A novel antiproliferative PKCα-Ras-ERK signaling axis in intestinal epithelial cells

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We have previously shown that the serine/threonine kinase PKCα triggers MAPK/ERK kinase (MEK)-dependent G₁→S cell cycle arrest in intestinal epithelial cells, characterized by downregulation of cyclin D1 and inhibitor of DNA-binding protein 1 (Id1) and upregulation of the cyclin-dependent kinase inhibitor p21Cip1. Here, we use pharmacological inhibitors, genetic approaches, siRNA-mediated knockdown, and immunoprecipitation to further characterize antiproliferative ERK signaling in intestinal cells. We show that PKCα signaling intersects the Ras-Raf-MEK-ERK kinase cascade at the level of Ras small GTPases and that antiproliferative effects of PKCα require active Ras, Raf, MEK, and ERK, core ERK pathway components that are also essential for pro-proliferative ERK signaling induced by epidermal growth factor (EGF). However, PKCα-induced antiproliferative signaling differs from EGF signaling in that it is independent of the Ras guanine nucleotide exchange factors (Ras-GEFs), SOS1/2, and involves prolonged rather than transient ERK activation. PKCα forms complexes with A-Raf, B-Raf, and C-Raf that dissociate upon pathway activation, and all three Raf isoforms can mediate PKCα-induced antiproliferative effects. At least two PKCα–ERK pathways that collaborate to promote growth arrest were identified: one pathway requiring the Ras-GEF, RasGRP3, and H-Ras, leads to p21Cip1 upregulation, while additional pathway(s) mediate PKCα-induced cyclin D1 and Id1 downregulation. PKCα also induces ERK-dependent SOS1 phosphorylation, indicating possible negative crosstalk between antiproliferative and growth-promoting ERK signaling. Importantly, the spatiotemporal activation of PKCα and ERK in the intestinal epithelium in vivo supports the physiological relevance of these pathways and highlights the importance of antiproliferative ERK signaling to tissue homeostasis in the intestine.

Self-renewal of the epithelial lining of the intestine requires precise coordination of rapid cell proliferation in the crypt with growth arrest at the crypt/villus junction (1, 2). Previous studies have determined that signaling downstream of extracellular signal-regulated kinase (ERK) 1 and 2 plays a central role in this process. Ligands such as epidermal growth factor (EGF), secreted by Paneth cells and mesenchymal cells, lead to activation of ERK in intestinal crypt cells (3). Multiple studies have established a growth stimulatory role for the ERK pathway in the intestinal epithelium. For example, inhibition of ERK signaling prevents cell cycle progression/proliferation in IEC-6 nontransformed intestinal epithelial cells, Caco-2 colon cancer cells (3), as well as Lgr5⁺ stem cells and transit amplifying cells in mouse intestinal organoids (4). However, evidence also points to important postmitotic roles of ERK signaling in the intestinal epithelium. The high levels of active ERK seen in proliferating cells of the crypt are maintained in nonproliferating cells of the villus in fetal and adult intestine (3, 5). In vitro, sustained activation of ERK correlates with G₁ arrest and is required for differentiation in postconfluent Caco-2 cells (3, 6). In vivo, deletion of ERKs in the murine intestinal epithelium leads to loss of intestinal epithelial function, increased proliferation, and crypt elongation (7, 8), pointing to a defect in growth arrest at the crypt–villus junction. At a mechanistic level, our previous studies have determined that activation of protein kinase C α (PKCα) induces a program of cell-cycle withdrawal in intestinal epithelial cells that involves sustained activation of ERK (9–11). Thus, studies in the intestinal epithelium have determined that, in addition to the well-established role of ERK signaling in supporting cell proliferation, maintenance of intestinal homeostasis requires antiproliferative ERK signaling that can be triggered by PKCα.

ERK signaling plays a major role in transmission of extracellular signals that regulate fundamental cellular processes such as proliferation, differentiation, development, stress responses, and apoptosis (12). Canonical ERK activation involves a signaling cascade in which membrane anchored Ras small GTPases (H-Ras, K-Ras, and N-Ras) recruit and activate Raf kinases (A-Raf, B-Raf, and C-Raf), which then phosphorylate and activate MEKs (MEK1 and MEK2); MEKs, in turn, phosphorylate and activate ERKs (ERK1 and ERK2) (13–16). Activity of this cascade is governed by cycling of the Ras GTPases between the inactive GDP-bound state and the active GTP-bound state. This process is predominantly regulated by Ras GTPase–activating proteins (Ras-GAPs) and Ras guanine nucleotide exchange factors (Ras-GEFs), with GEFs thought to play the major role in activation of Ras signaling (17, 18). Mammalian cells express three classes of Ras-GEFs: SOS

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proteins (SOS1, SOS2), the RasGRFs (RasGRF1 and RasGRF2), and the RasGRPs (RasGRP1, RasGRP3, and RasGRP4). Following pathway activation, ERK can phosphorylate multiple downstream targets in the cytoplasm and nucleus, including kinases such as RSK that further propagate the signal (19). This wide range of substrates allows ERKs to carry out their multiple roles in the cell. Several mechanisms have been implicated in directing ERK signaling to a particular physiological outcome, including signal strength and duration, scaffolding proteins, subcellular localization, and interaction with other signaling pathways (19). However, in most cases, these mechanisms remain poorly defined.

PKCa is a member of the classical subgroup (cPKCs) of the PKC family of serine/threonine kinases (20–22). Physiological activation of PKCa is calcium dependent and driven by membrane accumulation of diacylglycerol (DAG), generated by phospholipase C downstream of ligand binding to receptor tyrosine kinases (RTKs) or G protein–coupled receptors (20–22). DAG recruits PKCa to the membrane, leading to a conformational change that results in activation of the kinase (20, 21). In addition to physiological activation, membrane recruitment and activation of PKCa can be achieved experimentally using short chain DAGs, such as 1,2-dioctanoyl-sn-glycerol (DiC8), or pharmacological agents such as phorbol 12-myristate 13-acetate (PMA) (22–24).

Immunohistochemical analysis of intestinal tissue by our group demonstrated that PKCa is activated precisely at the point of growth arrest, as indicated by plasma membrane translocation of the enzyme at the crypt/villus junction (9, 25, 26). In vitro analysis using nontransformed intestinal epithelial cells confirmed that PKCa activation triggers a program of cell cycle withdrawal, involving downregulation of pro-proliferative cyclin D1 and inhibitor of DNA-binding 1 (Id1) and upregulation of the cyclin-dependent kinase (CDK) inhibitor p21Cip1 (9, 10, 26–28). Notably, these effects are dependent on MEK activation (11), indicating that PKCa initiates growth inhibitory ERK signaling in intestinal epithelial cells. While it has long been recognized that PKC signaling can activate the ERK pathway, there is controversy regarding the mechanism involved. While some studies have indicated that PKC activation of the pathway is Ras independent (29) and can be mediated by direct phosphorylation of C-Raf by PKCa (30), other studies indicate that Ras is required (31). Previous studies also support the ability of classical PKCs to activate ERK through a mechanism that does not require Raf phosphorylation (32) and that PKCa may directly activate MEK (33). Thus, mechanisms underly- ing PKCa-mediated activation of ERK remainder to be elucidated.

In the present study, we define crosstalk between PKCa and the ERK activation cascade during growth suppressive signaling in intestinal epithelial cells. Our analysis has determined that PKCa intersects the canonical ERK activation pathway at the level of Ras and that PKCa triggers at least two antiproliferative ERK signaling pathways. One pathway involves activation of RasGRP3 and H-Ras for ERK-dependent induction of p21Cip1, while at least one other PKCa–Ras–ERK pathway triggers downregulation of the pro-proliferative proteins, cyclin D1 and Id1.

Results

Activation of PKCa by PMA induces ERK-dependent antiproliferative signaling in intestinal epithelial cells

We have previously reported that pharmacological activation of PKCa in nontransformed IEC-18 intestinal crypt-like cells induces cell cycle arrest in association with activation of the ERK signaling pathway, as determined using antiphospho-ERK (pERK) immunoblotting (ref. (11) and Fig. 1A). Phosphorylation of the ERK substrate RSK in PMA-treated IEC-18 cells confirmed functional activation of ERK, an effect that was blocked by the selective ATP-competitive ERK inhibitor, SCH772984 (Fig. 1A). To determine the involvement of ERK in the antiproliferative activity of PKCa, we tested the effects of SCH772984 on PMA/PKCa-induced cell cycle arrest and downstream cell cycle regulatory targets. Treatment of IEC-18 cells with PMA for 6 h led to G1→S arrest, as indicated by a 50% increase in the percentage of cells in G1-phase and a >70% reduction in the percentage of cells in S-phase (Fig. 1B). Consistent with a role for pro-proliferative ERK signaling in intestinal epithelial cells, ERK inhibition with SCH772984 led to a modest decrease (~30%) in cells in S-phase. However, the ERK inhibitor also abrogated PMA/PKCa-induced G1→S arrest in these cells (Fig. 1B, right panels). PKCa-induced growth inhibition is associated with downregulation of the pro-proliferative proteins, cyclin D1 and Id1, and upregulation of the CDK inhibitor p21Cip1 (refs. (9, 26) and Fig. 1A). Notably, SCH772984 also blocked the ability of PMA to modulate the expression of these proteins (Fig. 1A), confirming that the effects of PKCa on these downstream molecules are dependent on ERK activity.

To further characterize PKCa-triggered antiproliferative ERK signaling, we compared PMA/PKCa-induced ERK activation with ERK activation induced by a known growth stimulatory factor, EGF. To avoid confounding effects of serum growth factors in the medium, these experiments were performed under serum-starved conditions (0.5% serum). As shown in Figure 1C1, EGF and PMA induced comparable levels of ERK phosphorylation in IEC-18 cells. Follow-up experiments tested the requirement for PKC activity in ERK activation by EGF and PMA using bisindolylmaleimide (BIM I), a pan-PKC inhibitor, or Gö6976, an inhibitor that selectively targets the cPKCs (PKCa, β, δ, and γ). The only PMA-responsive PKC isozymes expressed in IEC-18 cells are PKCa, PKCd, and PKCe (10, 11, 25); thus, Gö6976 selectively inhibits PKCa in these cells. The selectivity of Gö6976 for PKCa was confirmed by its ability to block PKC agonist (PMA) induced PKCa downregulation, which is dependent on PKCa activity, while not affecting the activity-dependent downregulation of PKCd or PKCe (Fig. 1Cii). Neither BIM I nor Gö6976 affected the ability of EGF to activate ERK, indicating that PKC activity is not involved in this effect of EGF (Fig. 1D). In contrast, Gö6976 largely prevented the increase in phospho-ERK induced by PMA (Fig. 1C1).
Differences were also observed in the duration of ERK activation elicited by PMA/PKC\(\alpha\) and EGF; while EGF-induced ERK activity returned to basal levels by 15 to 30 min, the ERK signal induced by PMA/PKC\(\alpha\) was sustained for longer than 2 h (Fig. 1E). We have previously demonstrated that sustained activation of ERK is required for the antiproliferative effects of PKC\(\alpha\) activation but not for pro-proliferative growth factor signaling (11). While 15 to 30 min of ERK activation is sufficient to promote proliferation in response to serum growth factors, we showed that 30 to 60 min of PKC\(\alpha\)-ERK signaling is required for G1→S phase arrest at 6 h, and maintenance of PKC\(\alpha\)-induced cell cycle blockade at 9 h requires continued PKC\(\alpha\)-ERK signaling for 60 to 90 min (11). Together, the data indicate that PKC\(\alpha\) activates antiproliferative ERK signaling that is qualitatively and temporally distinct from the canonical pro-proliferative ERK axis induced by growth factors.

**PKC\(\alpha\)-induced growth suppressive ERK signaling requires MEK, RAF, and Ras activity**

The canonical ERK signaling cascade involves sequential activation of Ras, Raf, and MEK (12), which stimulates ERK by phosphorylation. Subsequent experiments, therefore, investigated the requirement for canonical ERK pathway components in the antiproliferative effects of PKC\(\alpha\) activation. As seen with ERK inhibition using SCH772984, blockade of ERK activation using the MEK inhibitor, U0126, led to a 30% to 35% reduction in cells in S-phase. Consistent with our previous findings (11), U0126 also prevented PMA/PKC\(\alpha\)-induced G1→S-phase arrest (Fig. 2A, right panels). Further confirmation of the role of MEK in antiproliferative PKC\(\alpha\)-ERK signaling (11, 26) was provided by the ability of the second generation MEK1/2 selective inhibitor, PD0325901, to block PMA-induced ERK activation, downregulation of cyclin D1 and Id1, and upregulation of p21\(^{Cip1}\) (Fig. 2B).
To investigate if growth suppressive ERK signaling requires Raf activity, we used the pan-Raf inhibitor LY3009120, an ATP-competitive inhibitor that binds all Raf isoforms with the same affinity (34, 35). Inhibition of Raf activity by LY3009120 abrogated PMA/PKCα-induced G1 → S phase arrest (Fig. 2C), activation of ERK, and alterations in the expression of cyclin D1, Id1, and p21Cip1 (Fig. 2D). PMA treatment also led to a mobility shift in C-Raf on SDS-PAGE, suggestive of effects on C-Raf phosphorylation (Fig. 2E). Changes in activating and inhibitory phosphorylation of C-Raf (36) were, therefore, analyzed using phospho-specific antibodies, which detected a PMA-induced increase in activating phosphorylation at S338 and a reduction in inhibitory phosphorylation at S259 (Fig. 2Ei). Importantly, the change in C-Raf mobility was inhibited by Gö6976 (Fig. 2Eii), indicating that PMA-induced Raf activation is part of the same antiproliferative, PKCα-dependent pathway identified previously. Thus, as with growth promoting ERK pathways, PKCα-triggered growth suppressive ERK signaling requires MEK activity downstream of Raf activation.

To assess the role of Ras proteins in PKCα-induced activation of ERK, we tested the effects of salirasib, a farnesylcysteine mimetic that blocks membrane association of activated Ras proteins (37). As also shown in Figure 1C, both EGF and PMA/PKCa strongly activated ERK in serum-starved IEC-18 cells (Fig. 3A). As expected, salirasib abrogated EGF-induced activation of ERK, which is known to be dependent on Ras activity (38). Salirasib also blocked the ability of PMA to activate ERK (Fig. 3, A and B) and to modulate the expression of cyclin D1, Id1, and p21Cip1 (Fig. 3B), pointing to a requirement for Ras activity in PKCα-induced growth suppressive ERK signaling. Since salirasib can have off target effects (39–42), the requirement for Ras in PKC agonist-induced activation of ERK was further tested using “Rasless” N-Ras−/−; H-Ras−/−; K-RasD12; and Ubq-CreERT2 mouse embryonic stem cells (mESCs). Additional validation was particularly important because there has been considerable confusion in the field regarding the role of Ras in transducing signals from PKC to ERK, with some studies indicating that PKC-mediated activation of Rafs is Ras independent (29, 30, 33, 43). These mESCs
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The scaffold proteins KSR1 and KSR2 are not required for PKCa-mediated antiproliferative ERK signaling

The ERK scaffolding proteins, KSR1 and KSR2, play an important role in modulation of growth promoting and oncogenic ERK signaling and have been implicated in promoting ERK-dependent differentiation (46, 47). Therefore, the role of these scaffolding proteins in PKCa-induced growth inhibitory signaling was examined. Knockdown of KSR1 in IEC-18 cells did not affect the ability of PMA to induce a reduction of cells in S-phase (Fig. 4, A and B), indicating that KSR1 is not required for PKCa/ERK-mediated G1→S arrest in these cells. Similarly, KSR1 knockdown did not prevent PMA-induced downregulation of cyclin D1 and Id1 or upregulation of p21Cip1 (Fig. 4C). Since KSR2 is not expressed in IEC-18 cells (Fig. 4D), these data indicate that PKCa-induced growth inhibitory Ras-ERK signaling is not regulated by KSR scaffolding proteins.

Association of PKCa with ERK signaling pathway components

Having excluded a role for KSR scaffolds in regulation of growth suppressive ERK signaling, we examined the ability of PKCa to form complexes with components of the ERK pathway. Western blot analysis of PKCa immunoprecipitates using an antibody that recognizes all Ras isoforms failed to detect interaction between PKCa and Ras, either before or after activation of growth-inhibitory signaling with PMA (Fig. 5A). Similar analysis also failed to detect interaction between PKCa and MEK, total ERK, or pERK in unstimulated or PMA-treated cells (Fig. 5A).

Mammalian cells express three Raf isoforms, A-Raf, B-Raf, and C-Raf (48, 49). In contrast to the lack of interaction between PKCa and Ras, MEK, or ERK, immunoprecipitation experiments clearly detected association of both B-Raf and C-Raf with PKCa in unstimulated cells (Fig. 5B). These interactions were confirmed in reciprocal experiments in which B-Raf or C-Raf immunoprecipitates were probed for the presence of PKCa (Fig. 5C). Notably, PMA treatment reduced the interaction of PKCa with B-Raf and C-Raf by 10 min (Fig. 5, B and C), indicating that activation of growth-inhibitory ERK signaling is

can be used for this analysis because they express PKCa at equal levels before and after KO of K-Ras (Fig. 3C). N-Ras+/−; H-Ras+/−; K-Rasfllox, and Ubiq-CreERT2 mESCs are knocked out for H-Ras and N-Ras and allow for conditional KO of floxed K-Ras following activation of CreERT2; thus, they lack expression of all three Ras isoforms following treatment with 4-hydroxytamoxifen (4-OHT) (ref. (44) and Fig. 3C). As expected based on the established role of Ras in growth factor–induced ERK activation, complete loss of Ras in these cells is reflected in loss of basal ERK and downstream RSK phosphorylation (Fig. 3C). Prior to KO of K-Ras, PMA treatment readily increased ERK activation in these mESCs. A similar effect was seen with the short chain DAG, DiC8, a more physiological PKC/PKCα agonist that mimics endogenous DAG signaling (45) and elicits the same physiological responses as phorbol esters in IEC-18 cells (9, 11, 26). However, following KO of K-Ras to generate “Rasless” cells, neither PMA nor DiC8 was able to induce ERK phosphorylation (Fig. 3C), confirming a requirement for Ras in PKC agonist-induced ERK signaling. Collectively, the data indicate that PKCa-activated growth-inhibitory ERK signaling, like growth-promoting ERK signaling, is mediated by activation of Ras in addition to Raf and MEK.

Figure 3. PKCa-induced ERK activation and cell cycle arrest requires the activity of Ras. A, serum-starved IEC-18 cells were pretreated with vehicle (−) or 50 μM salirasib for 2 h, followed by 50 ng/ml EGF or 100 nM PMA for 10 min as indicated, and analyzed by Western blotting. B, cells were treated with PMA for 2 h in the absence or presence of salirasib and subjected to immunoblot analysis for the indicated proteins. C, N-Ras+/−; H-Ras+/−; K-Rasfllox, and Ubiq-CreERT2 mESCs were treated with vehicle (−) or 1 μM 4-hydroxytamoxifen (4-OHT) for 7 days to knock out K-Ras and generate “Rasless” mESCs. The cells were then treated with 100 nM PMA or 20 μg/μl DiC8 for 15 min as indicated, prior to Western blot analysis for PKCa, Ras, pERK, and pRSK. The data are representative of three independent experiments. mESC, mouse embryonic stem cell; PMA, phorbol 12-myristate 13-acetate.
Reciprocal immunoprecipitation experiments also consistently detected interaction between A-Raf and PKCα in unstimulated cells, although the signal was weaker than for the other Rafs (Fig. 5, B and C). It was not possible to determine the effects of PKCα activation on the strength of this interaction because of the ability of PMA to increase the levels of A-Raf in the lysates used for immunoprecipitation (Fig. 5, B and C, input). This increase, which occurred by 10 min of PMA treatment, was not seen in whole-cell lysates obtained using SDS solubilization buffer (e.g., Fig. 6) and, therefore, does not reflect an increase in A-Raf expression. Instead, the effect reflects increased solubility of PKCα-stimulated A-Raf in the NP-40/Igepal-630 containing buffer used for immunoprecipitation. While the mechanism(s) underlying the increased solubility of A-Raf remain to be determined, these data demonstrate that PKCα forms a complex with all three Raf isoforms and that, at least for B-Raf and C-Raf, these complexes dissociate during activation of growth-suppressive Ras-ERK signaling.

**Raf proteins have redundant functions in PKCα-induced growth inhibitory ERK signaling**

The three Raf isoforms function as homodimers and/or heterodimers to regulate downstream signaling (49). To determine if PKCα-induced antiproliferative ERK signaling is specified by differential activation of individual Raf isoforms and/or formation of specific Raf homodimers or heterodimers, siRNA knockdown experiments were performed. Single knockdown of A-Raf, B-Raf, or C-Raf did not affect the ability of PMA to activate ERK, downregulate cyclin D1 or Id1, or upregulate p21Cip1 (Fig. 6A), excluding a requirement for a specific Raf isoform or heterodimer for these effects. To test if specific Raf homodimer(s) mediate the effect, simultaneous knockdown of all possible Raf pairs was performed, effectively leaving the cells with only A-Raf, B-Raf, or C-Raf (Fig. 6A) (note that simultaneous knockdown of all three Raf isoforms was not possible due to toxicity). Interestingly, dual Raf knockdown also failed to block the effects of PMA treatment on cyclin D1, Id1, or p21Cip1 (Fig. 6A). Together, the data point to (a) redundancy in the ability of Raf isoforms to mediate growth suppressive ERK activation and (b) the ability of Raf homodimers to mediate PKCα-induced growth-suppressive signaling.

The ability of all Raf isoforms to mediate PMA/PKCα-induced antiproliferative ERK signaling was further supported by analysis of Raf heterodimer formation in immunoprecipitation experiments. Analysis of C-Raf immunoprecipitates revealed that PMA/PKCα promotes the association of C-Raf with both A-Raf and B-Raf (Fig. 6B, C-Raf IP right panel). A similar increase in C-Raf–A-Raf and B-Raf–A-RAF complexes following PMA treatment was detected in A-Raf immunoprecipitates (Fig. 6B, A-Raf IP left panel). Interpretation of the A-Raf immunoprecipitation results is complicated by the
ability of PKCα/PMA to increase the levels of A-Raf in lysates used for immunoprecipitation (see previous text). Nonetheless, these data indicate that PMA/PKCα increases the levels of A-Raf containing heterodimers in Igepal 630-soluble compartments. Although technical difficulties precluded analysis of B-Raf immunoprecipitates by Western blotting, the data collectively indicate that PMA/PKCα induces the formation of A-Raf/B-Raf, A-Raf/C-Raf, and B-Raf/C-Raf heterodimers and that there is redundancy in the ability of Raf isoforms and dimers to mediate PKCα-induced growth-suppressive ERK signaling. Thus, selective Raf activation does not appear to specify growth-suppressive ERK signaling pathways.

**Role of Ras guanine exchange factors in PKCα-induced growth inhibitory ERK signaling**

SOS1 and SOS2 are not required for PKCα-induced activation of the Ras-ERK pathway

Having established that PKCα signaling intersects the ERK pathway at Ras, we investigated the role of Ras-GEFs in PKCα-induced growth-inhibitory ERK activation. Since RNA-Seq analysis indicated that IEC-18 cells do not express RasGRFs (Table 1), these studies focused on SOS and RasGRP Ras-GEFs (50). Consistent with the involvement of SOS1 and SOS2 in growth-promoting signaling (51), combined knockdown of SOS1 and SOS2 markedly reduced the expression of cyclin D1 and Id1 (Fig. 7, A and B). However, single or double knockdown of SOS1 and SOS2 failed to prevent PMA-induced ERK activation (Fig. 7A) and did not affect the ability of PMA/PKCα to further downregulate cyclin D1 and Id1 or to upregulate p21Cip1 (Fig. 7B), indicating that PKCα induces growth-inhibitory Ras-ERK signaling independently of SOS1 Ras-GEFs.

PKCα signaling may promote ERK-dependent feedback inhibition of SOS1

While our analysis identified SOS1/2-independent growth suppressive PKCα-ERK signaling, the data did not exclude the possibility that antiproliferative PKCα-ERK signaling may also intersect growth-promoting ERK pathways through inhibition

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**Figure 5. Interaction of PKCα with components of the RAS-ERK pathway.** A and B, IEC-18 cells were treated with vehicle (C) or 100 nM PMA (P) for 10 min prior to lysis and immunoprecipitation (IP) of protein complexes using a monoclonal anti-PKCα antibody (PKCα IP). Immunoprecipitates were analyzed for the presence of the indicated proteins by Western blotting. Input represents 10% of the lysate used for immunoprecipitation. C, as in (A) except that complexes were immunoprecipitated with antibodies against A-Raf, B-Raf, or C-Raf as indicated. M: Mock immunoprecipitation using lysis buffer instead of cell extracts to detect antibody-derived nonspecific bands. NS: Nonspecific band. Data are representative of at least three independent experiments.
of SOS proteins. ERK-dependent phosphorylation of SOS1 is a major mechanism for negative feedback of growth factor–induced Ras-ERK signaling. ERK phosphorylates SOS1 on Ser1132, Ser1167, Ser1178, and Ser1193 in the C-terminal domain, which inhibits SOS1 function by reducing its association with Grb2, Shc, and the EGF receptor (51–53). Negative feedback of Ras stimulation can also result from ERK → Rsk-mediated phosphorylation of SOS1 (e.g., on Ser1134 and Ser1193) (54, 55). Follow-up studies, therefore, examined the ability of PMA/PKCα to induce ERK-dependent inhibitory phosphorylation of SOS1/2 (51). Since antibodies targeting these phosphosites are not commercially available, we used Phos-tag gels for the analysis. These SDS-polyacrylamide gels contain phosphate-binding metal chelate complexes that specifically retard migration of phosphorylated proteins during electrophoresis (56). While PMA/PKCα had no effect on SOS2 migration in Phos-tag gels (data not shown), PMA treatment induced a PKCα-dependent change in SOS1 phosphorylation, indicated by a mobility shift that was prevented by the PKCα selective inhibitor Gö6976 (Fig. 7Ci) and abrogated by λ-phosphatase treatment (Fig. 7Cii). Notably, PMA-induced SOS1 phosphorylation was also prevented by inhibition of ERK with SCH772984, indicating that the effect is mediated by ERK (Fig. 7Ciii). Since ERK-induced phosphorylation of SOS leads to downregulation of growth factor signaling (57), these data point to the possibility that PKCα can dampen pro-proliferative signaling in intestinal cells by promoting ERK-mediated inhibitory phosphorylation of SOS1. However,
it is clear that the activation of antiproliferative Ras-ERK by PKCα is SOS1 independent, as further supported by the ability of PMA to induce prolonged (>2 h) phosphorylation of ERK in cells with SOS1 knockdown (Fig. 7Civ).

PKCα-induced activation of the Ras-ERK pathway is partially dependent on RasGRP3

IEC-18 cells do not express RasGRP1 or RasGRP4 (Table 1 and Fig. 8Ai), but RasGRP2 and RasGRP3 were detected in these cells (Table 1 and Fig. 8Aii). Since RasGRP2 does not activate Ras (58), we focused our analysis on RasGRP3. In contrast to SOS1/2, siRNA-mediated knockdown of RasGRP3 consistently reduced the level of ERK activation seen following PMA treatment (Figs. 8, Aii, iii and B and 9Ai), indicating that this GEF is partially responsible for mediating PKCα activation of ERK. RasGRP3 deficiency reduced the ability of PKCα to induce ERK activation at early times (15 min) (Fig. 8Aii) and to sustain ERK activity at 2 h (Fig. 8Aiii), although ERK activity was still elevated relative to control cells at this later time. While RasGRP3 knockdown inhibited ERK activation by PMA/PKCα, the absence of this GEF did not affect ERK activation by EGF (Fig. 8B), supporting a role for RasGRP3 in antiproliferative rather than growth-promoting ERK signaling in intestinal epithelial cells. As mentioned previously, RasGRP1 is not expressed in control IEC-18 cells and a compensatory role for RasGRP1 that has been suggested in other systems (59, 60) was excluded since (a) Western blot analysis failed to detect RasGRP1 in control or RasGRP3 knockdown cells and (b) RasGRP1 siRNA, either alone or in combination with RasGRP3 knockdown, did not affect the ability of PMA to activate ERK (Fig. 8Aii).

Table 1

| Gene Name | Expression relative to Gapdh ($\times 10^3$) |
|-----------|------------------------------------------|
| Sos1      | 3.27                                     |
| Sos2      | 3.20                                     |
| Rasgrp1   | 0.04                                     |
| Rasgrp2   | 0.53                                     |
| Rasgrp3   | 4.13                                     |
| Rasgrf1   | 0.00                                     |
| Rasgrf2   | 0.00                                     |
| Hras      | 18.03                                    |
| Kras      | 11.73                                    |
| Nras      | 27.25                                    |

Figure 7. Role of SOS1/2 RasGEFs in PKCα-induced growth inhibitory ERK signaling. A and B, IEC-18 cells were transfected with nontargeting (NT) siRNA or siRNA targeting SOS1 and/or SOS2 as indicated. After 48 h, cells were treated with 100 nM PMA for 15 min (A) or 2 h (B) and analyzed for the indicated proteins by Western blotting. The graph to the right of the blots in (A) shows densitometric analysis of relative levels of pERK normalized to loading control ([SD, n = 3. *p ≤ 0.05; **p ≤ 0.01]. All data in (B) are from single immunoblots; the vertical lines indicate rearrangement of the blot for clarity. C, i, IEC-18 cells were pretreated with vehicle (--) or 4 μM Go6976 to inhibit PKCα activity, followed by 15 min treatment with vehicle (--) or 100 nM PMA as indicated. Lysates were run on Phos-tag gels and analyzed for SOS1 by immunoblotting. ii, IEC-18 cells were treated with 100 nM PMA for 15 min. Cells were then lysed, and half of each lysate was treated with lambda phosphatase (APP) as indicated before being subjected to Phos-tag gel analysis. iii, Phos-tag gel analysis of cells treated with PMA in the absence or presence of the ERK inhibitor SCH772984 as indicated. iv, as in (A), except that (NT) siRNA or SOS1 siRNA transfected cells were treated as indicated for 2 h. Data are representative of at least three independent experiments, except C(ii) and C(iii) which are representative of two independent experiments. PMA, phorbol 12-myristate 13-acetate.
Phosphorylation of RasGRP3 at Thr133 by conventional PKCs has been shown to be important for RasGRP3 activation in B cells (61, 62). Since a reliable reagent for detection of phospho-Thr133-RasGRP3 in rat cells is not available, we conducted Phos-tag gel analysis to determine if PMA/PKCα alters the phosphorylation of RasGRP3 in intestinal cells. Treatment of IEC-18 cells with PMA led to the appearance of a slower migrating RasGRP3 immunoreactive band following Phos-tag electrophoresis (Fig. 8, asterisk). This shift was inhibited in cells treated with Gö6976 and was not seen when the lysates were treated with λ-phosphatase; thus, these data indicate that activation of antiproliferative ERK signaling is associated with PKCα-induced phosphorylation of RasGRP3 (Fig. 8C).

As we have previously reported (11), PKCα signaling activates Ras in IEC-18 cells, as determined by Ras activity assays using GST-Raf1-RBD fusion protein to pull down the activated GTP-bound form of Ras (Fig. 8D). Importantly, active Ras pull-down assays confirmed a role for RasGRP3 in Ras activation, since knockdown of RasGRP3 reduced the level of activated Ras seen in PMA-treated cells (Fig. 8D).

RasGRP3 is partially responsible for PKCα-induced antiproliferative signaling in intestinal epithelial cells

Analysis of the effects of RasGRP3 knockdown revealed that distinct ERK-dependent mechanisms underlie the ability of...
PKCα to regulate pro-proliferative and antiproliferative proteins. While RasGRP3 knockdown had no effect on PMA-induced downregulation of cyclin D1 or Id1, knockdown of this Ras-GEF with two different siRNA reagents (OnTarget SMARTpool or Silencer Select) markedly impaired the ability of PMA to upregulate of p21<sup>Cip1</sup> (Fig. 9Ai–iv). These data indicate that induction of p21<sup>Cip1</sup> by PMA/PKCα is mediated by a RasGRP3-dependent ERK signaling pathway.

To confirm a role for RasGRP3 in growth-inhibitory PKCα-ERK signaling, we examined whether RasGRP3 knockdown inhibited the ability of PMA/PKCα to induce G<sub>1</sub>→S phase arrest, as determined by changes in the percentage of cells in S-Phase (Fig. 9B). Knockdown of RasGRP3 did not affect the level of S-phase in untreated cells and did not prevent the ability of PMA treatment to reduce the number of cells in S-phase, consistent with the presence of antiproliferative RasGRP3-independent PKCα signaling that channels through cyclin D1 and Id1. However, the growth-inhibitory effects of PMA/PKCα were significantly reduced by RasGRP3 deficiency, as indicated by a highly consistent increase in the proportion of S-phase cells in PMA-treated RasGRP3 knockdown cells compared with PMA-treated control cells (p = 0.003, n = 4; Fig. 9B). Thus, inhibition of the PKCα–RasGRP3–ERK–p21<sup>Cip1</sup> axis dampened the ability of PKCα signaling to induce G<sub>1</sub>→S phase arrest in intestinal cells. Together, the data indicate that there are at least two ERK signaling pathways that contribute to the growth-suppressive effects of PKCα in intestinal epithelial cells: a PKCα–RasGRP3–ERK signaling axis that induces upregulation of p21<sup>Cip1</sup> and additional RasGRP3-independent PKCα-ERK signaling module(s) that lead to downregulation of cyclin D1 and Id1. Consistent with the requirement for sustained ERK activity in PKCα-induced growth arrest, the RasGRP3-independent PKCα-ERK signaling module can also sustain enhanced ERK signaling for more than 2 h (Fig. 8Aiiii).

**H-Ras mediates PMA/PKCa-induced upregulation of p21<sup>Cip1</sup>**

To further characterize growth-suppressive ERK signaling, we next addressed the involvement of individual members of the Ras family. These studies focused on H-Ras based on evidence that this isoform is highly expressed in postmitotic/mature intestinal cells (63) and that H-Ras signaling reduces proliferation and induces the expression of differentiation markers (e.g., brush border hydrolases) in CaCo-2 colon cancer cells (3, 6). As shown in Figure 10A, active Ras pull-down assays confirmed that PMA/PKCa promotes GTP loading/activation of H-Ras in IEC-18 cells. siRNA-mediated knockdown of H-Ras in IEC-18 cells reduced ERK activation in response to PMA, confirmed by a reduction in phosphorylation of RSK (Fig. 10B). Thus, H-Ras appears to be partially responsible for activation of ERK by PKCα. Notably, PMA-induced H-Ras activation was dependent on RasGRP3, as indicated by the inability of PMA to activate H-Ras in RasGRP3 knockdown cells (Fig. 10A), pointing to a role for H-Ras in PKCα-induced p21<sup>Cip1</sup> upregulation. Remarkably, the effects of H-Ras knockdown on downstream proteins phenomenated those of loss of RasGRP3 on all growth regulatory targets analyzed: while no effect was seen on PMA-induced downregulation of cyclin D1 or Id1, PMA-induced upregulation of p21<sup>Cip1</sup> was abrogated in H-Ras knockdown cells (Fig. 10B). Together, these data further delineate growth-suppressive PKCα-ERK signaling by (a) indicating that upregulation of p21<sup>Cip1</sup> is mediated by RasGRP3-induced H-Ras activation and (b) determining that K-Ras and/or N-Ras can mediate downregulation of cyclin D1 and Id1.

**The growth inhibitory PKCα-RasGRP3-H-Ras-ERK-p21<sup>Cip1</sup> signaling module is also triggered by the PKCa agonist, DiC8, and is seen in other intestinal epithelial cells**

To exclude the possibility that the differential effects of RasGRP3 on downstream targets of growth-inhibitory PKCα-ERK signaling are restricted to phorbol ester treatment, we tested the effects of the PKC agonist, DiC8, a short chain DAG that, as discussed previously, mimics the effects of endogenous DAG and promotes PKCα-induced cell cycle arrest in IEC-18 cells (9, 10, 45). RasGRP3 knockdown reduced ERK activation and inhibited p21<sup>Cip1</sup> upregulation following DiC₈ treatment of IEC-18 cells, without affecting downregulation of cyclin D1 or Id1 (Fig. 11Ai, ii). DiC₈ also induced a slower migrating RasGRP3 band in Phos-tag gels (Fig. 11Aiii, asterisk) that was prevented by the PKCα-selective inhibitor G69676 and by λ-phosphatase, indicating that DiC₈ also recapitulates the effects of PMA on RasGRP3 phosphorylation. Finally, this short chain DAG recapitulated the ability of PMA to induce ERK-dependent phosphorylation of SOS1 (Fig. 11Av, cf. Fig. 7Ciii), demonstrating that none of the observed effects of PKCα activation are phorbol ester–specific but can also be elicited by this “physiological” agonist.

Importantly, the RasGRP3-dependent pathway that is required for upregulation of p21<sup>Cip1</sup>, but not downregulation of cyclin D1 or Id1, was also observed in PMA-treated IEC-6 cells (Fig. 11B), an independently derived nontransformed intestinal epithelial cell line (64, 65) that was previously used to demonstrate a role for growth-promoting ERK signaling in intestinal epithelial cells (3). Thus, the presence of a PKCα-induced RasGRP3–H-Ras–dependent growth-inhibitory ERK signaling axis that leads to upregulation of p21<sup>Cip1</sup>, as well as RasGRP3–H-Ras–independent ERK signaling module(s) that mediate PKCα-induced downregulation of cyclin D1 and Id1, appears to be a general characteristic of intestinal epithelial cells.

**Discussion**

In the present study, we have defined antiproliferative ERK signaling pathways downstream of PKCα using intestinal epithelial cells as a model. The finding that PKCα signaling intersects the ERK pathway by inducing the activation of Ras addresses longstanding confusion in the field regarding the requirement for Ras activity in PKC-mediated signaling to ERK. The dependence on Ras activity is consistent with findings from the Marshall group (31) showing that C-Raf activation by PKC requires the formation of Ras–GTP/C-Raf
complexes. Our data further exclude a role for direct activation of Raf \(^30\) or MEK \(^{33}\) in ERK activation by PKC\(\alpha\) in the models used in this study, while shedding new light on the involvement of individual Ras proteins in PKC\(\alpha\) signaling. We demonstrate for the first time that PKC\(\alpha\) specifically activates H-Ras for upregulation of the negative cell cycle regulator p21\(^{Cip1}\) and partial cell cycle arrest. An antiproliferative function of H-Ras in intestinal epithelial cells is consistent with high expression of this isoform in postmitotic/mature intestinal epithelial cells \(^{63}\) and the ability of H-Ras to reduce proliferation and induce differentiation markers in Caco-2 colon cancer cells \(^{3, 6}\). Interestingly, H-Ras is the least

**Figure 9.** RasGRP3 partially mediates PKC\(\alpha\)-induced antiproliferative signaling. A, loss of RasGRP3 significantly inhibits PKC\(\alpha\)-induced activation of ERK and upregulation of p21\(^{Cip1}\). IEC-18 cells were transfected with nontargeting siRNA (NT) or ON-TARGETplus SMARTpool siRNA targeting RasGRP3 (Dharmacon) as indicated. After 48 h, cells were treated with 100 nM PMA for 15 min (i) or 2 h (ii) and analyzed for the indicated proteins by Western blotting. iii, densitometric quantification of p21\(^{Cip1}\), cyclin D1, and Id1 expression (normalized to \(\beta\)-actin) from three independent experiments. *\(p = 0.03\). iv, cells were treated as in (A(ii)) except that the siRNA targeting RasGRP3 was from Invitrogen (Silencer Select predesigned siRNA Rasgrp3). Data are from single immunoblots; the vertical line indicates rearrangement of the blot for clarity. B, IEC-18 cells were transfected with nontargeting siRNA (NT) or siRNA targeting RasGRP3 (ON-TARGETplus SMARTpool, Dharmacon). After 24 h, cells were treated with 100 nM PMA for 6 h, as indicated, and the percentage of cells in S-phase was assessed by flow cytometry. **\(p = 0.003\), n = 4. PMA, phorbol 12-myristate 13-acetate.
frequently mutated Ras isoform in human cancer (4% versus 85% for K-Ras) and activating mutations in H-Ras are not observed in colorectal cancer (66), perhaps reflecting the growth-inhibitory function of this isoform in intestinal epithelial tissue.

The finding that H-Ras knockdown does not affect PKCα-induced downregulation of cyclin D1 and Id1 points to the existence of at least one additional growth-suppressive ERK signaling axis downstream of PKCα in intestinal cells. Data showing that PKCα-induced cyclin D1 and Id1 downregulation is Ras-ERK dependent support the conclusion that PKCα is also able to activate ERK through K-Ras and/or N-Ras. Furthermore, since loss of H-Ras only partially blocked the ability of PKCα to induce cell cycle arrest, these distinct ERK pathways appear to collaborate in mediating PKCα-induced cell cycle withdrawal in the intestinal epithelium. Engagement of multiple downstream pathways is a common feature of growth regulatory signaling. For example, anti-proliferative effects of TGFβ are mediated by multiple factors downstream of both canonical SMAD-mediated signaling and noncanonical signaling involving Ras-ERK, p38, and JNK cascades, among others (67). Thus, the identification of multiple signaling nodes downstream of PKCα supports the importance of PKCα in regulation of intestinal homeostasis, while pointing to its potential role as a signaling nexus for coordination of growth-suppressive pathways in this tissue.

The finding that the Ras-GEF, RasGRP3, is required for H-Ras activation and p21Cip1 induction in intestinal epithelial cells provides the first evidence for a PKCα–RasGRP3–H-Ras–Raf–MEK–ERK signaling axis. RasGRP3 has been shown to activate H-Ras in other systems (e.g., 293T cells), and in vitro studies with the RasGRP3 catalytic domain showed that RasGRP3 can promote GDP release from H-Ras (68–70). Like PKCs, the RasGRPs have a DAG/phorbol ester–binding C1 domain and DAG/PMA redistributes RasGRP3 to the plasma membrane to induce Ras-GTP loading (71, 72). However, studies in lymphocytes have revealed that PKCs can phosphorylate RasGRP3 at Thr133 (62, 73) and that PKC-mediated phosphorylation is required for RasGRP3 activation and downstream signaling (61). These published findings, combined with our demonstration of PKCα-dependent phosphorylation of RasGRP3 in intestinal epithelial cells (Figs. 8 and 11), suggest a model in which DAG/phorbol ester treatment leads to membrane concentration of PKCα and RasGRP3, facilitating RasGRP3 phosphorylation and activation by the kinase. Interestingly, RasGRP family members have been implicated in negative regulation of cell proliferation in intestinal epithelial cells, with RasGRP1 opposing EGFR–SOS1–Ras signals in crypt cells through a negative feedback loop (74, 75). Ras-GRP1 also restricts proliferation of colorectal cancer cells and low levels of RasGRP1 predict poor clinical outcome in colorectal cancer patients (74, 75).
RasGRP1 is not expressed in IEC-18 intestinal crypt-like cells (Fig. 8), the data collectively indicate that both RasGRP1 and RasGRP3 have anti-proliferative functions in the intestinal epithelium, functions that are consistent with frequent loss of both proteins in colorectal cancer cells (refs. (74, 75), and TCGA data).

Although details of the ERK pathway(s) that mediate downregulation of cyclin D1 and Id1 remain to be elucidated, a potential contributing mechanism involves the ability of PMA/PKCa to induce ERK-dependent phosphorylation of SOS1. SOS1 is subject to feedback phosphorylation by ERK or ERK→Rsk at multiple sites, and ERK-regulated phosphorylation negatively modulates SOS1 interaction with Grb2, Shc, and the EGF receptor to dampen growth factor signaling (57). Since SOS proteins are known to play a key role in maintaining cyclin D1 and Id1 levels in proliferating cells, as indicated by a reduction in expression of these proteins in IEC-18 cells following knockdown of SOS1 and SOS2 (Fig. 7), inhibition of SOS1 function through PKCa→ERK “feedback” phosphorylation is a potential mechanism for the loss of cyclin D1 and Id1 seen following PKCa activation (Fig. 12B). However, the ability of PKCa to further downregulate cyclin D1 and Id1 in SOS1/...
SOS2 double-knockdown cells (Fig. 7) indicates that other SOS-independent mechanism(s) are also involved.

While our studies revealed selectivity in the effects of Ras isoforms in mediating PKCa antiproliferative signaling, they revealed redundancy at the level of Raf (Fig. 6). Such redundancy has also been noted in pro-proliferative Raf signaling, although B-Raf/C-Raf heterodimers have the highest catalytic activity and appear to predominate in Ras-mediated signaling in many systems (49, 76, 77). While extensive evidence supports a role for Rafs in promoting cell proliferation (78, 79), antiproliferative roles have also been reported, with several studies linking high activity of Rafs with cell cycle arrest rather than proliferation (80, 81). Analysis of the Raf-interactome has identified PKCs among the proteins that form a complex with Raf proteins (78). To our knowledge, our studies provide the first evidence that PKCa forms complexes with all three Raf proteins in unstimulated cells that dissociate with PKCa activation (Fig. 5). While PKCa/Raf interactions are not sufficient to induce ERK activation, as indicated by the requirement for Ras in the pathway, they likely contribute to growth-inhibitory signaling. In unstimulated cells, both PKCa and Raf proteins reside in the cytoplasm in an inactive state.
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conformation and are activated by recruitment to the plasma membrane (22, 36). Thus, the interaction of PKCa with Rafs suggests the following model (Fig. 12, A and B): (a) in the resting state, inactive PKCa associates with inactive Raf proteins in the cytoplasm; (b) PKC agonists, such as PMA or DAG, recruit PKCa and associated Raf proteins to the plasma membrane; (c) PMA/DAG also recruit RasGRP3 to the plasma membrane, where it is phosphorylated and activated by membrane-localized PKCa; (d) activated RasGRP3 promotes GTP loading and activation of H-Ras and PKCa activates K-and/or N-Ras through at least one other mechanism; and (e) PKCa dissociates from Raf proteins, which can then interact with nearby activated Ras for efficient activation of growth-inhibitory ERK signaling. Our previous studies have also determined that PMA and DAG recruit PKCa to membrane subdomains such as lipid rafts (24, 82, 83); thus, the PKCa/Raf interaction may also ensure spatially restricted activation of ERK within a signaling milieu that supports growth-inhibitory signaling.

Although PKCa-induced growth inhibitory ERK signaling parallels growth-promoting ERK signaling in its requirement for canonical ERK pathway components, we show that these pathways differ in several respects. At the level of Ras activation, in contrast to the established role of SOS proteins in activation of Ras downstream of RTKs, SOS1/2 are dispensable for PKCa-induced ERK activation (Fig. 7). Conversely, while RasGRP3 contributed to ERK activation by PKCa, depletion of this Ras-GEF did not affect ERK activation by EGF (Fig. 8B). The duration of ERK activation following stimulation by PKC agonists or EGF also differentiates these pathways: PKCa signaling induces prolonged ERK activation (>2 h) while ERK activation induced by EGF is transient (ref. (11) and Fig. 1E). Interestingly, both RasGRP3-mediated and RasGRP3-independent pathways stimulated by PKCa induce prolonged activation of ERK (>2 h, Figs. 7Civ and 8iii). Sustained ERK activation is a hallmark of antiproliferative signaling mediated by the Ras–Raf–MEK–ERK cascade (11, 80, 84, 85), and our previous studies have confirmed that prolonged ERK pathway activation, which is dependent on sustained activation of PKCa, is required for PKCa-induced intestinal epithelial cell cycle arrest (11). The finding that PKCa-ERK signaling was not affected by knockdown of KSR1 also points to the possibility that differential utilization of scaffolding proteins may direct the ERK signaling pathway to growth inhibition.

While the current study defines the pathway by which PKCa activates ERK for growth inhibition in intestinal epithelial cells, the factor(s) regulating PKCa activation in this tissue remain to be identified. We have shown that PKCa is cytosolic and inactive in proliferating cells of the intestinal crypt and that the enzyme translocates to the plasma membrane for activation coincident with growth arrest near the crypt–villus junction, remaining membrane associated/active along the length of the villus (refs. (9, 24, 25, 27) and Fig. 13B). A systematic analysis of canonical and noncanonical Wnt pathway components in the murine gastrointestinal tract (86) points to several potential upstream regulators of PKCa including Wnt-2b, Wnt-4, Wnt-5a, Frizzled 4, Frizzled 6, and Dickkopf (DKK) family members, all of which are expressed in epithelial or mesenchymal cells of the villus/colonic surface. Notably, several of these candidates (Wnt-4 (87), Wnt-5a (88), Frizzled 6 (88), and DKK1 (89)) are also PKC/PKCα agonists in other systems. Like PKCa (90), these candidates have been reported to inhibit canonical Wnt signaling, which is required for intestinal epithelial cell proliferation and/or promote cell growth arrest/differentiation. Studies of the major signaling pathways regulating intestinal epithelial self-renewal also support a potential role for growth-suppressive members of the bone morphogenetic protein (BMP) and transforming growth factor β (TGFβ) families (91, 92). Although the role of these factors in upstream activation of PKCa remains to be defined, analysis of the self-renewing intestinal epithelium in vivo supports a role for growth-suppressive ERK signaling downstream of PKCa in this tissue. Activation of PKCa at the crypt/villus junction coincides with cyclin D1 and Id1 downregulation and p21Cip1 upregulation in the intestinal epithelium (9, 26). While phosphorylated active ERK is readily detected in the crypt epithelium (Fig. 13A), consistent with its established role in supporting proliferation within this compartment, a postmitotic role for the kinase is also indicated by the presence of phospho-ERK in the nonproliferating cells of the villus in both adult and fetal intestine (ref. (3, 5) and Fig. 13A). Notably, live imaging of the small intestine of transgenic mice ubiquitously expressing a FRET biosensor for ERK activity also detected active ERK in crypt and villus epithelial cells (93), with particularly strong ERK activity observed in the region of growth arrest at the crypt–villus transition (93). A role for ERK signaling in precise regulation of intestinal epithelial cytostasis is further supported by recent in vivo studies showing crypt cell hyperproliferation and crypt elongation following targeted deletion of ERK1 and ERK2 in the murine small intestinal epithelium (7, 8). Since strong ERK activity coincides with membrane translocation/activation of PKCa (24, 27), the spatiotemporal distribution of these molecules supports a model in which PKCa activates growth-suppressive ERK signaling to (a) drive cell cycle arrest at the crypt–villus junction and (b) maintain the postmitotic state on the villus (Fig. 13, A and B).

Experimental procedures

Cell culture

IEC-18 (ATCC CRL-1589) and IEC-6 (ATTC CRL-1592) rat intestinal crypt-like cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 4 mM L-glutamine, 1 mM sodium pyruvate, and 5 μg/ml insulin at 37 °C and in 5% CO2. For serum starvation, cells were cultured for 24 h in the same medium containing 0.5% FBS and lacking insulin. N-RasΔN-12; H-RasΔN-12; K-RasΔN-12; and UbicreER T2 mESCs (“Rasless” mESCs) were cultured at optimal density (2.5–3 × 106 in 10 cm dish) on 0.1% gelatin (Millipore Sigma)-coated plates in DMEM supplemented with 15% FBS, 1000 U/ml leukemia inhibitory factor (Millipore Sigma), 1% Glutamax (Invitrogen), 0.1 mM nonessential amino acids, 1% penicillin/streptomycin, and
55 mM β-mercaptoethanol (44). For KO of K-Ras, fresh medium containing 1 μM 4-OHT (Sigma) was added daily for 7 days. Medium was changed every day, and cells were subcultured every second or third day depending on confluency.

**Drug treatments and reagents**

For PKC/PKCα activation, cells were treated with 100 nM PMA (Sigma) dissolved in ethanol or 20 μg/ml DiC₈ (Cayman Chemicals) dissolved in acetonitrile. DiC₈ was added repeatedly (every hour) in fresh medium to compensate for its rapid metabolism in cells as we have described (24). EGF was dissolved in PBS and was added to the culture medium at 50 to 100 ng/ml for the indicated times. For inhibitor treatments, cells were pretreated for 1 h with the indicated concentrations of BIM I (Calbiochem), G66976 (Calbiochem), SCH772984 (SelleckChem), LY3009120 (Med Chem BioExpress), PD0325901 (SelleckChem), or salirasib (SelleckChem) dissolved in dimethyl sulfoxide before addition of PKCα agonist, unless otherwise stated. 4-OHT was dissolved in ethanol prior to addition to the culture medium. The relevant vehicle was added to controls and the final concentration of solvents was ≤0.2%.

**Antibodies**

Anti-KSR1 (ab68483) and anti-PKCα (ab32376 used for immunoblot analysis; ab221611 used for immunoprecipitation) antibodies were from Abcam; anti-H-Ras (A19619), anti-pan-Ras (A4735), anti-RasGRP1 (A10495), and anti-PKCε

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**Figure 13**. PKCα membrane translocation/activation coincides with strong ERK activity at the crypt/villus junction in the mouse intestinal epithelium. **A**, upper panel, phospho-ERK immunostaining of formaldehyde-fixed paraffin-embedded murine intestinal epithelial tissue shows that ERK is active in both proliferating crypt cells (C) and postmitotic villus cells (V). Strong pERK staining is detected at the crypt/villus junction (J) where cells undergo growth arrest (arrows). Lower panel, negative control in which primary antibody was omitted demonstrates specificity of staining. **B**, upper panel, immunohistochemical analysis shows that PKCα is diffusely distributed in the cytoplasm and inactive in proliferating crypt cells (C) and is recruited to the plasma membrane, a hallmark of PKC activation, coincident with growth arrest at the crypt/villus junction (J) (arrows). PKCα remains membrane-associated/activated in postmitotic cells of the villus (V) (arrows). Lower panel, negative control demonstrates specificity of staining. Magnification Bar: 50 μm.
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(A2110) antibodies were from Abclonal; anti-KSR2 (H00283455-M08) antibody was from Abnova; anti-A-Raf (75804S), anti-B-Raf (14814S), anti–phospho-Ser259 C-Raf (9421), anti–phospho-Ser338 C-Raf (9427), anti-cyclin D1 (2978), anti-Gapdh (5174), anti–pan-Ras (3339S), anti-pERK (9106 and 4370), anti-pMEK (9121), anti–total ERK (9102), anti–total MEK (9122), anti-SOS1 (D3T771), and anti-pRSK(S380) (9341) antibodies were from Cell Signaling Technology; anti-C-Raf (610151) and anti-p21Cip1 (556430) antibodies were from BD Pharma; anti-Id1 (BCH-1/195-14-50) antibody was from BioCheck; anti-pRSK(S380) (04-418) was from Millipore; anti-p21Cip1 (NBP2-29463) antibody was from Novus Biologicals; anti-RasGRP3 (sc-271068) and anti-SOS2 (sc-393667), and anti-PKC8 (sc-8402) antibodies were from Santa Cruz Biotechnology; and anti-actin (A2066) antibody was from Sigma–Aldrich. Secondary antibodies were horseradish peroxidase–conjugated goat anti-rabbit IgG (AP132P, Millipore), horseradish peroxidase–conjugated goat anti-rabbit IgG (111-035-144, Jackson Immunoresearch Laboratories), and horseradish peroxidase–conjugated goat antimouse IgG (170-6516, Bio-Rad).

Western blot analysis

Cells were washed twice with ice cold PBS and lysed in 1% SDS lysis buffer (1% SDS, 10 mM Tris–HCl, pH 7.4). Equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane as described previously (9). Membranes were stained with 0.1% fast green (Sigma–Aldrich) to confirm equal loading and even transfer. Following blocking with 5% milk or 5% bovine serum albumin (Sigma) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), membranes were incubated with primary antibody (in blocking buffer) overnight at 4 °C, washed (3 x 10 min in TBS-T), and incubated with horseradish peroxidase–conjugated anti-rabbit IgG or anti-mouse IgG secondary antibody for 2 h at room temperature. All antibodies were used at a dilution of 1:1000 except anti-actin, anti-Gapdh, and anti-PKCα antibodies (1:20,000); anti-C-Raf, anti-p-ERK, and anti-total ERK antibodies (1:2000); and the anti-RasGRP3, anti-SOS2, anti-H-Ras, and anti-p21Cip1 antibodies (1:500). Signal detection on washed membranes was performed using SuperSignal West (Pierce).

siRNA transfection

IEC-18 or IEC-6 cells were transfected with 100 pmol siRNA using Lipofectamine RNAiMAX reagent (Invitrogen). Analysis was performed 48 h after transfection, unless otherwise stated. siRNAs were as follows: ON-TARGETplus nontargeting siRNA #1 (D-001810-01-05; Dharmacon); ON-TARGETplus rat A-Raf SMARTpool siRNA (L-088540-02-0005; Dharmacon); ON-TARGETplus rat B-Raf SMARTpool siRNA (L-094802-02-0005; Dharmacon); ON-TARGETplus rat C-Raf SMARTpool siRNA (L-087699-02-0005; Dharmacon); ON-TARGETplus rat H-Ras SMARTpool siRNA (L-084782-01-0005;Dharmacon); ON-TARGETplus rat SMARTpool KSR1 siRNA (L-085072-02-0005; Dharmacon); ON-TARGETplus rat RasGRP1 SMARTpool siRNA; Dharmacon (L-100584-02-0005); ON-TARGETplus Rat RasGRP3 SMARTpool siRNA (L-098911-02-0005; Dharmacon); Silencer select predesigner siRNA RasGRP3, GGAAGCUAGUCAACUATT (4392420; Invitrogen), Silencer select predesigner siRNA SOS1, CAAGCAGUUUCGAAUATT (Invitrogen); Silencer select predesigner siRNA SOS2, GCUUUUGAUUUAUCCTATT (4390771; Invitrogen). ON-TARGETplus SMARTpool siRNAs are a pool of four siRNAs designed and modified to increase specificity and reduce off-target effects.

Flow cytometry

Subconfluent IEC-18 cells were briefly washed with PBS and treated with trypsin (Gibco) to obtain single cell suspensions as described (10). About 9 x 10^5 cells were fixed in 70% ethanol and stained overnight at 4 °C with Telford’s reagent (90 mM EDTA, 0.1% (v/v) Triton X-100, 50 µg/ml propidium iodide in PBS). DNA content was determined by flow cytometric analysis in the Flow Cytometry Research Facility at UNMC.

Immunoprecipitation

IEC-18 cells were treated with PMA or vehicle for 10 min, rinsed twice with ice-cold PBS, and lysed for 15 to 30 min on ice in immunoprecipitation buffer (1% NP-40/Igepal CA-630, 137 mM NaCl, 20 mM Tris–HCl, pH 8.0 with freshly added protease inhibitor cocktail and phosphatase inhibitor cocktails I and II [Sigma]). Lysates were centrifuged at 13,000 rpm for 15 min, and protein concentration of the supernatants was quantified using the bicinchoninic acid assay kit (Pierce). About 500 to 1000 µg of protein were incubated in immunoprecipitation buffer containing the specified antibody (diluted as recommended by the manufacturer) or isotype matched IgG (control) overnight with rocking at 4 °C. Antibody-protein complexes were incubated with protein A/G-agarose beads (Santa Cruz) for 2 h at 4 °C with rocking. Beads were collected by centrifugation, and washed three times with ice-cold immunoprecipitation buffer. Bound proteins were extracted with 2x SDS buffer (containing freshly added DTT, 350 mM final concentration) and subjected to Western blotting.

Active Ras pull-down assay

Active Ras pull-down assays were performed using an Active Ras Detection Kit (Cell Signaling Technology) according to the manufacturer’s instructions. Briefly, nontargeting siRNA or RasGRP3 siRNA transfected IEC-18 cells were treated with vehicle or 100 nM PMA for 15 min and lysed in 1x lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma). Lysates were cleared by centrifugation, and protein concentration in the supernatant was quantified using the bicinchoninic acid assay kit (Pierce). Active Ras in lysates (containing equal amounts of protein) was pulled down with GST-Raf1-RBD (Ras-binding domain of Raf) fusion protein and glutathione resin, and levels of total (pan) Ras or H-Ras in the pulldowns and original lysates were determined by

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Western blotting. Positive and negative controls for the pull-down procedures were generated by incubating lysates with GTPγS or GDP, respectively.

**Phos-tag gel analysis**

Cells were lysed in immunoprecipitation buffer or in immunoprecipitation buffer lacking phosphatase inhibitors, and lysates were cleared by centrifugation. For λ-phosphatase treatment, supernatants lacking phosphatase inhibitors were adjusted to 1 mM MnCl₂ and 800 units of λ-phosphatase (P0753, New England Biolabs) were added for 30 min at 30 °C. Samples were subjected to SDS-PAGE/Western blotting using a 6% acrylamide resolving gel containing 20 μg Phos-tag reagent (Wako Pure Chemical Industries; AAL-107) in the presence of 0.1 mmol/l MnCl₂. Following electrophoresis at 60 to 90 V, gels were equilibrated in transfer buffer containing 10 mM EDTA (2 × 10 min). The gels were then soaked in regular transfer buffer for another 10 min followed by immunoblotting.

**Immunohistochemistry**

Deparaffinized sections of formaldehyde-fixed, paraffin-embedded mouse intestinal epithelium were rehydrated with ddH₂O for 5 min. For antigen retrieval, slides were heated in preheated 1× target retrieval solution (DAKO) for 30 to 70 min at 90 °C, followed by cooling for 20 min. Endogenous peroxidase was inactivated with 3% H₂O₂. For pERK staining, sections were blocked with 5% normal goat serum in TBS-T. Sections were then incubated with primary anti-pERK antibody (Cell Signaling Technology antibody 4370, diluted 1:400) overnight at 4 °C in a humidified chamber, followed by SignalStain boost anti-rabbit IgG polymer (Cell Signaling Technologies) and diaminobenzidine (DAKO) as recommended by the manufacturer. Immunostaining for PKCa used anti-PKCa antibody from Abcam (ab32376) as we have described (26). Controls for staining specificity involved omission of primary antibody or replacement of the primary antibody with normal rabbit serum. Sections were counterstained with hematoxylin and dehydrated before mounting with coverslips.

**Software and statistical analysis**

Cell cycle analysis of flow cytometric data and generation of histograms were performed using FlowJo (FlowJo LLC) and Modfit (Verity Software) software. Densitometric analysis of scanned Western blot data was performed with ImageJ software (NIH) using multiple exposures of each blot. Levels of proteins of interest were normalized to the loading control and presented as relative expression levels. Due to very low levels of active ERK in many control samples, quantification of pERK and pRSK is expressed as relative to the levels in PMA-treated samples for consistency. Contrast and brightness of scanned images were adjusted using GMU Image Manipulation Program (GIMP), Adobe Photoshop, or Microsoft PowerPoint software. All adjustments to contrast and brightness were made equally across the entire blot and no individual lanes were treated differently than the rest of the blot. In some figures, dashed lines indicate where lanes have been rearranged for clarity: in all cases lanes in each panel are from the same exposure of a single blot except as indicated in Figure 6A. Graphs were generated using Microsoft Excel software, and figures were assembled and annotated in Microsoft PowerPoint software. Statistical analysis was performed using Microsoft Excel software. Statistical significance of differences was assessed using Student’s t-tests with p-values ≤0.05 considered significant.

**Data availability**

All data described in the article are contained within the article.

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**Abbreviations**—The abbreviations used are: 4-OHT, 4-hydroxytamoxifen; BIM I, bisindolylmaleimide I; DAG, diacylglycerol; DiC₈, 1,2-dioctanoyl-sn-glycerol; FBS, fetal bovine serum; mESC, mouse embryonic stem cell; PMA, phorbol 12-myristate 13-acetate.

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