Protein Kinase C-mediated Down-regulation of Cyclin D1 Involves Activation of the Translational Repressor 4E-BP1 via a Phosphoinositide 3-Kinase/Akt-independent, Protein Phosphatase 2A-dependent Mechanism in Intestinal Epithelial Cells*

Received for publication, November 13, 2006, and in revised form, March 8, 2007. Published, JBC Papers in Press, March 13, 2007, DOI 10.1074/jbc.M610513200

Lingjie Guan†, Kyung Song‡, Marybeth A. Pysz†‡, Kathryn J. Curry†, A. Asli Hizli‡, David Danielpour‡, Adrian R. Black‡, and Jennifer D. Black†‡

From the †Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263 and the ‡Case Comprehensive Cancer Center Research Laboratories and Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106

We reported previously that protein kinase Ca (PKCa), a negative regulator of cell growth in the intestinal epithelium, inhibits cyclin D1 translation by inducing hypophosphorylation/activation of the translational repressor 4E-BP1. The current study explores the molecular mechanisms underlying PKC/PKCa-induced activation of 4E-BP1 in IEC-18 nontransformed rat ileal crypt cells. PKC signaling is shown to promote dephosphorylation of Thr45 and Ser64 on 4E-BP1, residues directly involved in its association with eIF4E. Consistent with the known role of the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway in regulation of 4E-BP1, PKC signaling transiently inhibited PI3K activity and Akt phosphorylation in IEC-18 cells. However, PKC/PKCa-induced activation of 4E-BP1 was not prevented by constitutively active mutants of PI3K or Akt, indicating that blockade of PI3K/Akt signaling is not the primary effector of 4E-BP1 activation. This idea is supported by the fact that PKC activation did not alter S6 kinase activity in these cells. Further analysis indicated that PKC-mediated 4E-BP1 hypophosphorylation is dependent on the activity of protein phosphatase 2A (PP2A). PKC signaling induced an ~2-fold increase in PP2A activity, and phosphatase inhibition blocked the effects of PKC agonists on 4E-BP1 phosphorylation and cyclin D1 expression.

H2O2 and ceramide, two naturally occurring PKC agonists that promote growth arrest in intestinal cells, activate 4E-BP1 in PKC/PKCa-dependent manner, supporting the physiological significance of the findings. Together, our studies indicate that activation of PP2A is an important mechanism underlying PKC/PKCa-induced inhibition of cap-dependent translation and growth suppression in intestinal epithelial cells.

Members of the protein kinase C (PKC) family of signal transduction molecules have been implicated in regulation of fundamental cellular processes, including cell growth and cell cycle progression, differentiation, survival/apoptosis, protein translation, membrane trafficking, receptor desensitization, and migration (1–10). Increasing evidence supports a key role for PKC signaling in the maintenance of intestinal homeostasis (1, 11, 12), and aberrations in PKC expression and function occur early during intestinal tumorigenesis (13). Previous studies from this laboratory have demonstrated that PKC/PKCa signaling triggers a program of cell cycle withdrawal in intestinal epithelial cells characterized by rapid down-regulation of cyclin D1, increased expression of Cip/Kip cyclin-dependent kinase inhibitors, and activation of the growth suppressor function of members of the pocket protein family (14, 15). Down-regulation of cyclin D1 precedes various hallmark events of cell cycle exit, pointing to the importance of regulation of this cyclin in PKC-mediated growth arrest in intestinal epithelial cells.

Analysis of the mechanisms underlying PKC/PKCa-mediated down-regulation of cyclin D1 identified PKCa as a negative regulator of cyclin D1 translation (7). PKC/PKCa signaling activates the translational repressor 4E-BP1, which blocks cap-dependent translation initiation through association with eukaryotic translation initiation factor 4E (eIF4E). eIF4E is a key regulator of translation initiation that binds to the 5′-cap structure (m7GpppN, where N is any nucleotide) found in the majority of eukaryotic mRNAs. At the cap, eIF4E interacts with eIF4G and eIF4A to form eIF4F, the translation initiation complex that recruits the 40 S ribosomal subunit to mRNA (16). eIF4E activity is rate-limiting for translation and has been found to be particularly important for translation of mRNAs with highly structured 5′-untranslated regions, a characteristic of mRNAs of many growth-related genes such as cyclin D1 and c-myc (17).

* The abbreviations used are: PKC, protein kinase C; BIM, bisindolylmaleimide I; Bryo, bryostatin-1; CMV, cytomegalovirus; DiC8, 1,2-dioctanoyl-sn-glycerol-3-phosphate; 4E-BP1, eIF4E-binding protein 1; eIF4E, eukaryotic initiation factor 4E; GFP, green fluorescent protein; m.o.i., multiplicity of infection; mTOR, mammalian target of rapamycin; PMA, phorbol 12-myristate 13-acetate; PI3K, phosphoinositide 3-kinase; PP2A, protein phosphatase 2A; S6K, S6 kinase.

1 Supported by National Institutes of Health Postdoctoral Fellowship CA113048.
2 To whom correspondence should be addressed: Dept. of Pharmacology and Therapeutics, Roswell Park Cancer Inst., Elm and Carlton St, Buffalo, NY 14263. Tel: 716-845-5766; Fax: 716-845-8857; E-mail: jennifer.black@roswellpark.org.

3 The abbreviations used are: PKC, protein kinase C; BIM, bisindolylmaleimide I; Bryo, bryostatin-1; CMV, cytomegalovirus; DiC8, 1,2-dioctanoyl-sn-glycerol-3-phosphate; 4E-BP1, eIF4E-binding protein 1; eIF4E, eukaryotic initiation factor 4E; GFP, green fluorescent protein; m.o.i., multiplicity of infection; mTOR, mammalian target of rapamycin; PMA, phorbol 12-myristate 13-acetate; PI3K, phosphoinositide 3-kinase; PP2A, protein phosphatase 2A; S6K, S6 kinase.
PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

Thus, eIF4E activity is a primary target for control of gene expression, and its importance in maintenance of tissue homeostasis is highlighted by the fact that it is commonly overexpressed in tumors (17).

A major mechanism for regulation of eIF4E activity is through its interaction with a family of translational inhibitory proteins, the eIF4E-binding proteins (4E-BPs), the best characterized of which is 4E-BP1 (16). In their active forms, these molecules compete with eIF4G for binding to eIF4E, thereby preventing the assembly of eIF4F and thus inhibiting cap-dependent translation initiation. The activity of 4E-BPs is regulated by hierarchical phosphorylation at multiple sites (18). Although hypophosphorylated 4E-BPs bind with high affinity to eIF4E, hyperphosphorylation of these molecules results in loss of their eIF4E binding activity, thus promoting translation initiation. Importantly, through their inhibitory effects on protein translation, 4E-BPs have been identified as potent negative regulators of cell growth (19). Our studies have demonstrated that PKC/PKCα-induced activation of 4E-BP1 in intestinal epithelial cells promotes association of 4E-BP1 with eIF4E and sequestration of cyclin D1 message in 4E-BP1-associated complexes (7). These alterations ensure rapid disappearance of the potent mitogenic factor cyclin D1 and thus play an important role in PKC/PKCα-induced cell cycle withdrawal in intestinal epithelial cells.

The phosphorylation of 4E-BP1 is regulated by a variety of extracellular stimuli including mitogens, growth factors, cytokines, hormones, G-protein-coupled receptor agonists, and nutrient availability (16). A large number of studies in different systems point to the mTOR protein kinase as a major regulator of 4E-BP1 (20, 21). Inhibition of mTOR by rapamycin markedly impairs 4E-BP1 phosphorylation and blocks its release from eIF4E (22). Furthermore, phosphorylation of 4E-BP1 is strongly modulated by the phosphoinositide 3-kinase (PI3K)/Akt pathway and by nutrients/amino acids, both upstream regulators of mTOR signaling (20, 23, 24). In addition to mTOR, other factors that have been implicated in regulation of 4E-BP1 phosphorylation/activation include the kinases pim-2 (25), PKCβ (8), and c-Abl (26) and phosphatases such as serine/threonine protein phosphatase 2A (PP2A) (27–29). Thus, a complex system of signaling molecules, comprised of scaffold proteins, kinases, and phosphatases, appears to control the phosphorylation/activity of 4E-BP1.

In this study, we have explored the mechanism(s) underlying PKC/PKCα-induced hypophosphorylation/activation of 4E-BP1 in IEC-18 nontransformed rat intestinal crypt cells. Our analysis indicates that PP2A activity plays a predominant role in mediating PKC-induced dephosphorylation of 4E-BP1 in this system and points to a PKC-regulated, PI3K/Akt-independent pathway of 4E-BP1 control in intestinal cells.

**EXPERIMENTAL PROCEDURES**

**Materials and Antibodies**—Phorbol 12-myristate 13-acetate (PMA), 1,2-dioctanoyl-sn-glycerol (DiC8), N-hexanoyl-sphingosine (C6 ceramide), hydrogen peroxide (H2O2), and 1-α-phosphatidylinositol sodium salt were purchased from Sigma. Bryostatin-1 (Bryo), bisindolylmaleimide I (BIM), and rapamycin were from LC Laboratories and okadaic acid, Go6976, Ro-32-0432, and LY294002 were from Calbiochem. Calyculin A, tautomycin, and protein A/G-plus-agarose slurry were from Santa Cruz Biotechnology and silica gel H with 1% potassium oxalate was from Analtech. Anti-PKCa (sc-8393), -PKCB (sc-213), -PKCe (sc-214), -total Akt (sc-8312), and -phospho-Ser44-4E-BP1 (numbering based on the sequence of the rat protein) (sc-18091) antibodies were from Santa Cruz Biotechnology. Anti-cyclin D1 (rm-9104) antibody was from LabVision Corp. and anti-actin antibody was from Sigma. Anti-phospho-Thr308-Akt (9275), -phospho-Ser473-Akt (9271), -phospho-S6 ribosomal protein (2211), -4E-BP1 (9452), -phospho-Thr36/45-4E-BP1 (2855), nonphospho- Thr45-4E-BP1 (9423), phospho-Thr45-4E-BP1 (9455) (numbering for 4E-BP1 phosphorylation sites based on rat sequence), -phospho-GSK3α/β (9331), -total AMPKα (2532), and -phospho-Thr172-AMPKα (2535) antibodies were purchased from Cell Signaling Technology. Anti-4E-BP1 (AB3251) antibody was also obtained from Chemicon. Anti-PI3K p85 (06-195) antibody, anti-Myc tag (05-724) antibody, a PP2A immunoprecipitation antibody, a PP2A regulatory subunit antibody (ab26233) was from Abcam Biotechnology. Anti-cyclin D1 (rm-9104) antibody was from Vector Biolabs. [32P]ATP was obtained from PerkinElmer Life Sciences.

**Cell Culture and Drug Treatments**—IEC-18 nontransformed rat intestinal epithelial cells were maintained in Dulbecco’s modified Eagle’s supplemented with 5% fetal bovine serum, 4 mM 1-glutamine, and 0.15% insulin as described (14). PKCa, -δ, and -ε were activated in subconfluent cells by treatment with PMA (100 nM), Bryo (100 nM), or DiC8 (20 μg/ml). Control cells were treated with the appropriate vehicle (ethanol or Me2SO). Inhibition of PKCa, -δ, and -ε activity, or of PKCe activity alone, was achieved using 5 μM BIM, 10 μM Ro-32-0432 or 1–2 μM Go6976. PI3K activity was inhibited using 50 μM LY294002, and mTOR activity was inhibited using 10 nM rapamycin. Calyculin A (10 nM), okadaic acid (2.5 μM), and tautomycin (5 μM) were used to inhibit PP2A and/or PIK activity. All inhibitors were added 30 min prior to PKC agonist treatment except for calyculin A and okadaic acid, which were added 15 min prior to addition of PKC stimulation, and Ro-32-0432, which was added 1 h prior to PKC agonist treatment.

**In Vitro Cap Affinity Assay**—Cap affinity chromatography was performed as we have described (7). PMA- or vehicle-treated IEC-18 cells were harvested in lysis buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with protease/phosphatase inhibitor mixtures (Sigma), and particulate material was removed by centrifugation. Cell lysates (~400 μg of protein) were incubated with 50 μl of 7-methyl-GTP-Sepharose 4B slurry (Amersham Biosciences) for 16 h at 4 °C. Beads were washed three times with lysis buffer, and cap-bound protein was eluted with Laemmli sample buffer and subjected to immunoblot analysis.

**Analysis of 4E-BP1-eIF4E Interaction**—4E-BP1 was immunoprecipitated from extracts of PMA- or vehicle-treated cells using rabbit anti-4E-BP1 antibody (Cell Signaling) and protein A-Dynabeads (Dynal) as we have described (7). Immunopre-
PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

dipitates were boiled in 2× Laemml sample buffer and subjected to anti-4E-BP1 and anti-elf4E immunoblot analysis.

Analysis of Protein Expression—Cell lysis and immunoblot analysis were performed as described previously (7, 15). Blots were routinely stained with 0.1% Fast Green (Sigma) to confirm equal loading and even transfer. Primary antibody dilutions were as follows: anti-total Akt, -PKCδ, -PKCe, -4E-BP1 (Cell Signaling), anti-phospho-Thr38/44, -Thr45, -PP2A regulatory subunit antibodies, anti-phospho-Thr172, -AMPKα, -PKCα, -phospho-Thr308, -Akt, -phospho-Ser473-Akt, -phospho-Ser64-4E-BP1, and -phospho-Thr68-4E-BP1 antibodies, 1:500; anti-phospho-S6 ribosomal protein and -total AMPKα antibody, 1:5000;anti-PKCe, -phospho-GSK3α/β, and cyclin D1 antibodies, 1:1000; anti-Myc tag antibody, 1:8000; anti-PP2A catalytic subunit antibody, anti-4E-BP1 (Chemicon), and anti-actin antibody, 1:10,000.

PI3K Assay—PI3K activity was measured as described previously (30, 31). IEC-18 cells treated with LY294002 (50 μM) or PMA (100 nm) were lysed in buffer A (137 mM NaCl, 20 mM Tris HCl, pH 7.4, 1 mM MgCl2, 1 mM CaCl2, 1% Nonidet P-40, 100 mM NaVO4, and 1 mM phenylmethylsulfonyl fluoride) and sheared by several passes through a 27-gauge needle. The extract was then incubated on ice for 30 min and cleared by centrifugation (14,000 × g, 15 min) at 4 °C. lysates containing equal amounts of protein (500 μg) were incubated with 5 μl of anti-PI3K p85 antibody overnight at 4 °C followed by the addition of 25 μl of protein A/G plus-agarose slurry (Santa Cruz Biotechnology) for 2 h at 4 °C. The immunoprecipitates were then washed twice with wash buffer 1 (phosphate-buffered saline, 1% Nonidet P-40, 100 μM NaVO4), twice with wash buffer 2 (100 mM Tris-HCl, pH 7.4, 5 mM LiCl, 100 μM NaVO4), and twice with wash buffer 3 (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 100 μM NaVO4). Following the washes, immunoprecipitates were resuspended in 50 μl of kinase assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EGTA); 10 μg of phosphatidylinositol sodium salt was added, and the mixture was incubated at room temperature for 10 min. Kinase activity was measured by the addition of 2 μl of [32P]ATP (PerkinElmer Life Sciences) and 20 mM MgCl2 for 10 min at room temperature. The reaction was terminated by the addition of concentrated HCl and chloroform:methanol mix (0.2:20:20, v/v). The aqueous and organic phases were separated by centrifugation at 14,000 × g for 2 min. The organic phase containing the phosphatidylinositol phosphates was spotted onto a silica gel H TLC plate coated with 1% potassium oxalate and separated in a TLC chamber in developing buffer (chloroform: methanol:ammonium hydroxide: H2O at 129:114:15:21, v/v). The TLC plate was exposed to Kodak Biomax XAR film for 2–6 h at room temperature or overnight at −80 °C. Phosphorylated products were visualized, and the intensity of the spots was quantified by scanning densitometry.

Adenovirus Expansion and Transfection—Adenoviral vectors directing expression of constitutively membrane-associated Myr-HA-Akt1 (Ad-CMV-Akt1 (Myr)) or green fluorescent protein (GFP) reporter adenovirus (Ad-CMV-GFP) were purchased from Vector Biolabs. Constitutively active PI3K adenovirus (Myc-CA-PI3K(p110αCAAX)-AdV) and control adenovirus vectors were generated and characterized as described (32). Adenoviral vectors expressing full-length human PKCα or LacZ were a kind gift from Drs. Toshio Kuroki (Kobe University, Japan) and Marcelo G. Kazanietz (University of Pennsylvania) (33). Subconfluent IEC-18 cells in 6-well plates were infected with GFP or Myr-Akt adenoviruses for 16 h at a multiplicity of infection (m.o.i.) of 50 in Dulbecco’s modified Eagle’s medium supplemented with 2% fetal bovine serum, 4 mM l-glutamine, and 0.15% insulin. Analysis of cells
PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

infected with GFP reporter adenovirus revealed an infection efficiency of near 100%. IEC-18 cells were infected with control or CA-PK3 adenoviruses at an m.o.i. of 140 with >90% infection efficiency as determined by anti-Myc immunofluorescence staining. PKCα and LacZ adenoviruses were added at an m.o.i. of 5, with an infection efficiency of ~95%. After the virus was removed, cells were incubated in fresh complete culture medium for an additional 48 h. Following treatment with PKC agonists or vehicle, the cells were harvested and analyzed by immunoblotting as described above.

Phosphatase Activity Assay—PP2A activity was determined using a PP2A immunoprecipitation phosphatase assay from Upstate that measures free phosphate with a malachite green dye. After the indicated treatments, cells were washed twice in cold phosphate-buffered saline and lysed in lysis buffer (20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0, with 1 mM phenylmethylsulfonyl fluoride). To immunoprecipitate PP2A, lysates containing 200 μg of protein were incubated with 4 μg of anti-PP2A-C subunit antibody (clone 1D6) and 40 μl of protein A-agarose slurry for 2 h at 4 °C with constant rocking. The immunoprecipitates were washed three times in Tris-buffered saline and once with Ser/Thr assay buffer (50 mM Tris-HCl, pH 7.0, 100 μM CaCl2), and resuspended in 20 μl of Ser/Thr assay buffer. The reaction was initiated by the addition of 60 μl of phosphopeptide substrate (750 μM) (KRpTIRR). Following incubation for 10 min at 30 °C in a shaking incubator, the reaction mixture was centrifuged briefly and the supernatant transferred to a 96-well microtiter plate. The reaction was terminated by the addition of malachite green dye. After the indicated treatments, cells were washed twice in Tris-buffered saline and once with Ser/Thr assay buffer (50 mM Tris-HCl, pH 7.0, 100 μM CaCl2), and resuspended in 20 μl of Ser/Thr assay buffer. The reaction was initiated by the addition of 60 μl of phosphopeptide substrate (750 μM) (KRpTIRR). Following incubation for 10 min at 30 °C in a shaking incubator, the reaction mixture was centrifuged briefly and the supernatant transferred to a 96-well microtiter plate. The reaction was terminated by the addition of malachite green phosphate detection solution for 10–15 min at room temperature, and free phosphate was quantified by measuring the absorbance of the mixture at 650 nm using a microplate reader.

RESULTS

PKC Agonists Induce Hypophosphorylation of 4E-BP1 and Down-regulation of Cyclin D1 in IEC-18 Cells—Murine 4E-BP1 is detected as three distinct bands by Western blot analysis: a slow-migrating, hypophosphorylated γ-form; and two faster migrating, hypophosphorylated forms, designated α and β (22, 27). The γ-form does not interact with eIF4E and is, therefore, permissive for translation, whereas the α- and β-forms bind to eIF4E with high and moderate affinity, respectively, and are translational repressors. As shown in Fig. 1A (top panel), activation of PKC with a panel of PKC agonists, including the phorbol ester PMA, the diacylglycerol analog DiC8, and the macrocyclic lactone Bryo, leads to a reduction in the translation permissive γ-form of 4E-BP1 and an accumulation of the faster migrating α- and β-forms. The activity of these translation inhibitory forms was confirmed by cap affinity chromatography, which revealed a striking increase in cap-bound 4E-BP1/α/β-phosphoforms in PKC agonist-treated cells (Fig. 1A, middle panel). 4E-BP1 immunoprecipitation assays further confirmed increased association of 4E-BP1 with eIF4E in PMA-treated cells, comparable with that observed in serum-starved cells (Fig. 1A, bottom panel). Hypophosphorylation of 4E-BP1 by PKC agonists is accompanied by down-regulation of cyclin D1 protein (Fig. 1B), which can be seen by 30 min and is maximal by ~2 h.

As we have shown previously for cyclin D1 down-regulation (7), the effects of PKC agonists on 4E-BP1 are PKC-dependent, as indicated by the ability of the general PKC inhibitors BIM and Ro-32-0432 to block the accumulation of translational repressive forms of the protein (Fig. 2A, left and middle panels). Inhibition of PMA-induced 4E-BP1 activation by the PKCα-selective inhibitor Gö6976 points to a major role of PKCα in promoting translational repression (Fig. 2A; see Ref. 7). The PKCα-selectivity of Gö6976 in IEC-18 cells was confirmed by its ability to protect PKCα, but not PKCβ or ε, from PKC agonist-induced down-regulation (an effect that requires PKC catalytic activity (34)) (Fig. 2A, right panel). The role of PKCα in activation of 4E-BP1 is further supported by PKCα overexpression studies. We have previously demonstrated that PMA-induced 4E-BP1 activation is transient in IEC-18 cells, with reversal of the effect coinciding with down-regulation/desensitization of PKC/PKCα (Fig. 2B; see Ref. 7). Little 4E-BP1 translational inhibitory activity remains after 6 h of PMA treatment.
PKC/PKCα Mediates the Effects of H2O2 and Ceramide on 4E-BP1—To explore the physiological relevance of PKC/PKCα-mediated activation of 4E-BP1, we examined the involvement of PKC signaling in hypophosphorylation/activation of 4E-BP1 by H2O2 and ceramide. These agents were selected based on their participation in growth-inhibitory signaling systems that have been shown to increase diacylglycerol levels and/or activate PKCα in a number of cell types (H2O2 (35–39); ceramide (40–43)). As shown in Fig. 3, both H2O2 and ceramide induce hypophosphorylation of 4E-BP1 in IEC-18 cells. Notably, 4E-BP1 activation by these agents can be inhibited by the general PKC inhibitor Ro-32-0432 (data not shown) and the PKCα-selective inhibitor Gö6976 (Fig. 3). The demonstration that PKC/PKCα activity is required for induction of 4E-BP1 activity by two physiological signaling molecules in this system provides support for the biological significance of the effects observed using PKC agonists.

PKC/PKCα Signaling Promotes Dephosphorylation of Thr45 and Ser64 on 4E-BP1 in IEC-18 Cells—The interaction between 4E-BP1 and eIF4E is regulated by hierarchical multisite phosphorylation (18, 44, 45, 47). Key phosphorylation sites on 4E-BP1 include Thr36, Thr45, Ser64, and Thr69 (in the rodent sequence, +1 for the human sequence), and phosphorylation of Thr36/Thr45 appears to be required for modification of Thr45 and Ser64. To determine the phosphorylation sites on 4E-BP1 targeted by PKC/PKCα activation in IEC-18 cells, cell extracts from vehicle and PMA-treated cells were subjected to immunoblot analysis using phospho-specific antibodies. Effects were compared with those of the mTOR inhibitor rapamycin. As shown in Fig. 4, PMA treatment resulted in an increase in levels of nonphosphorylated Thr45 (detected using an antibody that specifically recognizes 4E-BP1 when it is not phosphorylated at Thr45). Although immunodetection with an antibody that recognizes phospho-Thr36/Thr45 on 4E-BP1 was consolidated in the β-form, overall levels of reactivity were not diminished indicating that phosphorylation at Thr36 is not significantly reduced by PKC signaling. Consistent with previous reports (18, 44, 45, 47), phospho-Ser64 was detected only in the inactive γ-form of 4E-BP1; PMA promoted a decrease in phosphorylation of this residue that paralleled the loss of the γ-form of the protein. PKC agonists do not induce significant accumulation of the α-form of 4E-BP1, a form that lacks phosphate at Thr69 (44, 45), indicating that phosphorylation at Thr69 is not a major target of PKC signaling in IEC-18 cells. This finding was confirmed using anti-phospho-Thr69 antibody; although phospho-Thr69 was consolidated in the β-form in PMA-treated cells, loss of phosphorylation at this site was not sufficient to allow detection by anti-phospho-Thr69 immunoblotting. Together, these data indicate that PKC-induced activation of 4E-BP1 primarily involves loss of phosphorylation at Thr45 and Ser64. Although rapamycin produced somewhat more robust hypophosphorylation of 4E-BP1, as indicated by more pronounced loss of the γ-form and greater accumulation of the β-form (i.e. Ser64 dephosphorylated) and α-form (i.e. Thr69 dephosphorylated), the overall pattern of dephosphorylation seen with this agent paralleled that induced by PMA in IEC-18 cells.

PKC Activation Induces Transient Inactivation of Akt—Based on the similarity in the patterns of 4E-BP1 dephosphorylation induced by PMA and rapamycin in IEC-18 cells (Fig. 4), we examined the effects of PKC signaling on the PI3K/Akt/mTOR signaling pathway, which plays an important role in the regulation of 4E-BP1 activity in many systems (20, 23). Serum or growth factors up-regulate the activity of Akt through a mechanism dependent on PI3K. Full activation of Akt is achieved by phosphorylation at two sites on the molecule, threonine 308 (Thr308) and serine 473 (Ser473) (48). The effects of PKC agonists on Akt phosphorylation were, therefore, evaluated by Western blot analysis using anti-phospho-Thr308-Akt and -phospho-Ser473-Akt antibodies; the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin were used as positive and negative controls for the assay, respectively (Fig. 5A, right panel). Treatment of IEC-18 cells with 100 nM PMA...

FIGURE 3. H2O2 and ceramide promote PKC/PKCα-dependent activation of 4E-BP1 in IEC-18 cells. Upper panel, IEC-18 cells were treated with 1 mM hydrogen peroxide (H) for 1 h in the absence or presence of the PKCα-selective inhibitor Gö6976 (Gö, 1 μM; added 30 min prior to hydrogen peroxide). 4E-BP1 expression was determined by immunoblot analysis (IB). C, vehicle-treated control; P, cells treated with 100 nM PMA for 1 h. Lower panel, cells were treated with 10 μM Gö6976 (Cö) and analyzed for 4E-BP1 expression. P, cells treated with 100 nM PMA for 3 h. Data are representative of at least three independent experiments.

FIGURE 4. PKC activation promotes dephosphorylation of 4E-BP1 at Thr45 and Ser64 in IEC-18 cells. Cells were treated with 10 nM rapamycin (Rap) or 100 nM PMA for 30 min. Equal amounts of protein (5 μg) were subjected to immunoblot analysis (IB) for total 4E-BP1, phospho-Thr45/Thr46-4E-BP1 (p-Thr45/46), nonphospho-Thr45/Thr46-4E-BP1 (non-p-Thr45/46), phospho-Ser64-4E-BP1 (p-Ser64), and phospho-Thr69-4E-BP1 (p-Thr69) as indicated. Arrows indicate the phosphoforms of 4E-BP1. C, vehicle-treated control. Data are representative of at least three independent experiments.
PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

resulted in a marked reduction in phosphorylation of Akt at both sites (Fig. 5A, left panel). The effect was transient, with maximal hypophosphorylation evident by 30 min after PMA administration and recovery to near basal levels by ~2 h. Rapid and transient down-regulation of phospho-Akt was also seen in response to PKC activation by Bryo, DiC₈, and the phorbol ester phorbol 12,13-dibutyrate (Fig. 5B; phorbol 12,13-dibutyrate data not shown), excluding potential nonspecific effects of PMA treatment (49). The PKC dependence of the effect was confirmed by using the general PKC inhibitor BIM, which blocked the effects of PMA, Bryo, and DiC₈ on Akt phosphorylation (Fig. 5C). Notably, BIM enhanced basal Akt phosphorylation in the absence of PKC agonists (Fig. 5C, lane 5), suggesting that PKC signaling has an inhibitory effect on Akt activity in unstimulated cells. Together, these results demonstrate that PKC signaling transiently inhibits Akt activity in IEC-18 cells and that basal PKC activity appears to act as a tonic repressor of the Akt pathway in unstimulated cells.

PKC Activation Induces Transient Down-regulation of PI3K Activity—PKC-mediated inhibition of Akt phosphorylation/activity could result from negative regulation of upstream input signals and/or through activation of an Akt phosphatase(s). Activation of Akt by growth factors and other stimuli is mediated by PI3K (50–55). To determine whether PKC signaling affects PI3K activity in IEC-18 cells, an in vitro PI3K activity assay was performed. LY294002-treated cells were included as a control for the assay. As shown in Fig. 6, PMA induces a transient inhibition of PI3K activity in IEC-18 cells. As observed with Akt phosphorylation (see Fig. 5), the effect is maximal by ~30 min of PMA treatment, and PI3K activity is restored to near basal levels by 2 h. The close correspondence between PMA-induced alterations in PI3K and Akt activity in IEC-18 cells indicates that PKC-induced inhibition of Akt is mediated, at least in part, by a reduction in upstream PI3K activity in this system.

PKC Activation Induces Hypophosphorylation/Activation of 4E-BP1 through a PI3K/Akt-independent Pathway—The finding that PKC-induced effects on PI3K/Akt signaling are short-lived compared with those on 4E-BP1 argued for the involvement of other factors in regulation of the translational repressor. To test this idea, we investigated the requirement for inhibition of Akt and/or PI3K in PKC agonist-induced hypophosphorylation/activation of 4E-BP1. Adenoviral constructs were used for efficient delivery of constitutively active Akt (Myr-HA-Akt1) or PI3K (Myc-CA-PI3K(p110CAAX)) into IEC-18 cells. Transduction of IEC-18 cells with Myr-HA-Akt1 resulted in a robust increase in basal Akt phosphorylation on both Thr³⁰⁸ (data not shown) and Ser⁴⁷³ (Fig. 7). Total Akt levels were also significantly augmented (Fig. 7). Immunoblot analysis further demonstrated increased phosphorylation of GSK3

![Figure 5](image_url) Activation of PKC induces transient inhibition of Akt phosphorylation in IEC-18 cells. A, left panel, IEC-18 cells were treated with 100 nM PMA for the indicated times. The expression of phospho-Thr³⁰⁸-Akt (p-Akt Thr308) (top panel), phospho-Ser⁴⁷³-Akt (middle panel), and total Akt (bottom panel) was determined by immunoblot analysis (IB). C, control treated with PMA for 2 h. Right panel, IEC-18 cells were treated with 10 nM rapamycin (Rap), 100 nM PMA, or 50 µM LY294002 (LY) for 30 min. The expression of phospho-Ser⁴⁷³-Akt (upper panel) and 4E-BP1 (middle panel) was determined by immunoblot analysis. Arrows indicate the phosphoforms of 4E-BP1. B and C, PMA-induced down-regulation of Akt phosphorylation is PKC-dependent in IEC-18 cells. B, effects of 100 nM PMA, 100 nM Bryo, or 20 µg/ml DiC₈ on Akt phosphorylation at serine 473. The expression of phospho-Ser⁴⁷³-Akt and total Akt was determined by immunoblot analysis. C, IEC-18 cells were preincubated with the general PKC inhibitor BIM (5 µM) for 30 min prior to the addition of 100 nM PMA (P), 20 µg/ml DiC₈ (D), or 100 nM Bryo (B) for 30 min. The expression of phospho-Ser⁴⁷³-Akt and total Akt was determined by immunoblot analysis. C, control cells treated with vehicle for 1 h. Data are representative of at least three independent experiments.

![Figure 6](image_url) PKC-induced down-regulation of phospho-Akt correlates with inhibition of PI3K activity. IEC-18 cells were treated with 50 µM LY294002 for 30 min or 100 nM PMA for the indicated times. Cell lysates were prepared, and PI3K was immunoprecipitated using anti-PI3K p85 antibody. The kinase activity of the immunocomplexes was then measured by an in vitro lipid kinase assay using phosphatidylinositol as substrate, as described under "Experimental Procedures." Phospholipids were extracted, separated by thin-layer chromatography, and visualized by autoradiography. Left panel, representative autoradiogram. The arrow indicates radioactive phosphatidylinositol phosphate (PIP). C, control cells treated with vehicle for 30 min. Right panel, densitometric analysis of four independent experiments. Bars represent relative PI3K activity normalized to untreated cells (basal activity). Values are mean ± S.D. (n = 4).
PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

| Time (min) | GFP-AdV | Myr-Akt-AdV |
|------------|---------|-------------|
| PMA, 100nM | -       | +           |
| -          | -       | +           |
| -          | -       | +           |

**FIGURE 7.** Activation of PKC in IEC-18 cells induces 4E-BP1 hypophosphorylation/activation through an Akt-independent pathway. IEC-18 cells were infected with control GFP-adenovirus (GFP-AdV) or constitutively membrane-associated Myr-HA-Akt1 adenovirus (Myc-CA-PI3K-AdV); infection efficiency was near 100%. The infected cells were then treated with 100 nM PMA for the indicated times. The expression of phospho-Ser473-Akt, total Akt, phospho-GSK3α/β (p-GSK3α/β), 4E-BP1, and cyclin D1 was determined by immunoblot analysis (IB). Arrows in the 4E-BP1 panel indicate the phosphoforms of 4E-BP1. Data are representative of more than three independent experiments.

**FIGURE 8.** Activation of PKC in IEC-18 cells induces 4E-BP1 hypophosphorylation/activation through a PI3K/Akt-independent pathway. IEC-18 cells were infected with control adenovirus (Control-AdV) or Myc-tagged constitutively active PI3K adenovirus (Myc-CA-PI3K-AdV). The infected cells (>90%) were then treated with 100 nM PMA for the indicated times. The expression of Myc tag, phospho-Ser473-Akt, total Akt, 4E-BP1, and cyclin D1 was determined by immunoblot analysis (IB). A single membrane was probed for each marker; dividing lines between lanes are included for clarity. Arrows indicate the phosphoforms of 4E-BP1. Data are representative of more than three independent experiments.

PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

Furthermore, Myc-CA-PI3K was resistant to down-regulation by PKC signaling, as indicated by the persistence of high levels of Akt phosphorylation in PMA-treated IEC-18 cells. Notably, as seen with Myr-Akt-expressing cells, the presence of constitutively active PI3K did not block the ability of PMA to induce hypophosphorylation of 4E-BP1 and down-regulation of cyclin D1 (Fig. 8). Together these data strongly support the notion that PKC/PKCα activation can promote 4E-BP1 hypophosphorylation/activation through a PI3K/Akt-independent pathway.

**PKC-induced Akt Dephosphorylation Does Not Affect S6K Signaling in IEC-18 Cells—**The unexpected finding that inhibition of the Akt/PI3K pathway is not required for PKC-induced activation of 4E-BP1 in intestinal epithelial cells led us to explore whether other downstream targets of the pathway are affected by PKC modulation in this system (e.g. mTOR). To address this question, we determined the ability of PKC signaling to affect S6K, a direct target of mTOR that is subject to regulation by the mTOR inhibitor rapamycin (59, 60). The S6 ribosomal protein is a physiological substrate of S6K, and its phosphorylation status can be used as an indicator of the activity of the mTOR-S6K module. In IEC-18 cells, both LY294002 and rapamycin completely ablated phosphorylation of S6, indicating that S6K activity is dependent on PI3K-mediated activation of mTOR in this system (Fig. 9A, lower panel). Notably, PMA had no effect on S6 phosphorylation, even at 30 min when inhibition of Akt was maximal (Fig. 9A, upper panel). Similarly, S6 ribosomal protein phosphorylation was abolished in the presence of PKC activators, further supporting the notion that PKC signaling does not affect S6K signaling in IEC-18 cells.
PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

**FIGURE 10.** PKC-induced 4E-BP1 hypophosphorylation/activation and down-regulation of cyclin D1 are mediated by PP2A activity. A, inhibition of PP2A activity prevents PMA-induced hypophosphorylation of 4E-BP1. IEC-18 cells were treated with 100 nM PMA for 30 min in the presence or absence of 10 nM calyculin A (CA), 2.5 μM okadaic acid (OA), or 5 μM tautomycin (TM) added 15 min (calyculin A, okadaic acid) or 30 min (tautomycin) before PMA. The expression of phospho-Ser473-Akt, total Akt, and 4E-BP1 was determined by immunoblot analysis (IB). The expression of phospho-Ser473-Akt, total Akt, and 4E-BP1 was determined by immunoblot analysis (IB). Arrows indicate the phosphoforms of 4E-BP1. Note the appearance of a new, slower migrating form of 4E-BP1 in CA- and OA-treated cells (*).

B, PKC signaling activates PP2A in intestinal epithelial cells. IEC-18 cells were treated with 100 nM PMA (P), 20 μg/ml DiC8 (D), or 100 nM Bryo (B) for 30 min in the presence or absence of 10 nM calyculin A added 15 min prior to PMA. The expression of phospho-Ser473-Akt (upper panel), 4E-BP1 (middle panel), and total Akt (lower panel) was determined by immunoblot analysis. Arrows indicate the phosphoforms of 4E-BP1. *, indicates a new form of 4E-BP1 evident in calyculin-treated cells; C, indicates control cells treated with vehicle for 45 min. C, PKC-induced down-regulation of cyclin D1 requires PP2A activity. IEC-18 were treated with 100 nM PMA in the presence or absence of 10 nM calyculin A for the indicated times. The expression of phospho-Ser473-Akt, 4E-BP1, and cyclin D1 was determined by immunoblot analysis. A single membrane was probed for each marker; dividing lines between lanes are included for clarity. *, new form of 4E-BP1. D, PKC signaling does not affect PP2A expression levels in IEC-18 cells. Cells were treated with 100 nM PMA, 20 μg/ml DiC8, or 10 nM calyculin A for the indicated times, and whole cell lysates were analyzed for the expression of a regulatory (PP2A-R) and catalytic (PP2A-C) subunit of PP2A by immunoblot analysis. E, PKC signaling activates PP2A in intestinal epithelial cells. IEC-18 cells were treated with 100 nM PMA for the indicated times or 10 nM calyculin A for 45 min. PP2A was immunoprecipitated from cell lysates and PP2A activity in the immunocomplexes was measured using RkpTIRR phosphopeptide as substrate. The mean ± S.D. of three independent experiments is shown in the bar diagram. All values are normalized to samples from untreated cells (basal activity) within each experiment. F, activation of PP2A by PMA or DiC8 is PKC/PKCα-dependent. IEC-18 cells were treated with 100 nM PMA or 20 μg/ml DiC8 for 30 min in the presence or absence of 5 μM BIM or 1 μM Go6976 added 30 min prior to PKC agonist treatment. Cells treated with 10 nM calyculin A for 45 min were used as a negative control. PP2A activity was measured and presented as in E. *, statistically different from control (p < 0.05, as determined by two-tailed Student’s t test). Data are representative of at least three independent experiments.

Protein phosphorylation levels were not altered by other PKC agonists, including Bryo and DiC8 (Fig. 9B), despite the ability of these agents to promote hypophosphorylation/activation of Akt in the cells (see Fig. 5, B and C). These data suggest that the inhibition of PI3K/Akt induced by PKC signaling may not be sustained long enough to affect S6K signaling (61, 62) and, perhaps, also the phosphorylation/activation of 4E-BP1.

Inhibition of PP2A Activity Prevents PKC Agonist-induced Hypophosphorylation of 4E-BP1—To explore the involvement of phosphatases in PKC-mediated hypophosphorylation of 4E-BP1, we used a pharmacological approach to inhibit PP1 and/or PP2A-type serine/threonine phosphatases. Three phosphatase inhibitors were evaluated for their ability to affect PKC agonist-induced 4E-BP1 and Akt hypophosphorylation: calyculin A, okadaic acid, and tautomycin. Although calyculin A has been shown to inhibit the activity of both PP1 and PP2A in cells, okadaic acid selectively inhibits PP2A, but not PP1, in vivo, and tautomycin predominantly inhibits PP1 activity (63). Treatment of IEC-18 cells with calyculin A (10 nM) or okadaic acid (2.5 μM) alone substantially enhanced the basal phosphorylation of Akt, consistent with the reported ability of PP2A to regulate phosphorylation of the kinase (64–67) (Fig. 10A). Relative levels of the γ-form of 4E-BP1 were also enhanced, an effect that could result from increased upstream Akt activity or from similar regulation of basal 4E-BP1 phosphorylation by phosphatases (28) (Fig. 10A). Notably, both calyculin A and okadaic acid blocked the ability of PMA to promote hypophosphorylation of 4E-BP1 and inactivation of Akt in IEC-18 cells. Although 10 nM calyculin A was effective in abrogating the effects of PMA, 2.5 μM okadaic acid was required to produce a similar level of inhibition, likely reflecting the slow penetration of okadaic acid through the cell membrane and the variable efflux of the compound in different cell lines (63, 68). In contrast, the PP1 inhibitor tautomycin was unable to enhance basal Akt or 4E-BP1 phosphorylation at concentrations as high as 5 μM (levels confirmed to be active against PP1 in IEC-18 cells) by their ability to enhance the phosphorylation of the PP1 substrate AMPKα (69); Fig. 10A, right panel) and failed to prevent PMA-induced alterations in Akt and 4E-BP1 phosphorylation. Together, these results point to PP2A-type phosphatase(s) as a requisite mediator of PKC-induced dephosphorylation of Akt and 4E-BP1 in IEC-18 cells, a conclusion that is further supported by the ability of calyculin A to block the effects of other PKC ago-
PKC Agonists Enhance PP2A Activity in IEC-18 Cells—The finding that PKC/PKCα signaling promotes dephosphorylation of 4E-BP1 and Akt by a PP2A-type serine/threonine phosphatase in IEC-18 cells led us to explore the possibility that PKC agonists increase the levels and/or activity of PP2A in these cells. As shown in Fig. 10D, total levels of a regulatory (PP2A-R) and catalytic (PP2A-C) subunit of PP2A remained unaffected in PMA- and DiC₈-treated cells. To determine the effects of PKC signaling on PP2A activity, IEC-18 cells were treated with PMA or DiC₈, and PP2A was immunoprecipitated from cell extracts using an anti-PP2A catalytic subunit antibody. PP2A activity in the immunoprecipitates was then measured using KRpTIRR phosphopeptide as a substrate. Fig. 10E shows that PP2A activity was increased more than 2-fold within 30 min of PMA administration. Phosphatase activity remained elevated for 2 h, although it gradually decreased with prolonged treatment. Increased PP2A activity was also seen with the diacylglycerol analog DiC₈ (Fig. 10F), excluding potential nonspecific effects of PMA treatment (49). The PKC dependence of the effect was further confirmed using the PKC inhibitors BIM and Gö6976 (Fig. 10F). As PKCα is the only classical PKC expressed in IEC-18 cells, the ability of Gö6976 to inhibit the effect suggests that PKCα, in particular, plays a predominant role in activating PP2A in this system. Combined treatment of IEC-18 cells with PMA or DiC₈ and the phosphatase inhibitor calyculin A also abrogated the effects of PKC signaling on PP2A activity (data not shown).

Together, these data indicate that PP2A, or a PP2A-like protein phosphatase, is activated by PKC/PKCα signaling in intestinal cells, leading to rapid dephosphorylation of Akt and 4E-BP1. These effects result in inactivation of Akt and Akt-independent activation of 4E-BP1. It is possible that PKC-induced inhibition of upstream PI3K/Akt signaling also impacts the phosphorylation status of 4E-BP1, although S6K activity, which like 4E-BP1 is a downstream target of Akt signaling, is not affected by PKC signaling in IEC-18 cells.

DISCUSSION

Previous studies from our laboratory have established that PKC/PKCα-induced cell cycle withdrawal in intestinal epithelial cells is associated with rapid down-regulation of cyclin D1 (14, 15), mediated by activation of 4E-BP1 and inhibition of cap-dependent translation initiation (7). In this study, we have identified Thr⁴⁵ and Ser⁶⁴ (equivalent to Thr⁴⁶ and Ser⁶⁵ in the human sequence) as the main phosphorylation sites on 4E-BP1 targeted by PKC signaling in intestinal cells. Phosphorylation of these two residues, which flank the conserved eIF4E binding motif (70), appears to be critically involved in regulating the interaction of 4E-BP1 with eIF4E; modification of either site blocks eIF4E binding in vitro (44). Although Thr⁴⁵ phosphorylation may be important in controlling the phosphorylation of other sites, Ser⁶⁴ is the last residue to be modified in the hier-
PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

architectural phosphorylation of the molecule (18, 44, 45, 47). Phospho-Ser64 is only found in the γ-form of 4E-BP1, and dephosphorylation of this residue is sufficient to allow interaction with eIF4E (44, 71). Thus, the ability of PKC/PKCa signaling to promote dephosphorylation of Thr47 and Ser46 provides an explanation for the increased association of 4E-BP1 with eIF4E in PKC agonist-treated cells (Fig. 1). The physiological significance of PKC agonist-induced 4E-BP1 hypophosphorylation/activation is supported by evidence that two natural PKC agonists, H2O2 (35–39) and ceramide (40–43), activate 4E-BP1 in a PKC/PKCa-dependent manner (Fig. 3) and produce growth arrest in intestinal cells (72–75).

Extensive evidence supports a role for the PI3K/Akt pathway in regulation of 4E-BP1 activity and translation initiation (20, 23). Growth factors such as insulin and PDGF (platelet-derived growth factor) activate PI3K/Akt signaling, leading to phosphorylation/inhibition of the TSC1/TSC2 heterodimer, stimulation of the small GTPase Rheb (Ras homolog enriched in brain), and subsequent activation of mTOR (76). 4E-BP1 is a major downstream target of mTOR that is inactivated upon mTOR-dependent phosphorylation. Our demonstration that treatment of IEC-18 cells with the PI3K inhibitor LY294002 or rapamycin results in hypophosphorylation of 4E-BP1 indicated that, under basal conditions, the translational repressor is (a) predominantly phosphorylated/inactive and (b) under negative control of the PI3K/Akt/mTOR pathway (Fig. 5). Thus, we explored the ability of PKC signaling to regulate PI3K/Akt activity in IEC-18 cells. Interestingly, treatment of these cells with the general PKC inhibitor BIM resulted in increased levels of Akt phosphorylation (Fig. 5), suggesting that a member(s) of the PKC enzyme family plays a repressive role on basal Akt activity in unstimulated cells. Consistent with this role, PKC agonists were found to reduce PI3K and Akt signaling in IEC-18 cells (Figs. 5 and 6). This effect was transient, with maximal inhibition observed at 30 min of PKC stimulation and recovery to near basal levels by 2 h. The ability of member(s) of the PKC family to negatively regulate PI3K and/or Akt activity has been noted in other systems, including adipocytes (77), phaeochromocytoma cells (78), vascular smooth muscle cells (79, 80), kidney epithelial cells (81, 82), human airway epithelial cells (82), prostate cancer cells (83), and keratinocytes (84), pointing to a widespread role of PKC signaling in control of this pathway. Interestingly, PKCα (but not PKCe) was recently reported to inhibit the lipid kinase activity of PI3K in vitro through direct phosphorylation of its p110α catalytic subunit (85). PKCs have also been shown to phosphorylate various upstream modulators of PI3K activity, including receptors and docking proteins, and thus attenuate downstream PI3K signaling (86–88). Importantly, however, our studies demonstrated a failure of constitutively active mutants of PI3K and Akt to prevent PKC-induced hypophosphorylation of 4E-BP1 or down-regulation of cyclin D1 in IEC-18 cells (Figs. 7 and 8). These findings provided further support for the importance of 4E-BP1 activity in PKC-induced cyclin D1 modulation and pointed to the existence of a PI3K/Akt-independent mechanism of 4E-BP1 activation in IEC-18 cells.

mTOR is subject to regulation by inputs that are independent of PI3K signaling (21). To explore the possibility that PKC/PKCa modulates mTOR by an alternative mechanism in intestinal cells, we evaluated the effects of PKC agonist treatment on the mTOR target, S6 kinase. Consistent with findings in other cell types, rapamycin treatment abrogated S6 kinase activity (as measured by S6 phosphorylation) in IEC-18 cells, confirming the mTOR dependence of S6 kinase in this system (Fig. 9). In contrast, PKC agonists failed to affect S6 kinase activity in these cells, arguing that mTOR remains active toward S6 kinase under conditions of enhanced PKC/PKCa signaling. Interestingly, pharmacological blockade of PI3K with LY294002 also abrogated S6 phosphorylation, indicating that S6 kinase is dependent on PI3K activity in IEC-18 cells. The basis for the failure of PKC agonists to affect S6 kinase activity despite inhibition of PI3K/Akt signaling may lie in the duration of the effect in IEC-18 cells. The transient inhibition of PI3K/Akt signaling produced by PKC agonists may be insufficient to affect the activity of the kinase, as noted in other systems (89). Also relevant to our observations is evidence that S6 phosphorylation does not always correlate with Akt activity, both in mammalian cells and in Drosophila (90, 91), and that the threshold of Akt activation required for maintenance of S6 kinase activity and S6 phosphorylation is relatively low (61, 92).

Having excluded a requirement for inhibition of PI3K/Akt activity in PKC agonist-induced hypophosphorylation/activation of 4E-BP1, we explored the possible role of phosphatases. Use of pharmacological inhibitors demonstrated that PKP2A activity, but not PP1, is required for PKC-induced hypophosphorylation of 4E-BP1 in IEC-18 cells (Fig. 10). Phosphatase activity assays further showed that PKC signaling promotes an ~2-fold increase in PP2A activity in intestinal cells (Fig. 10, E and F), an effect that may be mediated primarily by PKCα, because it is attenuated by the PKCα-selective inhibitor G6976 (PKCα selectivity in IEC-18 cells confirmed in Fig. 2A). Importantly, our data also demonstrate that inhibition of PP2A activity abolishes PKC/PKCa-induced down-regulation of cyclin D1, confirming the importance of phosphatase activity in the growth inhibitory effects of PKC signaling in intestinal epithelial cells (Fig. 10C).

PP2A accounts for a majority of the serine/threonine phosphatase activity in cells and has been implicated in the regulation of multiple cellular processes including cell cycle progression, DNA replication, gene transcription, and protein translation (68). PP2A plays a critical role in control of a variety of signaling pathways by opposing the activity of signaling kinases such as ERK/MAPK, Akt, PKC, p70S6 kinase, and cyclin-dependent kinases (68). Interestingly, PP2A has been implicated in regulation of 4E-BP1 phosphorylation in other systems (27, 28). A model has been suggested in which mTOR restrains the activity of PP2A, thus preventing the dephosphorylation/activation of 4E-BP1 (28, 29). In this model, increased PP2A activity, resulting from such signals as nutrient deprivation or rapamycin treatment, would overcome the inhibitory effects of mTOR and promote 4E-BP1 activation. The finding that the pattern of 4E-BP1 dephosphorylation induced by rapamycin in IEC-18 cells parallels that seen following PKC-induced activation of PP2A (Fig. 4) provides additional support for this model. We propose that the increased phosphatase activity promoted by PKC/PKCa signaling similarly overrides inhibitory effects of
PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

mTOR or other factors on 4E-BP1 in IEC-18 cells. In this regard, it is noteworthy that PP2A-mediated dephosphorylation of 4E-BP1 has been shown to override the inhibitory effects of insulin in cardiac myocytes (27).

PP2A phosphatases are subject to multiple levels of regulation (68, 94). The core enzyme, a heterodimer (PP2A) comprised of a catalytic (C) and scaffolding (A) subunit, is regulated by both phosphorylation and methylation of the C subunit. The core enzyme can also form heterotrimers (PP2A) by association with one of a number of regulatory B subunits, and catalytic subunit methylation controls this interaction (95). In addition to regulating the enzymatic activity of PP2A, B subunits influence its subcellular localization and substrate specificity. Although the catalytic and A subunits are each encoded by two closely related genes, there are at least four distinct families of regulatory subunits (B/PR110, B'/PR61, B'/PR72, B''/PR93/PR110), each of which contains multiple members that can be alternatively spliced. Thus, PP2A potentially can exist in more than 50 isoforms. PP2A is also regulated by association with other proteins, such as the mTOR target, 4E-BP1 (the mammalian homolog of yeast TAP42) (96, 97). Although the mechanism by which PKC signaling promotes PP2A-mediated dephosphorylation of 4E-BP1 is under investigation, evidence points to a potential role for alterations in PP2A holoenzyme composition and interaction with other proteins. B subunits broadly participate in negative regulation of cell cycle progression (98) and have been linked to tumor suppression. For example, B'/PR61 and B'/PR72 subunits have been shown to inhibit Wnt signaling (99, 100), and colon tumor-associated catalytic subunit mutations affect association of B'/PR72 with the core enzyme (101). Interestingly, SV40 small t antigen, which displaces certain B subunits from PP2A, affects the activity of the enzyme against 4E-BP1 and blocks H_2O_2-induced dephosphorylation of pocket proteins by the phosphatase (102). It is notable in regard to the findings in this study that 4E-BP1 overexpression enhances dephosphorylation of 4E-BP1 without affecting phosphorylation of S6K (103). Current studies are directed toward identifying the mechanism by which PKC up-regulates PP2A activity and the isoform(s) involved in observed effects on 4E-BP1.

The PKC dependence of the effects of H_2O_2 and ceramide on 4E-BP1 points to a role for PP2A in the growth inhibitory effects of these molecules in intestinal epithelial cells. In keeping with this idea, both H_2O_2 and ceramide induce PP2A activity in multiple systems (27, 102, 104). Although the mechanisms for PP2A activation by these agents remain to be defined, a signaling pathway can be envisioned in which certain growth inhibitory signals activate PKC/PKCα, leading to increased PP2A activity, hypophosphorylation of 4E-BP1, down-regulation of cyclin D1, and growth arrest (Fig. 11). PP2A activity has also been reported to regulate Akt and S6K phosphorylation. Consistent with these findings, inhibition of PP2A in IEC-18 cells by calyculin A or okadaic acid treatment resulted in increased basal phosphorylation of Akt at both Thr^308 and Ser^473 and attenuated PKC agonist-induced hypophosphorylation of the enzyme. Dephosphorylation by PP2A may, therefore, be a major mechanism underlying the reduced phosphorylation of Akt in PKC agonist-treated cells (as suggested previously (83)). In contrast, PKC-induced activation of PP2A did not alter the activity of S6K in IEC-18 cells. The failure of PP2A to modulate S6 kinase activity may reflect differential requirements for subunit composition/protein interactions (see above) and/or inappropriate localization of phosphatase pools, as it is becoming increasingly apparent that PP2A function requires specific protein-protein interactions that support favorable phosphatase-substrate proximity (62). It is interesting in this regard that PKC activation leads to a redistribution of PP2A in IEC-18 cells (34).

Taken together, our findings indicate that increased PP2A activity is an important component of PKC/PKCα-induced cell cycle arrest in intestinal epithelial cells. PP2A inhibits Akt activity and promotes the translational and growth repressor functions of 4E-BP1 (19), leading to inhibition of cap-dependent translation initiation and loss of the important mitogenic molecule cyclin D1 (Fig. 11). PKC-mediated inhibition of the PI3K/Akt module may also be involved in effects on 4E-BP1, although...
PKC-mediated Activation of 4E-BP1 Is PP2A-dependent

PP2A-type phosphatase activity is likely the predominant mediator of the effect in IEC-18 cells. It is noteworthy in regard to these findings that the growth suppressor TGF-β has recently been shown to inhibit protein translation via a PP2A-dependent mechanism to induce cell cycle arrest in mammary epithelial cells (105). Activation of phosphatase activity and inhibition of protein translation may, therefore, be important downstream consequences of many growth inhibitory signals. This notion is consistent with evidence that PP2A has tumor suppressor properties (94, 106) and that alterations in TGF-β and PKCa expression/activity are common characteristics of various tumor types, including those of the intestine. Loss of PKCa expression in colon adenomas and adenocarcinomas (13, 107–109) may contribute to intestinal tumorigenesis, at least in part, via loss of control of the tumor suppressive activity of PP2A. The importance of translational regulation to intestinal homeostasis is underscored by increasing evidence that overexpression of eIF4E, and the consequent up-regulation of cyclin D1, are important components of colon cancer development (17, 93, 110–115). Thus, translational effects are likely to be a major factor in the emerging role of PKC signaling in regulation of intestinal homeostasis and tumorigenesis (1, 11, 13).

Acknowledgment—We thank Dr. Jing Wang for help with the PI3K activity assays.

REFERENCES

1. Black, J. D. (2000) Front. Biosci. 5, D406–D423
2. Clemens, M. J., Trayner, L., and Menaya, J. (1992) J. Cell Sci. 103, 881–887
3. Gutcher, I., Webb, P. R., and Anderson, N. G. (2003) Cell. Mol. Life Sci. 60, 1061–1070
4. Brodie, C., and Blumberg, P. M. (2003) Apoptosis 8, 19–27
5. Ivaska, J., Kermorgant, S., Whelan, R., Parsons, M., Ng, T., and Parker, P. J. (2003) Biochem. Soc. Trans. 31, 90–93
6. Ivaska, J., Whelan, R. D., Watson, R., and Parker, P. J. (2002) EMBO J. 21, 3608–3619
7. Hizil, A. A., Black, A. R., Pysz, M. A., and Black, J. D. (2006) J. Biol. Chem. 281, 14596–14603
8. Kumar, V., Pandey, P., Sabatini, D., Kumar, M., Majumder, P. K., Bharti, A., Carmichael, G., Kufe, D., and Kharbanda, S. (2000) EMBO J. 19, 1087–1097
9. Palmitant, R., George, M. D., Akiyama, S. K., Wolber, F. M., Olden, K., and Roberts, J. D. (2001) Cancer Res. 61, 2445–2452
10. Alvi, F., Idkowiak-Baldys, J., Baldys, A., Raymond, J. R., and Hannun, Y. A. (2007) Cell. Mol. Life Sci. 64, 263–270
11. Di Mario, J. F., Miffin, R. C., and Powell, D. W. (2005) Gastroenterology 128, 2131–2146
12. Oster, H., and Leitges, M. (2006) Cancer Res. 66, 6955–6963
13. Black, J. D. (2001) Gastroenterology 120, 1868–1872
14. Frey, M. R., Clark, J. A., Leontieva, O., Uronis, J. M., Black, A. R., and Black, J. D. (2000) J. Cell Biol. 151, 763–778
15. Clark, J. A., Black, A. R., Leontieva, O. V., Frey, M. R., Pysz, M. A., Kunneva, L., Woloszynska-Read, A., Roy, D., and Black, J. D. (2004) J. Biol. Chem. 279, 9233–9247
16. Gingras, A. C., Raught, B., and Sonenberg, N. (1999) Annu. Rev. Biochem. 68, 913–936
17. De Benedetti, A., and Graff, J. R. (2004) Oncogene 23, 3189–3199
18. Gingras, A. C., Raught, B., Gygi, S. P., Niedzwiecka, A., Miron, M., Burlay, S. K., Polakiewicz, R. D., Wyslouch-Cieszynska, A., Aebersold, R., and Sonenberg, N. (2001) Genes Dev. 15, 2852–2864
19. Rousseau, D., Gingras, A. C., Pause, A., and Sonenberg, N. (1996) Onco-
