We have reported previously that protein kinase C (PKC) signaling can mediate a program of cell cycle withdrawal in IEC-18 nontransformed intestinal crypt cells, involving rapid disappearance of cyclin D1, increased expression of Cip/Kip cyclin-dependent kinase inhibitors, and activation of the growth suppressor function of pocket proteins (Frey, M. R., Clark, J. A., Leontieva, O., Uronis, J. M., Black, A. R., and Black, J. D. (2000) J. Cell Biol. 151, 763–777). In the current study, we present evidence to support a requisite role for PKC α in mediating these effects. Furthermore, analysis of the signaling events linking PKC/PKC activation to changes in the cell cycle regulatory machinery implicates the Ras/Raf/MEK/ERK cascade. PKC/PKC activation promoted GTP loading of Ras, activation of Raf-1, and phosphorylation/activation of ERK. ERK activation was found to be required for critical downstream effects of PKC/PKC α activation, including cyclin D1 down-regulation, induction, and cell cycle arrest. PKC-induced ERK activation was strong and sustained relative to that produced by proliferative signals, and the growth inhibitory effects of PKC agonists were dominant over proliferative events when these opposing stimuli were administered simultaneously. PKC signaling promoted cytoplasmic and nuclear accumulation of ERK activity, whereas growth factor-induced phospho-ERK was localized only in the cytoplasm. Comparison of the effects of PKC agonists that differ in their ability to sustain PKC α activation and growth arrest in IEC-18 cells, together with the use of selective kinase inhibitors, indicated that the length of PKC-mediated cell cycle exit is dictated by the magnitude/duration of input signal (i.e. PKC α activity) and of activation of the ERK cascade. The extent/duration of phospho-ERK nuclear localization may also be important determinants of the duration of PKC agonist-induced growth arrest in this system. Taken together, the data point to PKC α and the Ras/Raf/MEK/ERK cascade as key regulators of cell cycle withdrawal in intestinal epithelial cells.

Members of the protein kinase C (PKC) family of signal transduction molecules have been implicated in the regulation of a wide variety of cellular processes, including cell growth and cell cycle progression, differentiation, survival/apoptosis, and transformation (1–4). The PKC family consists of at least 10 distinct isozymes (α, β1, βII, γ, δ, ε, η, ζ, θ, and ϵ) that share the same basic structure but differ with respect to activator and cofactor requirements, substrate specificity, tissue expression, and subcellular distribution (3). Studies in several systems, including self-renewing epithelial tissues (i.e. intestinal mucosa and epidermis), several leukemic cell lines, and melanoma cells increasingly point to a role for sustained PKC signaling in mediating cell cycle exit and cell differentiation (see Ref. 1 for review). Mechanistic studies in intestinal (5) and epidermal (6) epithelial systems have shown that activation of PKC α, in particular, is sufficient to trigger a program of cell cycle withdrawal, involving inhibition of G1/S cyclin-dependent kinase activity and coordinated alterations in the expression/activity of members of the pocket protein family (i.e. p107, pRB, and p130). Studies in erythroleukemia cells (7), myeloid cells (8), and melanoma cells (9) have further demonstrated the importance of PKC α in cell differentiation. Whereas the cell cycle-specific and growth regulatory effects of PKC α signaling have been well characterized in these systems, the events linking PKC α activation to changes in the cell cycle regulatory machinery remain largely unknown.

The current study investigated the downstream events of negative growth regulatory PKC signaling using intestinal epithelial cells as a model system. Based on (a) evidence for the ability of PKC agonists (10) and individual members of the PKC family (11, 12) to activate the extracellular signal-regulated kinase (ERK) pathway, and (b) recent reports demonstrating a role for ERK activity in growth arrest/differentiation of intestinal epithelial cells (13–16), we focused on the ERK signaling cascade. This three-kinase cascade, consisting of Raf, MAP kinase/ERK kinase (MEK), and ERKs 1 and 2, is ubiquitously expressed in mammalian cells and, like PKC, has been widely implicated in control of cell proliferation, differentiation, survival, and transformation (10, 17, 18). ERK1/2 and their upstream regulators are acutely stimulated by the interaction of growth or differentiation factors with cell surface receptor tyrosine kinases, heterotrimeric G protein-coupled re-
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PKC-mediated IEC-18 cell cycle arrest involves strong and sustained PKC tyrosine (PDBu), and 1,2-dioctanoyl-sn-glycero-3-phosphoethanolamine (DiC8) signaling in this system correlates with the duration of activation of these molecules.

Other PKR targets include membrane and cytoplasmic proteins, such as downstream kinases and cytoskeletal proteins (10, 17).

Previous studies from our laboratory have demonstrated that activation of PKC/PKC α in IEC-18 nontransformed intestinal crypt cells results in cell cycle withdrawal. By using pharmacological inhibitors of PKC and ERK signaling, we now show that PKC α plays a key role in PKC agonist-induced cell cycle arrest in IEC-18 cells and that PKR signaling is required for critical downstream cell cycle-specific effects of PKC/PKC α activation, including down-regulation of cyclin D1, induction of p21\(^{Waf1/Cip1}\), and cell cycle exit. We further demonstrate that PKC-mediated IEC-18 cell cycle arrest involves strong and sustained PKR signaling and that the duration of growth arrest is determined by the extent/duration of input signal, i.e. PKC α activity, and of activation of the ERK pathway. The magnitude and duration of nuclear phospho-ERK activity also appear to be important determinants of the length of the effect. Notably, PKC/PKC α stimulation is shown to promote both Ras and Raf activity in IEC-18 cells, and maintenance of ERK signaling in this system correlates with the duration of activation of these molecules.

EXPERIMENTAL PROCEDURES

Materials—Mouse monoclonal antibody specific for PKC α was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal rabbit anti-PKC δ (C-17) and anti-PKC ε (C-15) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies used in this study have been characterized previously for the absence of cross-reactivity with other PKC isoforms (5, 24). Anti-phospho-p44/p42 ERK (anti-phospho-ERK1/2) monoclonal antibody (E10) was purchased from Cell Signaling Technology (Beverly, MA), and rabbit polyclonal antibody against total ERK1/2 was from Santa Cruz Biotechnology. Rabbit polyclonal anti-cyclin D1 (H-295) and anti-MAP kinase phosphatase 1 (anti-total ERK1/2) was from Santa Cruz Biotechnology. Rabbit polyclonal anti-cyclin D1 (H-295) and anti-MAP kinase phosphatase 1 (anti-total ERK1/2) was from Santa Cruz Biotechnology. Rabbit polyclonal anti-cyclin D1 (H-295) and anti-MAP kinase phosphatase 1 (antitotal ERK1/2) was obtained from Santa Cruz Biotechnology. Rabbit polyclonal anti-cyclin D1 (H-295) and anti-MAP kinase phosphatase 1 (anti-total ERK1/2) was obtained from Santa Cruz Biotechnology.

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Fig. 1. PKC activation inhibits cell cycle progression in IEC-18 nontransformed intestinal crypt cells. A, the PKC agonist PMA induces cell cycle arrest in IEC-18 cells. Subconfluent cultures of asynchronously growing IEC-18 cells were treated with 100 nM PMA for the indicated times, and cell cycle distribution was determined by flow cytometric analysis of DNA content in propidium iodide-stained cells. 

trifuged at 10,000 × g for 30 min at 4 °C. Soluble, membrane, and cytoskeletal fractions were boiled in Laemmli sample buffer (33) for 5 min before being subjected to SDS-PAGE and immunoblot analysis.

Ras Activation Assays—The activation state of Ras was determined using the EZ-Detect Ras Activation kit from Pierce, and data were confirmed using the Ras Activation assay kit from Upstate Biotechnology, Inc. These kits use a GST fusion protein containing the Ras-binding domain (RBD) of Ras (GST-Raf-RBD) to pull down active GTP-bound Ras. The pulled down active Ras is detected by anti-Ras immunoblotting. In vitro GTPysis or GDP treatments of lysates were performed to generate positive and negative controls for the pull-down procedures, according to the manufacturer’s instructions.

Raf-1 Activity Assay—Raf-1 kinase activity was measured using a Raf-1 Immunoprecipitation-Kinase Cascade Assay kit from Upstate Biotechnology, Inc. This kit determines Raf-1 activity through a cascade reaction that uses MEK activation and subsequent ERK phosphorylation as an end point. The assay was performed according to the manufacturer’s instructions. Briefly, Raf-1 was immunoprecipitated from IEC-18 cell lysates and incubated with MEK1 and ERK2 in the presence of ATP. Soluble products of this reaction were used to phosphorylate myelin basic protein substrate in the presence of [γ-32P]ATP. Samples were spotted onto phosphocellulose squares, and the incorporated radioactivity was measured using a Beckman LS6500 multipurpose scintillation counter.

Immunofluorescence Staining of Phospho-ERK in IEC-18 Cells—Cells were grown on glass coverslips and treated with PMA, Bryo, or 10% serum (double the normal content in growth medium) for various times. Coverslips were then washed in PBS, fixed in methanol/acetone (3:1) at −20 °C for 20 min, and air-dried. The cells were then incubated with anti-phospho-ERK1/2 mouse monoclonal antibody (1:30) in PBS containing 0.2% Triton X-100 (PBS/Triton) for 1 h at room temperature. Following washes in PBS/Triton, cells were incubated with TRITC-conjugated goat anti-mouse IgG (1:100) for 30 min. The coverslips were then washed in PBS, mounted with Aquamount (Polysciences, Inc., Warrington, PA), and viewed with a Zeiss epifluorescence microscope (OberKochen, Germany).

RESULTS

Previous studies in this laboratory have demonstrated that PKC activation in IEC-18 nontransformed intestinal crypt cells triggers hallmark events of cell cycle withdrawal into G0, including rapid disappearance of cyclin D1, increased expression of Cip/Kip cyclin-dependent kinase inhibitors, and coordinated alterations in the expression and phosphorylation state of pocket proteins (see Refs. 5 and 26). Treatment of IEC-18 cells with 100 nM PMA activates PKC α, δ, and ε (the only phorbol ester-responsive PKC isozymes expressed in IEC-18 cells) and results in transient cell cycle blockade (26) (Fig. 1A). Reversal of growth arrest at ~12 h after addition of PMA correlates with depletion of these phorbol ester-responsive PKC isozymes (cf. Figs. 1A and 5D and see Refs. 5 and 26). As confirmed in Fig. 1B, PKC-induced inhibition of IEC-18 cell cycle progression is PKC-dependent; consistent with published data using PKC-depleted cells (26), inhibition of PKC activity using the general PKC inhibitor BIM I (5 μM) blocked PMA-
induced cell cycle arrest. Treatment with BIM I also inhibited PMA-induced down-regulation of cyclin D1 and induction of p21Waf1/Cip1, verifying the PKC dependence of these key consequences of PMA treatment in IEC-18 cells (Fig. 1C). Notably, selective inhibition of PKC α (the only Ca2+-dependent PKC isoform in IEC-18 cells) by Gö6976 (28) also abrogated PMA-induced cell cycle block, providing the first evidence of a requisite role for PKC α in phorbol ester-induced IEC-18 intestinal epithelial cell cycle withdrawal (Fig. 1D). Consistent with this finding, knockdown of PKC δ or PKC ε using RNAi technology failed to prevent PMA-induced cell cycle blockage in this system (Fig. 1E).

**PMA-induced IEC-18 Cell Cycle Arrest Requires ERK Activity**—To gain insight into the signaling events that link PKC/ PKC α activation to the cell cycle regulatory machinery in IEC-18 cells, we investigated the involvement of the ERK signaling cascade. As shown in Fig. 2A, treatment of IEC-18 cells with 100 nM PMA resulted in marked phosphorylation/activation of ERK1/2 by 5 min, an effect that was inhibited by the MEK inhibitors U0126 and PD098059. The membrane-permeant diacylglycerol analog DiC8, a less potent but more physiological PKC agonist that also induces cell cycle arrest in IEC-18 cells (26), was similarly found to promote ERK activation in this system (Fig. 2A). To verify the PKC-dependence of PMA-induced ERK1/2 activation, the phorbol ester-responsive PKC isoforms, PKC α, δ, and ε, were depleted from IEC-18 cells by treatment with 1 μM PDBu for 24 h; cells expressing PKC α as the only phorbol ester-responsive PKC isozyme (lane α) were generated by treatment with 100 nM PMA for 15 min, followed by two washes in warm PBS, and return to complete medium for 24 h (26). ii, PKC-depleted cells were treated with 100 nM PMA for 30 min, and ERK1/2 activation and expression were assessed by immunoblot analysis as in A. iii, PKC δ/ε-depleted cells were treated with 100 nM PMA for 30 min, and ERK1/2 activation was assessed by immunoblot analysis as in ii. Note that the extent of PMA-induced ERK1/2 activation in PKC δ/ε-depleted cells varied slightly between experiments and correlated with the levels of PKC α remaining in the cells. Data are representative of at least three independent experiments.

**Fig. 2.** PKC signaling activates the ERK signaling cascade in IEC-18 cells. A, PMA and DiC8 induce ERK1/2 phosphorylation/activation in intestinal epithelial cells. IEC-18 cells were treated with 100 nM PMA for 5 min (upper panel) or 15 min (lower panel) in the presence or absence of 10 μM U0126 or 50 μM PD098059 (added 30 min prior to PMA). Alternatively, cells were treated with 20 μg/ml DiC8 for 5 min as indicated. ERK1/2 activation was assessed by immunoblot analysis using anti-phospho-ERK1/2 (anti-active ERK1/2). Total ERK1/2 levels were determined using anti-total ERK1/2 antibody. B, PMA-induced ERK1/2 activation is PKC-dependent and can be mediated by PKC α alone in IEC-18 cells. i, the phorbol ester-responsive PKC isoforms, PKC α, δ, and ε, were depleted (lane D) from IEC-18 cells by treatment with 1 μM PDBu for 24 h; cells expressing PKC α as the only phorbol ester-responsive PKC isozyme (lane α) were generated by treatment with 100 nM PMA for 15 min, followed by two washes in warm PBS, and return to complete medium for 24 h (26). ii, PKC-depleted cells were treated with 100 nM PMA for 30 min, and ERK1/2 activation and expression were assessed by immunoblot analysis as in A. iii, PKC δ/ε-depleted cells were treated with 100 nM PMA for 30 min, and ERK1/2 activation was assessed by immunoblot analysis as in ii. Note that the extent of PMA-induced ERK1/2 activation in PKC δ/ε-depleted cells varied slightly between experiments and correlated with the levels of PKC α remaining in the cells. Data are representative of at least three independent experiments.

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PKC-mediated negative growth regulatory signals involving the ERK cascade appear to be dominant over serum-induced proliferative ERK1/2 signaling pathways in this system.

The Duration of PKC-mediated Cell Cycle Arrest Is Linked to the Intensity/Duration of ERK1/2 Signaling in IEC-18 Cells—To characterize further the involvement of ERK signaling in PKC-mediated intestinal epithelial growth arrest, the cell cycle-specific effects of the macrocyclic lactone Bryo were examined and compared with those of PMA. Although a potent activator of PKC, studies in a variety of systems have shown that Bryo often produces only a subset of the typical phorbol ester responses and antagonizes those phorbol ester-mediated effects it cannot itself induce (35). Flow cytometric analysis revealed that, like PMA, Bryo is able to negatively regulate IEC-18 cell cycle progression (Fig. 5). Treatment of asynchronously growing IEC-18 cells with 100 nM Bryo inhibited G1 \rightarrow S phase progression by 6 h (Fig. 5A); this effect was PKC-dependent, as confirmed by the ability of the general PKC inhibitor BIM I to block Bryo-induced cell cycle arrest. The PKC \(\alpha\)-selective inhibitor Gö6976 also inhibited the cell cycle effects of Bryo in IEC-18 cells, indicating that PKC \(\alpha\) plays a key role in both PKC- and Bryo-induced negative growth regulatory effects in this system. Notably, comparison of the extent and duration of the effects of these agents revealed that the cell cycle arrest produced by Bryo is less complete and reverses more rapidly than that induced by PMA. In asynchronously growing cells, cell cycle arrest was evident by 5 h in both Bryo- and PMA-treated cells; however, whereas PMA-induced cell cycle arrest was maintained at 9 h (not reversing until \(\sim 12\) h, see Fig. 1A), the effect produced by Bryo had begun to reverse by 8 h of treatment (Fig. 5B, i). For analysis of the effects of these agents in synchronized cells, IEC-18 cells were arrested in \(G_0/G_1\) by serum deprivation and serum-stimulated for 8 h before addition of 100 nM PMA or 100 nM Bryo for the indicated times. Flow cytometric analysis of untreated cells revealed that cells progress into S phase \(\sim 15\) h after serum stimulation (data not shown). As shown in Fig. 5B, ii, treatment of IEC-18 cells with PMA in mid-G1 (8 h after serum stimulation) delayed S phase progression by \(\sim 6\) h; the release kinetics were similar to those seen in asynchronously growing cells with growth arrest maintained until \(\sim 12\) h after addition of phorbol ester (see Fig. 1). Bryo, on the other hand, produced a more short-lived delay in S phase entry lasting \(\sim 3\) h (from the addition of the agent).

To gain insight into the basis for the differential cell cycle effects of Bryo and PMA in IEC-18 cells, the ability of these agents to induce and sustain activation of PKC \(\alpha\), \(\delta\), and \(\epsilon\) in these cells was compared. PKC isozyme translocation (i.e. association with the particulate subcellular fraction) and down-regulation were used as measures of agonist-induced isozyme-specific effects (36, 37). As shown in Fig. 5C, Bryo promoted translocation of all three PKC isozymes to the membrane/cytoskeletal fractions by 15 min, paralleling the effects of PMA (26). Notably, however, membrane translocation of PKC \(\alpha\) was slower in Bryo-treated cells. Comparison of PKC isozyme expression and phosphorylation/activation state at later times after treatment revealed marked differences in the effects of these agents (Fig. 5D). Bryo produced significantly more rapid down-regulation/desensitization of PKC \(\alpha\) than PMA (Fig. 5D, i and ii), as indicated by more rapid disappearance of the fully phosphorylated slower migrating form of the enzyme (activated PKC \(\alpha\); top arrow) and accumulation of the higher mobility nonphosphorylated protein (bottom arrow) that is inactive (38, 39). This inactive form, which appeared following extensive caveolar internalization of the enzyme (39), was evident as early as 30 min after addition of Bryo (Fig. 5D, ii). Furthermore, whereas the slower migrating, activated form of PKC \(\alpha\)

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**Fig. 3.** The ERK pathway is required for PKC-mediated IEC-18 cell cycle arrest, cyclin D1 down-regulation, and p21\(^{Waf1/Cip1}\) induction in IEC-18 cells. A, inhibition of MEK activity blocks PMA-induced cell cycle arrest. IEC-18 cells were treated with 100 nM PMA for 6 h in the presence or absence of 10 \(\mu M\) U0126. DNA content/cell cycle distribution was determined by flow cytometric analysis. B, MEK inhibition blocks PMA-induced cyclin D1 down-regulation and p21\(^{Waf1/Cip1}\) induction in IEC-18 cells. IEC-18 cells were treated with 100 nM PMA for 2 h in the presence or absence of 10 \(\mu M\) U0126, and cyclin D1 and p21\(^{Waf1/Cip1}\) expression levels were assessed by immunoblot analysis. Data are representative of at least three independent experiments.
remained detectable following 9 h of PMA treatment, it was no longer evident by 4–6 h in Bryo-treated cells. In contrast, activated PKC δ was protected from down-regulation by Bryo (as observed in other systems, where the protected enzyme was confirmed to retain kinase activity (40)). As shown in Fig. 5D, iii, levels of PKC δ had changed little following 12 h of Bryo treatment, a time when Bryo-induced growth arrest had reversed and cells had progressed into S phase (Fig. 5B). Immunoblot analysis further demonstrated a little difference in the effects of PMA and Bryo on PKC ε expression over 9 h of treatment (Fig. 5D, i). Thus, the duration of PKC α activation, but not that of PKC δ or ε, correlated with the duration of cell cycle arrest produced by these agents, providing further support for a major role of PKC α signaling in PKC agonist-induced IEC-18 cell cycle arrest. Taken together, the data presented above demonstrated that both PMA and Bryo can induce PKC α-dependent cell cycle blockade in IEC-18 cells. However, differences are evident in the duration of PKC α activation and cell cycle arrest produced by these agents, with PMA inducing more sustained PKC α activity and more prolonged growth inhibition (3 h longer) than Bryo in these cells.

To examine the role of ERK signaling in Bryo-induced cell cycle arrest, asynchronously growing IEC-18 cells were treated with 100 nM Bryo in the presence or absence of the MEK inhibitor U0126. As seen in PMA-treated cells, 10 μM U0126 abrogated the inhibition of S phase progression produced by Bryo in these cells (Fig. 6A). Thus, the ERK signaling cascade appears to be required for Bryo-induced cell cycle inhibition in IEC-18 cells. Based on these findings, we next compared the extent and duration of ERK activation induced by PMA and Bryo by using Western blot analysis. As shown in Fig. 6B, i, PMA and Bryo strongly activated ERK1/2 by 5 min of treatment, although the levels of ERK1/2 activation were consistently slightly lower in Bryo-treated cells. While a noticeable decline in phospho-ERK was evident by 45 min in cells treated with Bryo (data not shown and Fig. 6B), cells treated with PMA showed a sustained activation of ERK lasting longer than 6 h (Fig. 6B, ii; also see Fig. 4). Bryo and PMA also differed in their effects on the expression of cyclin D1 and p21Waf1/Cip1 in IEC-18 cells. Bryo induced a less marked down-regulation of cyclin D1, and levels of p21Waf1/Cip1, although elevated, did not reach those produced by PMA in these cells (Fig. 6C). The duration of IEC-18 cell cycle arrest induced by PMA and Bryo treatment, therefore, correlated with the extent and duration of ERK1/2 activity produced by these agents which, in turn, was reflected in the extent of cyclin D1 down-regulation and p21Waf1/Cip1 induction in this system.

### The Duration of PKC α and ERK1/2 Activation Determines the Duration of PMA-induced Growth Arrest in IEC-18 Cells

Comparison of the effects of PMA and Bryo indicated that the duration of cell cycle arrest produced by these agents was determined by their ability to sustain PKC α and ERK1/2 activity in IEC-18 cells. To investigate this idea further, selective inhibitors were used to examine the effect of manipulating the duration of PKC α and ERK1/2 stimulation on PMA-induced growth arrest in this system. IEC-18 cells were treated with 100 nM PMA (or vehicle) for 6 or 9 h, and Go6976 (0.5 μM) or U0126 (10 μM) was added at various times to inhibit PKC α and ERK1/2 signaling, respectively. The inhibitors were added either 30 min prior, at the same time, or at different times after the addition of PMA (as indicated) and were left in the medium for the remainder of the 6 or 9 h PMA incubation. Cells were then subjected to flow cytometric analysis, and the degree of PMA-induced growth arrest was evaluated by changes in the percent of cells in S phase. As shown in Fig. 7, to obtain PMA-induced growth arrest at 6 h, less than 30 min of PKC α and ERK1/2 activation appears to be required. Addition of the inhibitors at 30 or 45 min provided partial protection from the growth inhibitory effects of PMA, whereas no protection was observed when the inhibitors were added at 60–75 min. In contrast, maintenance of PMA-induced cell cycle blockade at 9 h required continued PKC α and ERK1/2 signaling for 60–75 min. Addition of Go6976 or U0126 up to 60 min after treatment with PMA blocked the ability of the PKC agonist to reduce the
percentage of cells in S phase at 9 h; thus, activation of PKC for up to 1 h provided insufficient signal for maintenance of growth arrest at this time point. However, when the inhibitors were added at times later than 60 min, a progressive loss of cells in S phase was noted, with addition at times after 120 min offering no protection from the cell cycle effects of PMA. Thus, whereas elevated levels of Ras activity were maintained in Bryo-treated cells by 5 min; PMA- and Bryo-induced Ras activation was readily seen both in the presence and absence of serum. The activation of Ras by these agents was PKC-dependent, as confirmed by the inability of PMA or Bryo to increase Ras-GTP levels in PKC-depleted cells (Fig. 8B). Furthermore, both PMA and Bryo activated Ras in PKC-depleted IEC-18 cells, indicating that PKC α alone can induce GTP loading of Ras in intestinal epithelial cells (Fig. 8C). Consistent with their similar initial effects on ERK activity (see Fig. 6B), both agents initially activated Ras to a similar extent (Fig. 8, A and D). However, whereas elevated levels of Ras activity were main-
tained over a 6-h period in PMA-treated cells, there was a substantial decline in Bryo-induced Ras activity by 75 min of treatment (Fig. 8, D and E). Thus, Bryo-induced Ras activation was significantly more transient than that produced by PMA. IEC-18 cells were treated with 100 nM Bryo for 6 h in the presence or absence of 10 µM U0126. DNA content/cell cycle distribution was determined by flow cytometric analysis. B, i and ii, Bryo-induced ERK1/2 activation is more transient than that produced by PMA. IEC-18 cells were treated with 100 nM PMA (P) or 100 nM Bryo (B) for the indicated times, and cell lysates were subjected to immunoblot analysis using antibodies specific for phospho-ERK1/2 or total ERK1/2. Lane C, vehicle-treated cells. Control samples shown on the left and right ends of the panel in B, ii, were collected at 5 min and 16 h, respectively. C, the effects of Bryo on expression of cyclin D1 and p21Waf1/Cip1 in IEC-18 cells are weaker than those produced by PMA. Asynchronously growing IEC-18 cells were treated with 100 nM PMA or 100 nM Bryo for the indicated times, and expression of cyclin D1 and p21Waf1/Cip1 was determined by immunoblot analysis. The values below the blots represent the relative levels of cyclin D1 and p21Waf1/Cip1 determined by densitometry and normalized to levels of the loading control (a nonspecific band consistently seen with the p21Waf1/Cip1 antibody that was not affected by PKC agonist treatments; similar values were obtained using total ERK as a loading control, not shown). Data are representative of at least four independent experiments.

We next examined the ability of PMA and Bryo to activate Raf-1 in IEC-18 cells. Raf-1 is a serine-threonine kinase responsible for activation of MEK1/2. In many systems, Raf-1 activation appears to involve Ras-mediated recruitment of Raf-1 to the plasma membrane, as well as interaction with additional membrane-associated kinases and phospholipids (50). The activity of Raf-1 was determined by measuring its ability to activate MEK and ERK1/2 in a Raf-1 immunoprecipitation kinase cascade assay (Upstate Biotechnology, Inc.). Both PMA and Bryo stimulated Raf-1 kinase activity by 5 min of treatment (Fig. 9A). Notably, although PMA-induced Raf-1 activity was sustained at 75 min, levels in Bryo-treated cells had significantly decreased by this time. Bryo-induced activation of Ras, Raf-1, and ERK1/2 was, therefore, more transient than that induced by PMA in IEC-18 cells, consistent with the more short-lived cell growth arrest produced by this agent.

Treatment of PKC δ/ε-depleted cells with PMA also resulted in increased Raf-1 activity (Fig. 9B), demonstrating that PKC α is sufficient to mediate PKC agonist-induced Raf-1 activation in IEC-18 cells. The PKC dependence of PKC agonist-induced effects on Raf-1 was further investigated by taking advantage of a mobility shift in Raf-1 protein associated with activation of the kinase (51). Anti-Raf-1 immunoblot analysis revealed that both PMA and Bryo induce a slower migrating form of Raf-1 in IEC-18 cells (Fig. 9C; data not shown for Bryo-treated cells). This form of Raf-1 was also produced in response to DiC 8 treatment (data not shown). Depletion of PKC δ, ε and δ from IEC-18 cells by prolonged treatment with 1 µM PDBu abolished the PKC agonist-induced mobility shift in Raf-1, demonstrating that PKC signaling is required for the effect (Fig. 9C). Consistent
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with data from Raf-1 kinase assays, depletion of PKC δ and ε from these cells failed to affect PKA-induced post-translational modification of Raf-1, further indicating that PKC α is sufficient to induce phosphorylation/activation of Raf-1 in response to PKA (Fig. 9C).

PKA- and Bryo-induced ERK Activity Accumulates in the Cytoplasm and Nucleus, whereas Serum-induced Phospho-ERK Is Detected Only in the Cytoplasm—To compare the subcellular localization of phospho-ERK1/2 induced by growth factors, PKA, or Bryo, IEC-18 cells grown on glass coverslips were given a serum boost or exposed to PKC agonists for various times. Cells were then fixed and processed for immunofluorescence analysis of phospho-ERK1/2 distribution as described under “Experimental Procedures.” As shown in Fig. 10A, un-treated cells exhibited low levels of phospho-ERK staining, predominantly in the cytoplasm. Following addition of serum, there was a marked increase in cytoplasmic phospho-ERK fluorescence; this increase was detectable by 20 s of treatment (data not shown) and maintained for 30–45 min (Fig. 10B). Nuclear localization of phospho-ERK1/2 was not detected at any time following addition of growth factors to the cells. PKA and Bryo, on the other hand, produced a more complex pattern of phospho-ERK1/2 compartmentalization (Fig. 10C and Table I). Whereas both agents first promoted cytoplasmic accumulation of activated ERK, by 2–5 min (Fig. 10C, a–d) nuclear phospho-ERK1/2 staining became evident in many of the cells; importantly, the effects of Bryo were significantly slower than those of PKA, consistent with the slower membrane translocation of PKC α induced by this agent (see Fig. 5C and Ref. 39). The overall intensity of phospho-ERK staining was also slightly lower in Bryo-treated cells. By 15–30 min of treatment

The current report demonstrates that the ERK signaling cascade plays a requisite role in linking PKC/PKC α activity to regulation of the cell cycle machinery in IEC-18 nontransformed intestinal epithelial cells. We have reported previously (5, 26) that sustained activation of PKC α signaling is sufficient to induce and maintain a program of cell cycle exit in these cells, although a role for PKC δ and/or ε in this effect was not excluded in those studies. By using the inhibitor Go6976 (28) to selectively block PKC α activity in IEC-18 cells, we now provide the first evidence for a requisite role of PKC α signaling in mediating PKC agonist (PKA or Bryo)-induced cell cycle blockade in intestinal epithelial cells. Consistent with this finding, selective knockdown of PKC δ or PKC ε using RNAi technology did not affect PKA-induced cell cycle arrest in IEC-18 cells. Additional evidence for the involvement of PKC α was provided by the demonstration that the differential kinetics of PKA- and Bryo-induced cell cycle arrest correlate with the differential ability of these agents to sustain PKC α activity in the cells. Thus, Bryo produced a delayed and more transient activation of PKC α associated with a more transient cell cycle blockade. The physiological significance of our in vitro findings in intestinal epithelial cells is emphasized by the demonstration that (a) PKC α undergoes membrane translocation/activation precisely at the point within intestinal crypts at which cells cease dividing (5, 24), and (b) the program of cell cycle regulatory events triggered by PKC α activation in IEC-18 cells is representative of the changes seen coincident with growth arrest in intestinal crypts in situ (5, 24).

A growing body of evidence from other systems supports the ability of sustained PKC α activity to mediate negative growth
FIG. 8. PKC/PKC α stimulation by PMA or Bryo activates Ras in IEC-18 cells; Ras activation by Bryo is more transient than that induced by PMA. A, PMA and Bryo activate Ras in IEC-18 cells. Asynchronously growing or serum-starved (0.5% serum for 24 h, no insulin) IEC-18 cells were treated with 100 nM PMA (P) or 100 nM Bryo (B) for 5 min. Cell lysates were prepared according to the instructions provided in the Pierce EZ-Detect Ras Activation kit, and affinity precipitation of GTP-bound Ras was performed using GST-tagged Raf-RBD. Levels of Ras in cell lysates (total Ras) or pulled down Ras (Ras-GTP) were determined by anti-Ras immunoblotting. Positive (+) and negative (−) controls for the pull-down procedure were generated by in vitro GTPγS or GDP lysate treatments, respectively. Lane C, vehicle-treated cells. Data in asynchronously growing and serum-starved cells are representative of six and two independent experiments, respectively. B, activation of Ras by PMA or Bryo is PKC-dependent. i, the phorbol ester-responsive PKC isozymes, PKC α, δ, and ε, were depleted from IEC-18 cells by treatment with 1 μM PDBu for 24 h (26), as shown by Western blot analysis. ii, PKC-depleted cells were subsequently treated with 100 nM PMA or 100 nM Bryo for 5 min, and the activation state of Ras was determined as in A. Lane C, vehicle-treated cells. C, PKC α is sufficient to mediate Ras activation by PMA or Bryo. Cells expressing PKC α as the only phorbol ester/Bryo-responsive PKC isozyme were generated by pulse treatment with PMA for
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Fig. 9. PKC/PKC α stimulation by PMA or Bryo activates Raf-1 in IEC-18 cells; Raf-1 activation by Bryo is more transient than that induced by PMA. A, PMA and Bryo activate Raf-1 in IEC-18 cells. Asynchronously growing IEC-18 cells were treated with vehicle (Control), 100 nM PMA, or 100 nM Bryo for 5 or 75 min. Cell lysates were prepared according to the Raf-1 Immunoprecipitation Kinase Cascade Assay kit protocol from Upstate Biotechnology, Inc., and Raf-1-induced phosphorylation of myelin basic protein was determined by scintillation counting. 5- and 75-min data are the average of four and two independent experiments, respectively. B, PKC α is sufficient to mediate PMA-induced activation of Raf-1. PKC δε-depleted cells (α, Pulse), generated by pulse treatment with PMA as described under “Experimental Procedures” (see immunoblot), were treated with 100 nM PMA for 5 min, and cell lysates were examined for Raf-1 activity as described in A. C, PMA-induced activation of Raf-1 is PKC/PKC α-dependent. Asynchronously growing IEC-18 cells (i.e., control cells), PKC α, δ, and ε-depleted cells, and PKC δε-depleted (PKC α-expressing) cells were incubated with 100 nM PMA for 2 h. Raf-1 was detected in cell lysates by anti-Raf-1 immunoblotting. PMA treatment of cells expressing the full profile of PKC isozymes resulted in a change in the mobility of Raf-1 from a faster migrating form to a slower migrating form (arrow). This mobility shift was abrogated by PKC depletion (D) and could be induced by PKC α alone (α) in these cells. Lane C, control cells; P, control cells treated with PMA; D, PKC-depleted cells; D/P, PKC-depleted cells treated with PMA; α, PKC δε-depleted cells; α/P, PKC δε-depleted cells treated with PMA. Data in B and C are representative of two independent experiments.

regulatory signaling and cell differentiation (for review, see Ref. 1). Examples include megakaryocytic differentiation of K562 cells (7, 54), differentiation of HL-60 cells to macrophages (55), melanoma cell differentiation by retinoids (9), vitamin D-regulated differentiation in various cell systems (56, 57), and irreversible cell cycle withdrawal of keratinocytes (6). Consistent with our findings in intestinal epithelial cells: (a) PKC/PKC α inhibitors protected normal keratinocytes from suspension-induced growth arrest and inhibited coordinated changes in cell cycle regulators associated with cell cycle withdrawal (6), and (b) PKC α is activated in post-mitotic suprabasal keratinocytes of human epidermis in situ (6), coincident with growth arrest-associated alterations in pocket protein regulation (58). Taken together, these findings point to a widespread role for PKC α in growth arrest and post-mitotic signaling.

The present study implicates the ERK signaling cascade in PKC/PKC α-mediated IEC-18 cell cycle blockade in intestinal epithelial cells. Both PMA and Bryo were shown to induce PKC-dependent ERK1/2 activation in IEC-18 cells, as demonstrated by using phospho-ERK-specific antibodies, and PKC α signaling was shown to be sufficient to mediate this effect. A requisite role for the ERK signaling pathway in PKC-mediated cell cycle regulation was confirmed by the ability of pharmacological inhibitors of MEK (U0126 and PD098059) to block cyclin D1 down-regulation, p21WAF1/CIP1 induction, and cell cycle blockade induced by PKC agonists. A role for the ERK pathway in promoting the expression of p21WAF1/CIP1 has been described in other systems (22, 59–62), and accumulating evidence indicates that the ability of sustained ERK activity to produce growth arrest/differentiation is linked to the induction of this growth inhibitory protein. The finding that PKC-induced cyclin D1 down-regulation is also dependent on ERK1/2 activity, however, was unexpected in view of evidence that ERK signaling is critically required for mitogen-induced expression of cyclin D1 and association of newly synthesized cyclin D1 with cdk4 (63). Thus, consistent with the opposing roles of the ERK pathway in regulation of cell growth (20), ERK1/2 signaling also appears to have opposing roles in control of the expression of the key mitogenic molecule, cyclin D1.

PKC agonist-induced ERK activation in IEC-18 cells was strong and sustained relative to that produced by proliferative signals in these cells (PMA > Bryo > serum boost). Furthermore, as would be expected for a negative growth regulatory stimulus (64), the growth inhibitory effects of PKC agonists were dominant over proliferative signaling by serum/growth factors when both stimuli were administered simultaneously. These findings parallel those seen in studies of young adult mouse colon cells where simultaneous exposure to proliferative epidermal growth factor and anti-proliferative TNF-α signals resulted in replacement of the transient pattern of epidermal growth factor-stimulated ERK activity by the sustained pattern seen with TNF-α (15). We further demonstrate that, in IEC-18 cells, the sustained activation of the ERK cascade produced by the combined treatment with growth factors and PKA resulted in growth inhibitory cell cycle regulatory effects, including down-regulation of cyclin D1 and increased expression of p21WAF1/CIP1.

Comparison of the effects of PMA and Bryo pointed to the strength and duration of ERK activation as important determinants of the extent and duration of PKC-mediated cell cycle blockade in IEC-18 cells. PMA sustained robust ERK1/2 activation for longer than Bryo and produced more pronounced cyclin D1 down-regulation and p21WAF1/CIP1 induction, as well as...

15 min, followed by two washes in warm PBS, and return to complete medium for 24 h (26). PKC δε-depleted cells were subsequently treated with 100 nM PMA or 100 nM Bryo for 5 min, and the activation state of Ras and expression of PKC isozymes were determined as in B. D, Bryo-induced Ras activity is down-regulated more rapidly than that induced by PMA. Asynchronously growing IEC-18 cells were treated with 100 nM PMA (P) or 100 nM Bryo (B) for 5 or 75 min, and Ras activity and expression were determined as in A. E, PMA-induced Ras activity is maintained for at least 6 h in IEC-18 cells. Asynchronously growing cells were treated with 100 nM PMA for 1, 2, 4, or 6 h and Ras activity was determined as in A. Data in B–E are representative of at least two independent experiments.
as more prolonged cell cycle arrest (~3 h longer). Notably, the ability of PMA and Bryo to sustain PKC activity, but not their effects on PKC δ or ε, correlated with their ability to maintain ERK1/2 activity, pointing to a link between these events. Thus, the duration of the input signal appears to play a major role in determining both the duration of ERK1/2 signaling and growth

FIG. 10. Immunofluorescence localization of phospho-ERK in IEC-18 cells. IEC-18 cells were given a serum boost (10% FBS, double the normal content in growth medium) or treated with 100 nM PMA or 100 nM Bryo for the indicated times. Cells were fixed in methanol/acetone and immunostained for phospho-ERK as described under “Experimental Procedures.” A, untreated cells exhibit low levels of cytoplasmic phospho-ERK. IEC-18 cells were treated with vehicle for 5 min (a) or 6 h (b). B, growth factors transiently induce ERK activity in the cytoplasm of IEC-18 cells; prolonged cytoplasmic phospho-ERK activity is seen only in PMA-treated cells. IEC-18 cells were treated with PMA (a, e, g, i, and k) or Bryo (b, d, f, h, j, and l) for 2 min (a and b), 5 min (c and d), 15 min (e and f), 1 h (g and h), 2 h (i and j), or 6 h (k and l). Note that by 2 min many PMA- and some Bryo-treated cells are beginning to accumulate phospho-ERK in the nucleus (for example, see arrowhead in a). Although the majority of PMA- or Bryo-treated cells shows nuclear accumulation of phospho-ERK by 15 min, the proportion of cells with predominant nuclear staining was significantly higher in cells exposed to PMA (see Table I). By 1 h of PKC agonist treatment, some cells show uniform phospho-ERK staining throughout the nucleus and cytoplasm (closed arrows in g and h), whereas others show predominantly cytoplasmic staining (open arrow in g). A marked decline in Bryo-induced phospho-ERK staining is seen by this time (also see Table I). See text for details.

TABLE I
Analysis of the subcellular localization of PMA- and Bryo-induced phospho-ERK in IEC-18 cells

| Length of treatment | PMA Cyto Cyto + Nuclear Nuclear | Bryo Cyto Cyto + Nuclear Nuclear |
|---------------------|---------------------------------|---------------------------------|
| 2 min               | 56 38 6 | 83 14 3 |
| 5 min               | 14 63 23 | 49 43 8 |
| 15 min              | 0 37 63 | 9 52 39 |
| 30 min              | 0 35 65 | 0 50 50 |
| 45 min              | 16 57 27 | 36 52 12 |
| 1 h                 | 61 35 4 | 73 27 0 |
| 2 h                 | 69 31 0 | 85 15 0 |
| 6 h                 | 85 15 0 | Barely detectable cyto staining |

Bryo slightly weaker than PMA
Bryo much weaker than PMA
Barely detectable cyto staining

as more prolonged cell cycle arrest (~3 h longer). Notably, the ability of PMA and Bryo to sustain PKC activity, but not their effects on PKC δ or ε, correlated with their ability to maintain ERK1/2 activity, pointing to a link between these events. Thus, the duration of the input signal appears to play a major role in determining both the duration of ERK1/2 signaling and growth
arrest in this system. Similar findings have been reported in PC12 cells (20), K562 cells (65), and LNCaP cells (51). In LNCaP cells, PMA produced prolonged membrane translocation of PKC α and sustained ERK activation, whereas Bryo produced only transient PKC α translocation followed by rapid down-regulation of the enzyme and transient activation of the ERK cascade (51). The fact that both PMA and Bryo produced prolonged membrane translocation of PKC δ and ε in LNCaP cells suggests that, as observed in IEC-18 cells, these PKC isozymes are not major players in sustaining PKC agonist-induced ERK activity in prostate cancer cells. It is noteworthy in regard to the link between the duration of PKC α activity, ERK signaling, and maintenance of cell growth arrest/differentiation that K562 cells, which sustain ERK activity for >24 h and undergo differentiation in response to PMA, progressively accumulate PKC α protein (but not PKC βII) over 24 h of phorbol ester treatment (7). It should be noted that the ability to activate the ERK cascade is widespread among individual members of the PKC family (11, 42, 66–68), and evidence has been presented for isozyme-specific mechanisms of activation of this pathway (11). In IEC-18 cells, there appears to be some specificity in the ability of PKC α to signal prolonged activation of the ERK cascade and induction/maintenance of cell cycle arrest.

The idea that the duration of both the input signal and of downstream ERK activation is critical in determining the length of PKC agonist-induced growth arrest was confirmed by pharmacological inhibition of the activity of PKC α and ERK1/2 at various times before and after treatment with PMA. These studies clearly demonstrated that a more prolonged cell cycle blockade requires a more sustained stimulation of both signals. Thus, while <30 min of PKC α and ERK activity produces growth arrest at 6 h, at least 60 min of continued signaling is required to sustain growth inhibition at 9 h. These findings are consistent with evidence that, in Bryo-treated cells, a noticeable decline in levels of ERK activity by 45 min of treatment, coincident with detectable desensitization of PKC α signaling, is associated with reversal of cell cycle arrest by ~8–9 h.

Immunofluorescence analysis of the subcellular distribution of phospho-ERK1/2 in IEC-18 cells provided important insight into the temporal and positional requirements for ERK signaling in PKC-induced growth arrest. First, these studies revealed major differences in the localization of ERK activity induced by signals promoting cell proliferation or cell growth arrest. Whereas serum-induced ERK activity was retained in the cytoplasm, PMA- and Bryo-induced phospho-ERK transiently accumulated in the nucleus. Thus, as noted in other systems (21, 65, 69, 70), entry of the active kinases into the nucleus may be critical to initiate transcriptional events that are required for intestinal epithelial growth arrest. Second, our data with PMA and Bryo indicate that the ability to maintain growth arrest may be dependent on the strength and duration of nuclear ERK activity. Whereas both PMA and Bryo promoted nuclear accumulation of active ERK in IEC-18 cells, the magnitude and kinetics of enzyme redistribution by the two agents were significantly different. Thus, coincident with a more complete and prolonged growth arrest, PMA treatment resulted in more rapid nuclear translocation of phospho-ERK1/2 and longer nuclear retention of the activated enzyme; the effect produced by PMA was, therefore, more pronounced than that seen in Bryo-treated cells. Importantly, however, the nuclear accumulation seen with both agents was lost in the majority of cells by ~1–1.5 h of treatment, with the remaining phospho-ERK detected only in the cytoplasm (high levels in PMA-treated cells and very low levels in cells treated with Bryo). The timing of the disappearance of PMA-induced nuclear ERK activity in IEC-18 cells coincided with the time of ERK activation required to sustain growth arrest for 9 h; thus, the cytoplasmic ERK activity remaining after 1 h of treatment does not appear to be required to maintain growth arrest by this agent. Because PMA treatment inhibits cell cycle progression for ~12 h in IEC-18 cells, the ~1–1.5 h of nuclear ERK activation appears to be sufficient to sustain growth arrest for 11–12 h in this system (a time that is in keeping with the 2-h ERK activation required for nerve growth factor-induced differentiation of PC-12 cells (71)). The data also suggest that more prolonged nuclear accumulation of phospho-ERK would lead to more sustained growth arrest in this system, a notion that will be addressed in future studies.

The loss of nuclear ERK activity and the subsequent sustained cytoplasmic activity in PMA-treated IEC-18 cells likely reflects the combined activities of continued PKC α input signal together with negative feedback control mechanisms such as expression of nuclear MAP kinase phosphatases (e.g., MKP-1) and alterations in nuclear transport functions that act to prevent nuclear accumulation of the enzyme (17, 19, 52, 53). Protection of cytoplasmic ERK activity may reflect transient induction/down-regulation of cytoplasmic ERK phosphatases (53), as seen with MKP-1. The fact that the duration of nuclear ERK activation appears to be important in PKC-mediated growth arrest in IEC-18 cells is in keeping with the idea that the ultimate cellular response to ERK signaling is not only dependent on induction of immediate early proteins but also on whether activation of the pathway is prolonged enough to allow ERK to subsequently directly modify and stabilize these proteins (70).

The present study also examined the ability of PKC signaling to activate upstream regulators of ERK1/2 activity. There has been some controversy in the literature (for example, see Refs. 42–49) regarding the ability of PKC signaling to activate Ras. Our study confirms the ability of PMA to promote PKC-dependent GTP loading of Ras and, to our knowledge, provides the first evidence that Bryo can also produce this effect. Both PMA and Bryo were shown to activate Ras in PKC δ/ε-depleted cells, indicating that PKC α alone is capable of promoting Ras GTP loading in this system. Notably, the duration of Ras activation by these agents correlated with their ability to sustain PKC α and ERK1/2 activity, as well as to maintain IEC-18 cell cycle arrest. Both PMA and Bryo also activated Raf in IEC-18 cells, albeit with markedly different kinetics. While PMA-induced Raf activity was sustained for at least 75 min, Bryo-induced Raf activity had significantly declined by this time. Similar findings have been reported with these agents in LNCaP cells (51). Thus, following PMA or Bryo treatment, the duration of PKC-induced Raf activation correlated with the duration of both Ras and ERK activity, further emphasizing a link between these events. A recent report (72) points to an additional mechanism of PKC regulation of the Raf/ERK signaling cascade that could be involved in the effects of PKC agonists in IEC-18 cells. Classical and atypical, but not novel, PKC isozymes were shown to phosphorylate Raf kinase inhibitory protein at serine 153 causing its release from Raf-1. Whether this mechanism participates in induction and/or maintenance of ERK activity by PKC signaling in IEC-18 cells remains to be determined.

To our knowledge, the data presented in the current report are the first to demonstrate a link between PKC α activity, ERK signaling, and cell cycle arrest in nontransformed intestinal epithelial cells. The finding that the negative growth regulatory effects of PKC/PKC α signaling in IEC-18 cells require strong/sustained activation of the ERK cascade provides additional support for the paradigm that prolonged activation
of this pathway can play a key role in controlling cell cycle arrest/cell differentiation. Prolonged activity of Ras (73, 74), Raf-1 (61, 62, 75), and/or ERK (10) has been associated with negative growth regulatory events in a number of systems. ERK activity is also critical for certain developmental processes including photoreceptor cell specification and anterior-posterior patterning in Drosophila melanogaster, maturation of vulval precursor cells in Caenorhabditis elegans, and T cell selection (10). Notably, several recent studies point to the importance of ERK signaling in enterocyte growth arrest and maturation. Significant levels of ERK activity are maintained in the nucleus of differentiated enterocytes and appear to be required for G1 arrest and expression of differentiation-related genes in this tissue (14). Sustained activation of the ERK pathway is also required for TNF-α-stimulated intestinal epithelial cell cycle arrest, and TNF-α can be converted from an antiproliferative to a proliferative ligand by manipulating the duration of ERK activation (15). Expression of the v-rasH oncogene in CaCo-2 colon carcinoma cells resulted in induction of multiple differentiation markers (i.e., sucrose, alkaline phosphatase, and transforming growth factor-β), a finding consistent with evidence that Ha-ras is highly expressed in most differentiated cells of the intestinal mucosa (16). These findings, together with evidence that PKC δ is activated coincident with growth arrest within intestinal crypts and that its activity is sustained along the length of the villus (5, 24), suggest that PKC δ and ERK signaling play an important role in mucosal self-renewal in situ. Future studies will determine the mechanisms underlying Ras activation by PKC signaling as well as downstream targets of ERK activity in this system.

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