Protein Kinase C α Signaling Inhibits Cyclin D1 Translation in Intestinal Epithelial Cells

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Cyclin D1 is a key regulator of cell proliferation, acting as a mitogen sensor and linking extracellular signaling to the cell cycle machinery. Strict control of cyclin D1 levels is critical for maintenance of tissue homeostasis. We have reported previously that protein kinase C α (PKCα), a negative regulator of cell growth in the intestinal epithelium, promotes rapid down-regulation of cyclin D1 (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). The current study explores the mechanisms underlying PKCα-induced loss of cyclin D1 protein in non-transformed intestinal epithelial cells. Our findings exclude several mechanisms previously implicated in down-regulation of cyclin D1 during cell cycle exit/differentiation, including alterations in cyclin D1 mRNA expression and protein turnover. Instead, we identify PKCα as a novel repressor of cyclin D1 translation, acting at the level of cap-dependent initiation. Inhibition of cyclin D1 translation initiation is mediated by PKCα-induced hypophosphorylation/activation of the translational suppressor 4E-BP1, association of 4E-BP1 with the mRNA cap-binding protein eIF4E, and sequestration of cyclin D1 mRNA in 4E-BP1-associated complexes. Together, these post-transcriptional effects ensure rapid disappearance of the potent mitogenic molecule cyclin D1 during PKCα-induced cell cycle withdrawal in the intestinal epithelium.

Tight control of cell proliferation is essential for maintenance of normal homeostasis in self-renewing tissues such as the intestinal epithelium. Previous studies have identified protein kinase C (PKC) signaling as an important negative regulator of cell growth/cell cycle progression in intestinal epithelial cells (1–3). Several members of the PKC family are predominantly expressed/activated in non-proliferating and terminally differentiated intestinal cells (4–6), pointing to a major function in regulation of post-mitotic events in this tissue. Consistent with this role, we have demonstrated that PKCα signaling triggers a program of cell cycle withdrawal in non-transformed intestinal crypt cells, paralleling the membrane translocation/activation of this enzyme precisely at the point of growth arrest within intestinal crypts in situ (3, 4, 7, 8). Although the extracellular signals that trigger growth arrest in the intestine in situ remain poorly characterized, a possible physiological activator of PKCα in this system is transforming growth factor β. This potent growth inhibitory factor is known to promote G0/G1 arrest in intestinal epithelial cells (9–11) and to activate PKCα signaling in other systems (12).

One of the earliest events following PKCα activation in intestinal epithelial cells is down-regulation of cyclin D1 (3, 7, 8), indicating that this molecule is an important target of PKCα control. Cyclin D1 is a key regulator of cell proliferation, acting as a mitogen sensor and linking extracellular signaling to the cell cycle machinery (13). Progression through early G1 involves the activity of holoenzymes consisting of cyclin D (D1, D2, or D3 depending on the cell type) in association with cdk4 or cdk6. Cyclin D-dependent kinases promote cell cycle progression by initiating phosphorylation/inactivation of the retinoblastoma growth suppressor protein in mid G1, a process that is completed later in G1 by cyclin E/cdk2. Cyclin D/cdk complexes also have an important non-catalytic function that involves sequestration of cdk-inhibitory proteins of the Cip/Kip family, thus relieving repression of cyclin E- and cyclin A-cdk2 activity.

Precise regulation of cyclin D1 accumulation is of critical importance. Increased expression of the molecule shortens the G1 interval in many cell types and can reduce/overcome dependence on physiological growth stimuli (14). Decreased levels of the protein, on the other hand, lengthen G1 phase and reduce proliferation (14, 15). Thus, cyclin D1 expression is subject to strict control at multiple levels, including transcription, message stability and nucleocytoplasmic transport, protein synthesis, and protein turnover (14, 16–21). Notably, aberrant overexpression of cyclin D1 is a key component of tumor development in various tissues, including the intestine (14, 22, 23), and both transcriptional and post-transcriptional mechanisms have been implicated in deregulation of cyclin D1 expression in tumors (14).

In this study, we have explored the mechanisms underlying PKCα-induced down-regulation of cyclin D1 in IEC-18 non-transformed intestinal crypt cells. Our analysis of cross-talk between PKC signaling and cyclin D1 control unveiled a novel function for PKCα as a negative regulator of cyclin D1 translation initiation. PKCα modulates the activity of key translational regulators, including eukaryotic translation initiation factor eIF4E and eIF4E-binding protein 1 (4E-BP1), to repress cyclin D1 protein synthesis during intestinal epithelial cell cycle withdrawal. Importantly, the engagement of translational rather than transcriptional mechanisms ensures a rapid effect (24), with disappearance of cyclin D1 protein preceding other hallmark events of cell cycle withdrawal, including induction of Cip/Kip cyclin-dependent kinase inhibitors and activation of the growth suppressor function of pocket proteins (3, 7, 8).

EXPERIMENTAL PROCEDURES
Materials—Anti-cyclin D1 (sc-753, sc-450), -PKCα (sc-8393), -PKCδ (sc-213), and -PKCe (sc-214) antibodies were from Santa Cruz Biotechnology. Antibodies to eIF4E, phospho-Ser-9-GSK-3β, 4E-BP1, and
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We have previously shown that activation of PKCa signaling in IEC-18 non-transformed intestinal crypt cells results in cell cycle withdrawal (3, 8). Maintenance of cell cycle arrest requires sustained activation of PKCa, and the effect is reversed coincident with down-regulation of the enzyme. As shown in Fig. 1, PKC-induced cell cycle blockade is associated with marked changes in the levels of cyclin D1. Activation of PKCa, -δ, and/or -ε (the only phorbol ester-responsive PKC isozymes

eIF2α were from Cell Signaling Technology. Anti-phospho-Ser-51-eIF2α antibody was from Stressgen, and anti-FLAG M2 Affinity Gel was from Sigma. Phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), 1,2-dioctanoyl-sn-glycero (DiC2), and lithium were from Sigma, and bryostatin 1 (Bryo) was from Biomol or LC laboratories. Bisindolylmaleimide I and Go6976 were from Alexis and Calbiochem, respectively.

Cell Culture and Drug Treatments—IEC-18 non-transformed rat intestinal epithelial cells were maintained as described (3). PKCa, -δ, and -ε were activated in subconfluent cells by treatment with PMA (100 nM), PDBu (1 μM), Bryo (100 nM), or DiC2 (20 μg/mL). Control cells were treated with the appropriate vehicle (ethanol or Me2SO). Membrane translocation/activation of PKC isozymes was analyzed by subcellular fractionation and anti-PKC isozyme immunoblotting as previously described (7, 8). For depletion of PKCα, -δ, and -ε, cells were treated with 1 μM PDBu for 16–24 h (7, 8). Selective down-regulation of PKCδ and -ε was accomplished by pulse treatment with 10 nM PMA for 15 min, followed by two washes in PBS and return to fresh medium for 24 h (7, 8). Inhibition of PKCα, -δ, and -ε activity, or of PKCα activity alone, was achieved using 5 μM bisindolylmaleimide I or 1 μM Go6976, respectively (8).

Analysis of Protein Expression—Cell lysis and immunoblot analysis were performed as described previously (4, 8). Blots were routinely stained with 0.1% Fast Green (Sigma) to confirm equal loading and even transfer. Primary antibody dilutions were: 1:1000 for cyclin D1, p-GSK-3β, p-eIF2α, PKCa, and PKCe; 1:2000 for eIF4E, 4E-BP1, total eIF2α, and PKCδ.

Northern Blot Analysis—Total cellular and cytoplasmic RNA were isolated using the RNaseasy system (Qiagen) as recommended by the manufacturer. Nuclear RNA was similarly isolated from the nuclear pellet generated during the cytoplasmic RNA isolation procedure. Northern blot analysis of RNA samples (10 μg) was performed using randomly primed, 32P-labeled probe corresponding to mouse cyclin D1 cDNA, and specific hybridization was detected by phosphorimaging as we have described (25).

Immunoprecipitation—Immunoprecipitation using 2–4 μg of sc-753 anti-cyclin D1 antibody and Protein A/G-plus-agarose slurry (Santa Cruz Biotechnology) was performed essentially as described (26). In some experiments, cells were lysed in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease/phosphatase inhibitor cocktails (Sigma), and extracts were incubated with 2–3 μg of sc-450 anti-cyclin D1 antibody. For immunoprecipitation of FLAG-tagged cyclin D1, transfected cells were lysed in 20 mM Tris, pH 7.6, 120 mM NaCl, 0.5% Nonidet P-40, and protease/phosphatase inhibitor cocktails, and lysates were incubated with 50 μl of anti-FLAG M2 Affinity Gel.

Analysis of Protein Stability—Cells were incubated in methionine-free Dulbecco’s modified Eagle’s medium containing 4 mM glutamine, 5 μg/mL of insulin, and 5% dialyzed fetal bovine serum for 30 min and then labeled with 94 μCi of [35S]Met/Cys Expre32P3S protein-labeling mixture (PerkinElmer Life Sciences) for 1 h, prior to the addition of PMA or vehicle (ethanol) for 1 h. [35S]Met/Cys was chased for various times in label-free complete medium containing PMA or vehicle, and cells were harvested for cyclin D1 immunoprecipitation using sc-753 anti-cyclin D1 antibody. Immunoprecipitates were resolved on 20% SDS-PAGE gels, transferred to nitrocellulose membrane, and 35S-labeled protein was detected and quantified using the Storm/ImagelQuant PhosphorImaging system (Amersham Biosciences). For analysis of cyclin D1 decay following protein synthesis inhibition, cells were treated with 50 μg/mL of cycloheximide after 1 h of PMA/vehicle treatment and harvested for Western blot analysis at various times thereafter.

Biosynthetic Labeling—IEC-18 cells were incubated in methionine-free medium as described above and treated with PMA or ethanol for 30 min. Cells were then incubated in [35S]Met/Cys and PMA/vehicle for the indicated times as described (19). Cyclin D1 was immunoprecipitated using sc-450 antibody, and immunoprecipitates were analyzed as above.

Plasmids and Transfection—FLAG-tagged wild-type and T286A mutant cyclin D1 constructs were gifts from Dr. C. Sheri (St. Jude Children’s Research Hospital, Memphis, TN). Destruction box mutants (R92Q and L32A) were generated from the wild-type cyclin D1 construct using the QuikChange site-directed mutagenesis kit (Stratagene) and appropriate primers (sense strands: GGGTGTGTCAGGCGATGTCAGAAG; GCGAGCATGGCGCAACGAG). Cells in 60-mm plates were transfected with 5 μg of each construct (0.5 μg for the T286A mutant) using Lipofectamine 2000 (Invitrogen).

In Vitro Cap Affinity Assay—Cap affinity chromatography was performed essentially as described (27). 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 cocktails), and particulate material was removed by centrifugation. Cell lysates (350–400 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.

Immunoprecipitation-RT-PCR Assay—PMA- or vehicle-treated cells were harvested in detergent-free lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM MgCl2, and 10% glycerol, containing protease/ phosphatase inhibitors and 56 units/ml of RNase inhibitor (Sigma)) (28). Following removal of particulate material by centrifugation, lysates were supplemented with Tween 20 (0.5%), RNase inhibitor (53 units/ml), and dithiothreitol (0.1 mM), and 4E-BP1-containing complexes were immunoprecipitated using rabbit anti-4E-BP1 antibody (1.8 μg) and Protein A Dynabeads (Dynal). Beads were then washed twice (5 min) with lysis buffer containing 0.5% Tween 20 and once with lysis buffer without Tween 20 and resuspended in 45 μl of lysis buffer. 25 μl were taken for RNA isolation as described (28), and 20 μl were boiled in 2× Laemmli sample buffer for immuno blot analysis. Levels of cyclin D1 mRNA were assessed by RT-PCR in the presence of 50 μCi of [α-32P]CTP using the Titanium one-step RT-PCR kit (Clonetech) and specific primers (GTCTTCCCGCTGGCCATGAACTAC; AAGAAAGTGCGAGCCATGGCGCAACGAG). PCR cycle number was empirically determined to be in the linear range of amplification. PCR products were resolved on 5% PAGE gels and visualized by phosphorimaging. Quantitative RT-PCR analysis of cyclin D1 mRNA levels in immunoprecipitates was performed using the Full Velocity SYBR Green QRT-PCR Master Mix (Stratagene) and a 7300 real-time PCR system (Applied Biosciences).

RESULTS

PKC/PKCα Signaling Promotes Down-regulation of Cyclin D1 in Intestinal Epithelial Cells

We have previously shown that activation of PKC signaling in IEC-18 non-transformed intestinal crypt cells results in cell cycle withdrawal (3, 8). Maintenance of cell cycle arrest requires sustained activation of PKCα, and the effect is reversed coincident with down-regulation of the enzyme. As shown in Fig. 1, PKC-induced cell cycle blockade is associated with marked changes in the levels of cyclin D1. Activation of PKCα, -δ, and/or -ε (the only phorbol ester-responsive PKC isozymes...
PKCa Inhibits Cyclin D1 Translation Initiation

FIGURE 1. Activation of PKC/PKCα in IEC-18 cells promotes down-regulation of cyclin D1. A, IEC-18 cells were treated with 100 nM PMA for the indicated times, and cyclin D1 expression was determined by immunoblot analysis. The loading control is a nonspecific band consistently seen on cyclin D1 immunoblots that is not affected by PKC agonist treatment. C, control cells; IB, immunoblot; B, effects of 100 nM PMA (P), 1 μM PdBu (PB), 100 nM Bryo (B), or 20 μg/ml Dic8 (D) on cyclin D1 protein levels in IEC-18 cells. C, control cells. C and D, PMA-induced down-regulation of cyclin D1 is PKC/PKCα dependent. C, cells were pretreated (30 min) with the general PKC inhibitor bisindolylmaleimide I (BIM) (5 μM) or were depleted of PKCα,-δ, and -ε by prolonged incubation with PdBu as described (7) (PKC-depleted) prior to addition of PMA for 2 h. D, cells were pretreated (30 min) with the PKCα-selective inhibitor G66976 (1 μM) and treated with PMA as in panel C. The increased levels of cyclin D1 seen in PKC-depleted and PKC inhibitor-treated IEC-18 cells reflects relief of a repressive effect of unstimulated/basal PKC/PKCa signaling on cyclin D1 accumulation in cycling cells. Data are representative of at least three independent experiments.

Degradation of cyclin D1 occurs via ubiquitin/proteasome-mediated proteolysis, triggered by its phosphorylation on Thr-286 by GSK-3β (20). An N-terminal (amino acids 29–32) destruction box (RXQXL) has also been implicated in regulating proteasomal degradation of cyclin D1 (18). Consistent with the absence of alterations in cyclin D1 stability, PKC activation did not decrease the levels of inhibitory phosphorylation on Ser-9 (Fig. 3B, upper panel), and inhibition of GSK-3β activity with Li⁺ did not block PMA-induced loss of cyclin D1 (although its accumulation following Li⁺ treatment confirmed a role for GSK-3β in normal turnover of cyclin D1 in IEC-18 cells) (middle panel). These data, together with the fact that PKC activation promoted the down-regulation of degradation-resistant cyclin D1 mutants, including the GSK-3-β phosphosite mutant T286A (TA, lower panel) and the destruction box mutants R29Q (RQ) and L32A (LA) (Fig. 3C), confirmed that PKC-induced loss of cyclin D1 protein does not involve acceleration of cyclin D1 turnover.

PKC/PKCa Signaling Inhibits Translation of Cyclin D1 in IEC-18 Cells

Activation of PKC/PKCa Inhibits Cyclin D1 Synthesis—[35S]Methionine biosynthetic labeling was performed to determine whether PKC signaling inhibits translation of cyclin D1. A reduction in radiolabeled cyclin D1 was detected in PMA-treated cells relative to vehicle-treated cells at each time point examined (Fig. 4). These data, together with the
FIGURE 3. PKC/PKCa activation does not alter the stability of cyclin D1 protein in IEC-18 cells. A, [35S]Met/Cys pulse-chase analysis of cyclin D1 protein degradation. Cells were labeled with [35S]Met/Cys and, following 1 h of PMA or vehicle treatment, were transferred to chase medium in the presence or absence of PMA. Cyclin D1 was immunoprecipitated at the indicated times of chase, and [35S]-labeling was quantified by SDS-PAGE and phosphorimaging (lower panel). B, PKC agonist-induced cyclin D1 down-regulation does not involve GSK-3β. Upper panel, IEC-18 cells were treated with PMA for the indicated times and subjected to immunoblot analysis of inhibitory phosphorylation of GSK-3β on Ser-9. Middle panel, immunoblot analysis of cyclin D1 levels in cells pretreated with the GSK-3β inhibitor LiCl (30 or 50 mM) for 16 h prior to addition of PMA for 2 h. Lower panel, immunoblot analysis of cyclin D1 levels in vehicle- or PMA-treated cells (2 h) expressing FLAG-tagged wild-type (wt) or T286A mutant (TA) cyclin D1. The identity of the slower migrating band as FLAG-tagged cyclin D1 was confirmed by comparison of its mobility with that of FLAG-tagged cyclin D1 immunoprecipitated with anti-FLAG M2 antibody (IP: Flag). The Riol destruction box is not required for PKC-induced loss of cyclin D1. Immunoblot analysis of cyclin D1 levels in whole cell lysates (upper panel) or FLAG immunoprecipitates (lower panel) from cells expressing FLAG-tagged wild-type (wt) or destruction box mutant (R29Q/RQ) or L32A (L41) cyclin D1 following treatment with PMA or vehicle for 2 h. All data are representative of at least three independent experiments.

failure of PMA to affect cyclin D1 mRNA levels or protein stability (Figs. 2 and 3), point to repression of cyclin D1 translation as a mechanism underlying loss of cyclin D1 in PKC agonist-treated cells.

PKC/PKCa Activates 4E-BP1, Inhibits Cap-dependent Translation Initiation, and Leads to Sequestration of Cyclin D1 mRNA in 4E-BP1-associated Complexes—To gain insight into the molecular basis for PKC-mediated inhibition of cyclin D1 synthesis, we examined the effects of PKC activation on key regulators of translation initiation, including eIF4E and eIF2α. eIF4E binds the 5′-cap structure (m7GpppN, where N is any nucleotide) found in the majority of eukaryotic mRNAs and associates with eIF4G and eIF4A to form eIF4F, a translation initiation complex that mediates recruitment of ribosomes to mRNA (31). A major mechanism for control of eIF4E function 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 (31). The hypophosphorylated α and β forms of 4E-BP1 compete with eIF4G for binding to eIF4E, thereby preventing eIF4F assembly and inhibiting cap-dependent translation. In contrast, the hyperphosphorylated γ form is unable to bind eIF4E. Immunoblot analysis revealed that PMA treatment leads to rapid hypophosphorylation/activation of 4E-BP1 (Fig. 5A), evident by the appearance of the faster migrating α/β forms within 15 min. 4E-BP1 hypophosphorylation was also seen with other PKC agonists including PdBu, Bryo, and DiC8 (Fig. 5B), and the PKC dependence of the effect was confirmed using PKCα-, PKCδ-, and PKCε-depleted cells (Fig. 5C). Use of cells depleted of PKCδ and γ, but not PKCα, by PMA pulse treatment (7) demonstrated that PKCα can mediate PMA-induced hypophosphorylation of 4E-BP1 (Fig. 5D), and a requisite role for PKCα signaling was confirmed using the PKCα-selective inhibitor Gö6976 (Fig. 5E).

To determine whether the PKC-induced accumulation of 4E-BP1 α/β phosphoforms reflected an increase in 4E-BP1 activity, the interaction of 4E-BP1 with eIF4E was examined using cap affinity chromatography. Extracts from IEC-18 cells treated with vehicle or PMA were incubated with Sepharose 4B-immobilized 7-methyl-GTP cap analog to capture eIF4E and its binding partners. Serum-stimulated and serum-starved cells were included as negative and positive controls for 4E-BP1/
eIF4E interaction, respectively. Immunoblot analysis of cap analog-bound proteins revealed a striking increase in levels of cap-bound 4E-BP1/H9251/H9252 phosphoforms in PKC agonist-treated cells, comparable with those in serum-starved cells (Fig. 5F). Consistent with inhibition of cap-dependent translation initiation, metabolic labeling studies revealed that PKC agonist treatment results in a reduction in overall protein synthesis in IEC-18 cells (supplemental Fig. S1).

To examine the relevance of changes in 4E-BP1 activity to cyclin D1 translation, an immunoprecipitation RT-PCR protocol was used to assess sequestration of cyclin D1 mRNA in 4E-BP1-associated complexes. 4E-BP1 was immunoprecipitated from control, PMA-treated, serum-stimulated, or serum-starved cells. WCL and immunoprecipitates were subjected to immunoblot analysis for eIF4E and 4E-BP1 (upper panel). N.S., nonspecific band; M, MW markers. Levels of cyclin D1 RNA in immunoprecipitates were analyzed by semiquantitative RT-PCR (middle panel). Parallel immunoprecipitation was performed with anti-PKCα antibody (confirmed by immunoblot analysis, middle panel) as a negative control for 4E-BP1/cyclin D1 mRNA complex formation. Levels of cyclin D1 RNA (relative to control) in cell extracts (Input) and immunoprecipitates (I.P.) were also assessed by quantitative real-time RT-PCR (lower panel). This analysis revealed an ~3-fold increase in levels of 4E-BP1-associated cyclin D1 mRNA in PMA-treated and serum-starved cells. All data are representative of three or more independent experiments.
PKCα Inhibits Cyclin D1 Translation Initiation

Loss of PKC/PKCα Signaling Results in Recovery of the Translational Machinery and Restoration of Cyclin D1 Levels

Members of the PKC family undergo down-regulation following activation (33). In IEC-18 cells, PMA treatment results in progressive down-regulation of phorbol ester-responsive PKC isozymes, albeit with different kinetics (Fig. 7A). PKCβ is down-regulated first, followed by PKCα and then PKCε. Activation-induced depletion/de-sensitization of PKC isoforms was associated with a gradual restoration of cyclin D1 protein expression, first noted at \( \sim 3 \) h of PMA treatment (Fig. 7B). Comparison of the timing of cyclin D1 recovery with the kinetics of PKC isoforms down-regulation demonstrated that the reappearance of cyclin D1 coincides more closely with loss of PKCα (see 3-h time point), a finding that further supports the role of PKCα as a key negative regulator of cyclin D1 accumulation in IEC-18 cells. As shown in Fig. 7, A and B, PKCα expression remains steady for \( \sim 2 \) h of PMA treatment, paralleling the loss of cyclin D1 protein, and levels of the enzyme are considerably reduced by 3 h, coincident with restoration of cyclin D1 levels. In contrast, PKCβ is markedly down-regulated by 1 h of PMA addition, and PKCε expression is maintained for longer than 4 h in these cells. Loss of PKC/PKCα signaling was also accompanied by reversal of PMA-induced hypophosphorylation/activation of 4E-BP1 (Fig. 7C, upper panel) and decreased association of the protein with cap analog (lower panel). Thus, PKC down-regulation is accompanied by recovery of the translational machinery and restoration of cyclin D1 levels in IEC-18 cells.

DISCUSSION

Cyclin D1 levels are low in proliferating intestinal crypt cells and are markedly decreased in post-mitotic cells (3, 34–36). This expression profile points to the existence of mechanisms for tight control of the accumulation of this key G1 progression factor in normal intestinal epithelial cells. Here we have identified PKCα, an important mediator of growth inhibitory signaling in a variety of biological systems (e.g. Refs. 1, 37, 38), as a negative regulator of cyclin D1 expression in the intestinal epithelium. The involvement of PKCα in mediating down-regulation of cyclin D1 was established by demonstrating that (a) PKC agonists promote loss of cyclin D1 in IEC-18 cells expressing PKCα as the only phorbol ester-responsive isozyme (3), (b) the PKCα-selective inhibitor Go6976 blocks PMA-induced cyclin D1 down-regulation (Fig. 1), and (c) recovery of cyclin D1 levels closely correlates with loss of PKCα expression/activity in this system (Fig. 7).

Down-regulation of cyclin D1 is an early consequence of PKCα activation in IEC-18 cells, evident by 15–30 min of PKC agonist treatment (Figs. 1, 7). Analysis of the molecular mechanisms underlying the effect excluded several pathways previously implicated in the disappearance of cyclin D1 during cell cycle arrest/cell differentiation. Northern blot analysis revealed no change in cyclin D1 mRNA levels or subcellular distribution during the initial phase of the response (Fig. 2), indicating that PKC-induced down-regulation of cyclin D1 does not involve altered availability of cyclin D1 mRNA (21, 39). Pulse-chase analysis further excluded a role for alterations in protein turnover (20), a conclusion that was substantiated by the ability of PKC/PKCα to promote down-regulation of GSK-3β phosphorylation site (T286A) and destruction box (R29Q and L32A) cyclin D1 mutants in IEC-18 cells (Fig. 3).

Instead, our findings provide the first evidence for the ability of

PKCα

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PKCα signaling to repress cyclin D1 synthesis by targeting cap-dependent initiation, a key point of transition control (Fig. 5). Inhibition of cap-dependent initiation is accomplished by rapid, PKCα-dependent hypophosphorylation/activation of the translational repressor 4E-BP1 and increased association of the α/β phosphoforms of the protein with eIF4E (Fig. 5), thus preventing assembly of the eIF4F complex that mediates recruitment of ribosomes to mRNA (31). Accumulation of cyclin D1 mRNA in 4E-BP1-associated complexes provides support for a direct effect on translation of cyclin D1, consistent with findings that active 4E-BP1 represses cyclin D1 synthesis in other systems (40). The marked effects of 4E-BP1 on cyclin D1 synthesis are also consistent with evidence that limited availability of eIF4F complexes selectively impairs translation of several strong growth promoters, including cyclin D1, whose mRNAs are weak for translation due to lengthy and highly structured 5′-untranslated regions (41).

A number of studies have reported effects of PKC signaling on translation initiation factors and modulators, including 4E-BP1 (42–44). However, in direct contrast to our findings, these studies have linked PKC signaling to enhanced translation initiation and phosphorylation/inactivation of 4E-BP1. This discrepancy likely reflects different functions of individual PKC family members. For example, in 293T and glioblastoma cells, inactivation of 4E-BP1 was attributed to the novel PKC signaling to enhanced translation initiation and phosphorylation/initiation factors and modulators, including 4E-BP1 (42–44).

During intestinal epithelial growth arrest in situ ([3, 34, 36, 48], migreted onto the villus (3, 34, 36, 48). Because PKC isozyme analysis, and Drs. E. Berleth, G. Das, and A. Karpf for critical review of the manuscript. We also thank Dr. C. Sherr for providing FLAG-tagged cyclin D1 constructs.

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