies to elucidate the mechanisms regulating PKC-ζ expression and activity and to identify PKC-ζ targets will be needed.

Tight junctions contain potential PKC-ζ targets that may participate in the growth phenotypic alterations induced by this PKC isoform. PKC-ζ localizes to tight junctions in Caco-2 cells and regulates their assembly in other epithelial cells (11, 52). Integrins may mediate collagen activation of PKC-ζ (46, 47, 53). Interestingly, integrin engagement of Caco-2 cells can also induce cell growth and increase differentiation, mimicking the effects induced by PKC-ζ in these cells (53). We speculate that PKC-ζ mediates interactions between integrins and extracellular matrix that participate in cell-cell and cell-basement membrane signaling. Because PKC-ζ plays a critical role in tight junction biogenesis in other cells, down-regulation of this atypical isoform in colonic carcinogenesis might abrogate normal growth inhibition mediated by cell-cell contact. Loss of this growth regulation is a characteristic feature of malignant transformation. Future studies to elucidate growth-inhibitory pathways regulated by PKC-ζ may suggest novel approaches to prevent colon cancer.

Materials and Methods

**Materials**

Electrophoretic-grade acrylamide, bisacrylamide, Tris, SDS, prelabeled molecular weight markers, and RC-DC protein reagent were from Bio-Rad Labs (Richmond, CA). Kodak (Rochester, NY) supplied the X-OMAT AR film. Polyvinylidene difluoride membranes (Immobilon-P) were purchased from Millipore, Inc. (Bedford, MA). QIAQuick PCR purification columns were obtained from Qiagen, Inc. (Santa Clarita, CA). New England Nuclear (Boston, MA) provided [γ^32P]ATP. LipofectAMINE was purchased from Life Technologies (Gaithersburg, MD). The ecysosyme-regulated vector system and V5 antibodies were obtained from Invitrogen (Carlsbad, CA). Anti-PKC-α (2056) polyclonal antibodies were obtained from Cell Signaling (Danvers, MA). Isoform-specific anti-rabbit antibodies to PKC-βI, PKC-βII, PKC-δ, PKC-ε, and PKC-ζ and polyclonal goat antibodies to PKC-ζ were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Universal labeled streptavidin biotin horseradish peroxidase system (LSAB) was obtained from DAKOCytomation (Carpinteria, CA). Collagen and Matrigel-coated tissue culture plates were purchased from Becton Dickinson (Palo Alto, CA). Silencer siRNA construction and transfection kit with PKC-ζ or empty vector transfectants (5 × 10^6 per well) were plated in triplicate in six-well collagen I-coated plates. Twenty-four hours later, cells were treated with 5 μmol/L ponasterone A or DMSO (vehicle). On the indicated days, cells were collected and assayed for alkaline phosphatase activity as described in Materials and Methods. Specific activity was expressed as units of alkaline phosphatase/mg protein/min. Columns, means of two independent experiments each carried out in triplicate; bars, SD. *, P < 0.05, compared with vehicle-treated PKC-ζ cells.

FIGURE 7. PKC-ζ up-regulation enhances Caco-2 cell differentiation. Inducible PKC-ζ or empty vector transfectants (5 × 10^6 per well) were plated in triplicate in six-well collagen I–coated plates. Twenty-four hours later, cells were treated with 5 μmol/L ponasterone A or DMSO (vehicle). On the indicated days, cells were collected and assayed for alkaline phosphatase activity as described in Materials and Methods. Specific activity was expressed as units of alkaline phosphatase/mg protein/min. Columns, means of two independent experiments each carried out in triplicate; bars, SD. *, P < 0.05, compared with vehicle-treated PKC-ζ cells.
Human Colonic Tumors

The study was approved by the University of Chicago Hospitals Institutional Review Board for Human Investigations. Surgical specimens from fresh colonic cancers and polyps were obtained from Surgical Pathology under protocols approved by the Institutional Review Board. Tumors were divided and a portion fixed in 10% buffered formalin for histologic classification. Another fraction was flash frozen in liquid nitrogen and stored at $-80^\circ C$ until analysis. Tumor histology was graded by an expert gastrointestinal pathologist (Dr. John Hart, University of Chicago, Chicago, IL).

Western Blotting

Human colonic cancers, polyps, and normal mucosa were homogenized in 2× SDS Laemmli buffer using a Polytron and a probe sonicator. Samples were boiled at 100°C for 5 minutes and proteins quantified using the RC-DC protein assay. Western blotting was done as previously described (54). Briefly, SDS-treated samples (20 μg protein) were separated by SDS-PAGE using a 10% resolving gel. Prestained molecular weight markers were included on each gel. Gels were electroblotted to polyvinylidene difluoride membranes following the method of Towbin et al. (55). Nonspecific antibody binding was blocked by incubating the blots for 2 hours at room temperature in 50 mmol/L Tris-HCl, 150 mmol/L NaCl with 0.05% Tween 20 [TBS/0.05% Tween 20 (TBST)] containing 5% nonfat dry milk. After blocking, the blots were incubated overnight at 4°C in TBST made 1% in bovine serum albumin with isoform-specific antibodies to PKC-ζ, PKC-α, PKC-βI, PKC-βII, or PKC-δ at final concentrations of 0.1 μg/mL. After four washes in 10 mL TBST, the blots were incubated with 1:3,000 final dilution of appropriate peroxidase-coupled secondary antibodies. The blots were washed in TBST and the PKC isoforms were detected by xerography on X-OMAT AR film using an enhanced chemiluminescence system. The xerograms were digitized with an Epson flat bed scanner and quantitated using Scanalytic IP Lab gel software (Fairfax, VA) for data acquisition and analysis.

PKC-ζ Immunohistochemical Staining

Surgically resected colon cancer specimens with adjacent normal-appearing mucosa were fixed in 10% buffered formalin and paraffin embedded. Paraffin sections (5 μm) were deparaffinized in xylenes, rehydrated through graded ethanol solutions to distilled water, and washed in TBS. Antigen retrieval was carried out by heating sections in EDTA/Tris buffer (pH 9) for 45 minutes in an electric pressure cooker (Decloaking Chamber, Biocare Medical, Concord, CA). Endogenous peroxidase activity was quenched by incubation in 3% H2O2 in methanol for 5 minutes. Nonspecific binding sites were blocked using protein block for 20 minutes. Sections were then incubated for 1 hour at room temperature with 1:100 dilution of goat polyclonal antibodies against PKC-ζ. Primary antibodies were detected with the LSAB kit (DAKO, Carpinteria, CA). Nonimmune rabbit immunoglobulin was substituted for primary antibodies as a negative control.
**Cell Culture**

Caco-2 cells, derived from a human colonic adenocarcinoma, were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂:95% air as previously described (56). Cells were maintained in standard DMEM, containing 4.5 g/L glucose, 10 mmol/L HEPES (pH 7.4), 2 mmol/L l-glutamine, 50 units/mL penicillin G, 50 μg/mL streptomycin, 2 μg/mL gentamycin, 1% essential and nonessential amino acids, and 20% fetal bovine serum. Caco-2 cells were plated in multiwell biocotted or uncoated plates. In preliminary experiments, we examined PKC-ζ expression on several substrates, including plastic, collagen I, collagen IV, and Matrigel. Collagen I provided the greatest ponasterone A–inducible expression of PKC-ζ and was, therefore, chosen for the remainder of these studies. There were no differences in expression levels of nontargeted PKC isoforms α, β1, βII, or δ in cells grown on plastic compared with those on collagen I. Where indicated, cells were incubated with the indicated concentrations of insulin, PKC-ζ pseudosubstrate, or PKC-ζ siRNA oligonucleotides for the times shown.

**Inducible PKC-ζ Caco-2 Stable Transfectants**

Full-length cDNA encoding human PKC-ζ in pBluescript was kindly provided by Dr. David J. Burns (Abbott Laboratories, Abbott Park, IL). PKC-ζ cDNA, lacking the stop codon, was amplified by PCR and subcloned in frame with the COOH-terminal V5 epitope tag into pLND/V5-His A, a commercial ponasterone A–inducible, G418-selectable mammalian expression vector (38). PKC-ζ cDNA was verified by direct sequencing. The Zeocin-selectable pVgRXR plasmid of the two-component ecodyes system encodes the regulatory ecodyeson receptor and the retinoid X receptor. Caco-2 cells transfected with pVgRXR and demonstrating high β-galactosidase inducibility (≥3-fold) were stably transfected with pLND/V5-HisA-PKC-ζ or pLND/V5-HisA alone (empty vector). Zeocin/G418–resistant clones were pooled (≥25 clones) and expanded for further studies. Stable polyclonal transfectants were selected and maintained in complete Caco-2 medium containing 250 μg/mL Zeocin and 400 μg/mL G418.

**Table 1. Effect of PKC-ζ on Caco-2 Cell Apoptosis**

<table>
<thead>
<tr>
<th>Transfectants</th>
<th>Treatment</th>
<th>DAPI (%)</th>
<th>TUNEL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV</td>
<td>−PA</td>
<td>1.9 ± 0.2</td>
<td>7.6 ± 2.6</td>
</tr>
<tr>
<td>PKC-ζ</td>
<td>−PA</td>
<td>3.0 ± 0.4</td>
<td>9.8 ± 5.6</td>
</tr>
<tr>
<td>EV</td>
<td>+PA</td>
<td>1.5 ± 0.2</td>
<td>7.1 ± 1.9</td>
</tr>
<tr>
<td>PKC-ζ</td>
<td>+PA</td>
<td>9.0 ± 0.8*</td>
<td>24.6 ± 4.0*</td>
</tr>
</tbody>
</table>

**Constitutive PKC-ζ Caco-2 Transfectants**

Full-length human cDNA for PKC-ζ, lacking the stop codon and subcloned into the pLND/V5-His A vector, was mutagenized from AAA (lysine281) to TGG (tryptophan281) to destroy the ATP binding site as previously described (57). Wild-type and mutagenized PKC-ζ were then subcloned into pcDNA3.1/Myc-His (+) A in frame with a COOH-terminal sequence for a myc tag and a polyhistidine metal-binding peptide (Invitrogen). Plasmid DNA identities and mutations were confirmed by sequencing. Caco-2 cells were transfected with pcDNA3.1/Myc-His (+) A alone (empty vector), or vector containing wild-type or kinase-dead PKC-ζ. Kinase-dead PKC-ζ enhanced Caco-2 cell growth, whereas wild-type PKC-ζ inhibited proliferation in soft agar.

**FIGURE 9.** PKC-ζ inhibits Caco-2 cell growth in soft agar. Transfectants were plated in 0.35% soft agar and analyzed for their ability to form visible colonies (≥0.2 mm) as described in Materials and Methods. Columns, means of three independent experiments in duplicate; bars, SD. A, Inducible PKC-ζ transfectants. Ecdysone-regulated transfectants were treated with ponasterone A or vehicle. Ponasterone A–induced, PKC-ζ–transfected cells grew significantly slower on soft agar compared with vehicle-treated -transfectants or empty vector cells. *, P < 0.01, compared with empty vector cells. B, constitutive PKC-ζ transfectants. Caco-2 cells were transfected with expression vectors regulated by the viral cytomegalovirus promoter alone (empty vector) or containing cDNA encoding wild-type or kinase-dead PKC-ζ. Kinase-dead PKC-ζ, enhanced Caco-2 cell growth, whereas wild-type PKC-ζ inhibited proliferation in soft agar. *, P < 0.05, compared with empty vector cells.
PKC-ζ Biochemical Activity

Inducible PKC-ζ and empty vector transfectants were seeded on collagen I–coated, six-well multiwell plates at a density of 50,000 cells per well cultured in 250 μg/mL Zeocin and 400 μg/mL G418. Preconfluent cells (40-50%) were treated for 2 days with ponasterone A or vehicle (DMSO), and then stimulated with 10 mmol/L insulin or PBS for 10 minutes. Cells were collected in 500 μL ice-cold lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 2 mmol/L EDTA, 1 mmol/L EGTA, 10 μg/mL aprotinin, and 1 mmol/L benzamidine. PKC-ζ was isolated from the soluble fraction (200 μg protein) by immunoprecipitation with anti PKC-ζ antibodies and A/G Sepharose beads. Immunoprecipitates were equilibrated in kinase buffer containing 35 mmol/L Tris-HCl (pH 7.5), 15 mmol/L MgCl2, 1 mmol/L MnCl2, 0.5 mmol/L EGTA, 0.1 mmol/L CaCl2, 1 mmol/L DTT, 10 mmol/L p-nitrophenylphosphate 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, and 20 μg/mL leupeptin. Reactions were carried out under linear conditions for 30 minutes at 30°C in 50 μL of kinase buffer containing 3 μCi [γ32P]ATP, 10 μmol/L ATP, and 50 μmol/L PKC-ζ pseudosubstrate. Aliquots (25 μL), spotted on P81 filter paper, were washed thrice in 75 mL phosphoric acid. Radioactivity was determined by Cherenkov counting. For the cytomegalovirus-regulated transfectants, myc-tagged PKC-ζ was immunoprecipitated with myc antibodies and the kinase-dead or wild-type nature of these kinases was confirmed.

PKC-ζ Knockdown with siRNA

siRNA transfections were done as recommended by the manufacturer (Ambion). The following oligonucleotides were used: PKC-ζ, 5′-GGAAGUGAG-AGAACAUGUGUt-3′ (complementary strand not shown); an irrelevant control sequence was also used (42). Parental Caco-2 cells were plated at 10% serum on collagen I–coated 96-well plates. When 50% confluent, cells were transfected in replicates of six in serum-free conditions with 0 to 50 mmol/L double-stranded PKC-ζ–specific siRNA, or an irrelevant control sequence complexed with LipofectAMINE in 200 μL OPTI-MEM I essential medium. PKC-ζ knockdown was assessed by Western blotting 48 hours later. PKC-ζ expression was also measured to confirm isomeric specificity of the ζ-siRNA sequences.

Assay of Anchorage-Dependent Growth

Cell growth was quantified by Coulter Counter (Coulter Electronics, Miami, FL) or WST-1 colorimetric assay as previously described (34, 35). In preliminary studies, we examined growth on plastic, collagen I, collagen IV, and Matrigel. Because PKC-ζ up-regulation and cell growth inhibition were greater on collagen I compared with other substrates, we carried out the remainder of our studies on collagen I–coated plates. For cell counting, inducible PKC-ζ and empty vector–transfected Caco-2 cells were seeded on six-well collagen I–coated tissue culture plates at a density of 5 × 104 per well. Cells were incubated in DMEM medium containing 250 μg/mL Zeocin and 400 μg/mL G418, as well as 5 μmol/L ponasterone A or vehicle (DMSO). Medium was changed every other day. For WST-1 colorimetric assays (Roche Molecular Biochemicals), empty vector, and PKC-ζ–transfected Caco-2 cells were seeded on collagen I–coated, 96-well microtiter tissue culture plates at a density of 5 × 103 per well, and 5 μmol/L ponasterone A or DMSO was added 24 hours later. At the indicated days after plating, WST-1 reagent (20 μL) was added and the plate was incubated at 37°C for 1 to 2 hours. Sample absorbance was measured at 450 nm using a microtiter plate (ELISA) reader and cell proliferation rates were calculated from absorbance changes.

Alkaline Phosphatase Assay

Inducible PKC-ζ and empty vector–transfected Caco-2 cells were seeded at a density of 5 × 106 per well on collagen I–coated six-well plates in DMEM medium containing 20% fetal bovine serum, 250 μg/mL Zeocin, and 400 μg/mL G418. Cells were allowed to attach overnight. Cells were treated with 5 μmol/L ponasterone A or vehicle (DMSO) and medium was changed every other day. At the indicated times, cells from triplicate wells were rinsed with cold PBS buffer and scrapped into 300 μL of cold 2 mmol/L Tris-50 mmol/L mannitol buffer (pH 7.4) and stored at −80°C until analysis. Cells were sonicated and proteins were determined using RC-DC protein assay reagent. Total cell lysates (100 μL) were assayed for alkaline phosphatase activity with an alkaline phosphatase kit (Sigma Diagnostics, St. Louis, MO), using p-nitrophenyl phosphate as substrate and p-nitrophenol for the standard curve. Specific activity was expressed as units of alkaline phosphatase/mg protein/min.

Apoptosis Assays

Inducible PKC-ζ and empty vector transfectants were seeded at a density of 1.5 × 105 cells in collagen I–coated slide flasks in DMEM containing 20% fetal bovine serum, 250 μg/mL Zeocin, and 400 μg/mL G418. Cells were then treated with 5 μmol/L ponasterone A or vehicle (DMSO) for 48 hours in serum-free medium. Cells were washed twice with HBSS and fixed for 10 minutes at room temperature with 4% paraformaldehyde in PBS. Fixative was aspirated and cells were washed twice in PBS. For DAPI staining, DNA was stained for 1 minute with 2 μg/mL DAPI at room temperature. Excess DAPI stain was removed and the monolayer was extensively washed with water. A coverslip was then secured with Vectashield mounting medium. Stained nuclei were viewed at ×200 using an Olympus BH-2 fluorescence microscope and photographed using Kodak Ektachrome 400 film.

As an independent assessment of apoptosis, fragmented nuclear DNA was identified in situ using the TUNEL assay. Forty-eight hours after plating, floating cells were collected by cytospin (Shandon, Pittsburgh, PA). Floating and attached cells were fixed in 4% paraformaldehyde. DNA strand breaks were end conjugated with digoxigenin-labeled nucleotide triphosphates by terminal deoxynucleotidyl transferase, using ApoTag Peroxidase In situ Apoptosis Detection kit (Chemicon International). Labeled DNA fragments, bound to peroxidase-conjugated, anti-digoxigenin antibodies, were detected using the chromogenic substrate (3,3′-diaminobenzidine). TUNEL-stained nuclei were viewed by light microscopy. For both DAPI

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and TUNEL, apoptotic cells were quantified in duplicate plating per sample for each experiment. Slides were coded before counting and the results were confirmed by two independent observers. Apoptotic rates were expressed as the percentage of total cells (means ± SD) with condensed or fragmented nuclei (DAPI), or labeled DNA strand breaks in floaters (TUNEL).

**Assay of Anchorage-Independent Growth in Soft Agar**

Caco-2 transfectants were assayed for growth in soft agar as previously described (48). For inducible Caco-2 transfectants, P100 tissue culture dishes were precoated with 5 mL of 0.7% Bactoagar, containing 250 µg/mL Zeocin and 400 µg/mL G418, as well as 5 µg/mL ponasterone A or vehicle (DMSO). For constitutive Caco-2 transfectants, expressing wild-type or kinase dead PKC-ζ, Zeocin and ponasterone A/DMSO were omitted. The agar layer was allowed to harden and 8 mL DMEM medium containing 20% fetal bovine serum, 0.35% DMEM, and 5 g/mL Zeocin and 400 g/mL G418, as well as 5 µg/mL ponasterone A or vehicle (DMSO). For constitutive Caco-2 transfectants, expressing wild-type or kinase dead PKC-ζ, Zeocin and ponasterone A/DMSO were omitted. The agar layer was allowed to harden and 8 mL DMEM medium containing 20% fetal bovine serum, 0.35% Bactoagar, and 5 × 10^5 Caco-2 transfectants were overlaid on the agar-coated dishes. All dishes were incubated at 37 °C in a humidified atmosphere of 5%CO2-95% air. Fresh medium was added every 3 to 4 days for 4 to 6 weeks. Colonies were stained with 1% Giemsa and those >0.2 mm were quantified as added every 3 to 4 days for 4 to 6 weeks. Colonies were stained with 1% Giemsa and those >0.2 mm were quantified as previously described (48).

**Statistical Analysis**

Results are expressed as the means ± SD or means ± SE as indicated. Data were analyzed using Student’s t test for unpaired samples or ANOVA as appropriate. For post hoc analysis of multiple treatments, Dunnett’s test was used for comparisons of treated to control samples. Values of P < 0.05 were considered statistically significant.

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Protein Kinase-ζ Inhibits Collagen I–Dependent and Anchorage-Independent Growth and Enhances Apoptosis of Human Caco-2 Cells

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