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PKC α TUMOR SUPPRESSION IN THE INTESTINE IS ASSOCIATED WITH TRANSCRIPTIONAL AND TRANSLATIONAL INHIBITION OF CYCLIN D1

Marybeth A. Pysz¹, Olga V. Leontieva¹, Nicholas W. Bateman¹, Joshua M. Uronis², Kathryn J. Curry¹, David W. Threadgill², Klaus-Peter Janssen³, Sylvie Robine⁴, Anna Velcich⁵, Leonard Augenlicht⁵, Adrian R. Black¹, and Jennifer D. Black^{1,*}

¹Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York, 14263

²Department of Genetics and the Lineberger Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599, USA

³Klinikum rechts der Isar, TUM, Munich, Germany

⁴UMR144 Institut Curie, Paris, France

⁵Department of Oncology, Albert Einstein Cancer Center, Montefiore Medical Center, Bronx, New York 10467, USA

Abstract

Alterations in PKC isozyme expression and aberrant induction of cyclin D1 are early events in intestinal tumorigenesis. Previous studies have identified cyclin D1 as a major target in the antiproliferative effects of PKC α in non-transformed intestinal cells; however, a link between PKC signaling and cyclin D1 in colon cancer remained to be established. The current study further characterized PKC isozyme expression in intestinal neoplasms and explored the consequences of restoring PKC α or PKC δ in a panel of colon carcinoma cell lines. Consistent with patterns of PKC expression in primary tumors, PKC α and δ levels were generally reduced in colon carcinoma cell lines, PKC β II was elevated and PKC ϵ showed variable expression, thus establishing the suitability of these models for analysis of PKC signaling. While colon cancer cells were insensitive to the effects of PKC agonists on cyclin D1 levels, restoration of PKC α downregulated cyclin D1 by two independent mechanisms. PKC α expression consistently (a) reduced steady-state levels of cyclin D1 by a novel transcriptional mechanism not previously seen in non-transformed cells, and (b) re-established the ability of PKC agonists to activate the translational repressor 4E-BP1 and inhibit cyclin D1 translation. In contrast, PKC δ had modest and variable effects on cyclin D1 steady state levels and failed to restore responsiveness to PKC agonists. Notably, PKC α expression blocked anchorage-independent growth in colon cancer cells via a mechanism partially dependent on cyclin D1 deficiency, while PKC δ had only minor effects. Loss of PKC α and effects of its re-expression were independent of the status of the APC/ β -catenin signaling pathway or known genetic alterations, indicating that they are a general characteristic of colon tumors. Thus, PKC α is a potent negative

*Address correspondence to: Jennifer D. Black, Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, New York 14263, Tel. 716 845-5766; Fax.716 845-8857; E-mail: E-mail: jennifer.black@roswellpark.org.

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regulator of cyclin D1 expression and anchorage-independent cell growth in colon tumor cells, findings that offer important perspectives on the frequent loss of this isozyme during intestinal carcinogenesis.

Keywords

Protein Kinase C; Cyclin D1; Transcriptional Control; Colon Cancer; 4E-BP1; β -Catenin; Mouse Models

Introduction

Disruption of the tight control of cell proliferation in self-renewing tissues such as the intestinal epithelium is a key step in tumor development. Increasing evidence points to protein kinase C (PKC) isozymes as important regulators of homeostasis in the intestine [1-3]. PKC is a family of at least 10 serine/threonine kinases that differ in cofactor requirements, tissue distribution, and substrate specificity. These differences likely contribute to isozyme-specific regulation of various cellular processes, including proliferation, differentiation, apoptosis/survival, and transformation [1-5]. The importance of PKC signaling in maintenance of intestinal homeostasis is underscored by frequent deregulation of this enzyme system in intestinal neoplasms [2,4]. Decreased abundance of PKC α , β I, δ , ζ , and η has been noted in human and rodent intestinal tumors, while levels of PKC β II and ι appear to be elevated during neoplastic transformation [2,4]. Notably, downregulation of PKC α is an early event in intestinal tumorigenesis, evident in preneoplastic aberrant crypt foci of azoxymethane-treated mice [6] and adenomas of *Apc*^{Min/+} mice [7,8].

The demonstration that PKC α is activated at the point of growth arrest in intestinal crypts *in situ* [5,9], and that PKC α signaling promotes cell cycle withdrawal in non-transformed intestinal epithelial cells (IECs) [3], points to a key role of this isozyme in regulating intestinal self-renewal. Consistent with these findings, increased expression of PKC α in APC mutant CaCo-2 colorectal cancer (CRC) cells decreased proliferation, increased differentiation, and attenuated the transformed phenotype, while reduced expression resulted in enhanced growth, decreased differentiation, and a more aggressive tumor phenotype [10,11]. Strong support for a tumor suppressor role of PKC α in the intestine comes from studies in PKC α knockout mice, which showed increased proliferative activity within intestinal crypts and spontaneous intestinal adenoma formation. Importantly, PKC α -deficient *Apc*^{Min/+} mice develop more aggressive tumors and exhibit reduced survival relative to PKC α -expressing littermates [8]. Although PKC α deficiency has been noted in APC/ β -catenin mutant intestinal tumors, it remains to be determined if loss of PKC α is directly linked to alterations in β -catenin signaling or is a more general characteristic of intestinal carcinogenesis. Moreover, the molecular basis for loss of PKC signaling during intestinal tumorigenesis has not been defined.

Downregulation of cyclin D1 is one of the earliest consequences of PKC α activation in non-transformed IECs, preceding other hallmark events of cell cycle withdrawal [3,12]. Thus, cyclin D1 appears to be a critical target of PKC α in intestinal crypt cells. Cyclin D1 is a potent pro-proliferative molecule that links extracellular signaling to the cell cycle machinery [13]. Aberrant expression of cyclin D1 is one of the most common abnormalities in cancer and a key component of tumor development in various tissues [14-16]. Importantly, cyclin D1 deficiency inhibits formation of APC-mutant intestinal tumors in mice [14,17], pointing to a direct role in intestinal tumorigenesis. However, a link between PKC α deficiency and cyclin D1 in intestinal tumors remains to be established.

To gain further insight into the role of PKC α in intestinal tumorigenesis, this study (a) compared the expression of PKC isozymes and cyclin D1 in a panel of murine and human intestinal tumors and human CRC cell lines, and (b) analyzed the effects of PKC α and PKC δ on cyclin D1 levels and anchorage-independent growth in various CRC cell lines. Our data demonstrate that PKC α is broadly lost in intestinal tumors of diverse genetic backgrounds and point to a specific role for PKC α in regulation of cyclin D1 accumulation in CRC cells, irrespective of β -catenin signaling status. Notably, PKC α suppresses cyclin D1 expression in colon cancer cells by two independent mechanisms: transcriptional repression and blockade of cap-dependent translation. Together, these findings provide a new perspective on the frequent loss of PKC α in intestinal neoplasia.

Experimental Procedures

Materials and Cell Lines

Antibodies were obtained from: Santa Cruz Biotechnology [anti-cyclin D1 (sc-753, sc-450), -PKC α (sc-8393, sc-208), -PKC β II (sc-210), -PKC δ (sc-213 and sc-213P blocking peptide), -PKC ϵ (sc-214), -GAPDH (sc-25778), -phospho-EGFR (Tyr¹¹⁷³) (sc-12351-R), -total EGFR (sc-03), and -actin (sc-7210) antibody]; Lab Vision Corp [anti-cyclin D1 antibody (clone SP4)]; BD Transduction Labs [anti-PKC δ antibody (610397) used for immunohistochemical analysis]; Chemicon [anti-total eIF4E-binding protein 1 (4E-BP1) antibody and peroxidase-conjugated goat anti-rabbit IgG]; Cell Signaling Technology [anti-nonphospho-Thr⁴⁶-4E-BP1 antibody]; Bio-Rad [peroxidase-conjugated goat anti-mouse IgG]; Epitomics Inc [anti-PKC α rabbit polyclonal antibody (1510-1) used for immunofluorescence]; Jackson ImmunoResearch [TRITC-conjugated donkey anti-rabbit IgG (711-025-152)]; and Sigma-Aldrich [anti-actin (A-2066)]. Phorbol 12-myristate 13-acetate (PMA), propidium iodide, cycloheximide (CHX), actinomycin D (ActD), and *p*-iodonitrotetrazolium violet were from Sigma-Aldrich. LY294002 was from Calbiochem.

Human CRC cell lines were from Dr. M.G. Brattain, except for DLD-1 and HCT-15 cells (Dr. R.J. Bernacki, RPCI), LS180 cells (Dr. Y. Ionov, RPCI), and Colo205, SW620, and HCT116 cells (ATCC). Normal Fetal Human Colon (FHC) cells and IEC-18 non-transformed rat intestinal crypt cells were from ATCC. Human cell lines were cultured in RPMI 1640, 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine. FHC cells were also cultured in ATCC Complete Culture Medium. IEC-18 cells were cultured as described [3].

Analysis of Protein and RNA Expression

Immunoblot analysis was performed as described [3,5]. Antibody dilutions were 1:1000 or 1:2000 for anti-cyclin D1; 1:1000 for anti-PKC β II and -PKC ϵ ; 1:1000 or 1:5000 for anti-PKC α and -PKC δ ; 1:1000 (sc-7210) or 1:10000 (A-2066) for anti-actin; 1:5000 for anti-4E-BP1; 1:2000 for anti-phospho-Thr⁴⁶-4E-BP1; 1:2000 for anti-GAPDH; and 1:100 for anti-phospho-EGFR (Tyr¹¹⁷³) and anti-total EGFR. In competition experiments, anti-PKC δ was incubated with blocking antigenic peptide at 5:1 (wt/wt) peptide to antibody ratio for 15 min prior to addition to the membrane. Northern blot analysis of total cellular RNA using randomly primed ³²P-labeled probe corresponding to mouse cyclin D1 cDNA and quantification of phosphorimaging data with ImageQuant (GE Healthcare) were as described [18].

Human Tissues

CRC tissues and corresponding normal mucosa were collected from 10 patients at RPCI, with written informed consent and approval by the Institutional Review Board.

Adenoviral Transduction and Anchorage-Independent Growth Assays

Adenoviruses expressing LacZ, PKC α , PKC δ , or kinase-dead PKC α (kdPKC α) were from Drs. T. Kuroki (Kobe University, Japan) and M.G. Kazanietz (University of Pennsylvania) [19]. Cyclin D1 adenovirus was from Vector Biolabs. Adenoviruses were amplified in HEK293 packaging cells using standard techniques [20] and titers were determined using the Adeno-X Rapid Titer kit (Clonetechn). Cells ($2 - 4 \times 10^5$), in reduced serum growth medium (2% FBS), were infected with adenovirus in suspension in 6-well plates. After 16 h, cells were rinsed with phosphate buffered saline (PBS), and incubated in complete growth medium for 48 h prior to analysis. LacZ, PKC α , or PKC δ adenoviruses were used at a multiplicity of infection (moi) of 20 infection units/cell (except RKO cells, which required 150 moi to achieve expression comparable with other cell lines). To ensure similar levels of kdPKC α (which is unstable in cells [21]) and wild-type PKC α , kdPKC α adenovirus was added at 500 moi. In PKC α /cyclin D1 co-expression experiments, each adenovirus was added at 20 or 40 moi.

Flow cytometry

Cell cycle distribution of adenovirus-infected cells was determined by flow cytometry of propidium iodide-stained cells and analysis with Modfit (Verity Software House) as described [3].

Anchorage-Independent Growth Assays

Cells (5×10^3) were plated in soft agarose as described [22]. After 1-2 weeks, colonies were stained with *p*-iodonitrotetrazolium violet (1 mg/mL) for 24 h and photographed on a transilluminator using a digital camera.

β -catenin silencing

β -catenin was silenced in HCT116 or DLD-1 cells by transient transfection with 100 nM ON-TARGET $plus$ SMARTpool β -catenin siRNA (Dharmacon) or non-silencing siRNA (Qiagen), according to manufacturer protocols (using Lipofectamine 2000, Invitrogen). β -catenin-silenced cells were infected with 20 moi LacZ or PKC α adenovirus 72 h post-transfection.

Carcinogen Treatment of Mice and Immunohistochemistry

Experiments involving mice were in accordance with institutional and national guidelines/regulations, with appropriate approvals. A/J and KK/HIJ mice (2-4 months old, Jackson Laboratories) received azoxymethane (10 mg/kg) in 4 weekly IP injections, and were euthanized 5-6 months after the first injection. Tissues were fixed in 10% neutral buffered formalin overnight and embedded in paraffin. Paraffin-embedded tissues were sectioned (7 μ m), deparaffinized, rehydrated, and incubated in 3% H₂O₂ (15 min) to block endogenous peroxidase activity. Antigen retrieval involved microwaving (2 \times 10 min) in citrate buffer. Sections were "blocked" with 0.03% casein (30 min) and incubated with Santa Cruz Biotechnology sc-208 anti-PKC α antibody [1 μ g/mL in PBS/Tween-20 buffer], BD Biosciences anti-PKC δ antibody (10 μ g/mL), or Lab Vision Corp SP4 anti-cyclin D1 antibody (1:100) for 1 h, followed by biotinylated anti-rabbit secondary antibody (Vector Labs, 1:250) and streptavidin HRP (1:20, Zymed). Sections were then incubated with Vectastain Elite ABC reagent (Vector Laboratories) and DAKO DAB chromogen solution (K3466), counterstained with hematoxylin, and imaged with an Olympus BPX41 microscope and DP70 digital camera.

Luciferase Reporter Assays

-1745CD1Luc and -163CD1Luc cyclin D1 promoter constructs were from Dr. R. Pestell (Kimmel Cancer Center) [23]. Adenovirus-infected DLD-1 cells were transfected and assayed for luciferase activity as described [18].

Immunofluorescence

Cells plated on glass coverslips were infected with adenovirus and incubated in fresh medium for 24 h prior to fixation in 2% formaldehyde/PBS and permeabilization in 0.2% Triton X-100 (PBS/Triton) as described [5]. Anti-PKC α antibody (Epitomics) and TRITC-conjugated donkey anti-rabbit secondary antibody were applied at 1:1000 and 1:100 dilution in PBS/Triton, respectively. Cells were imaged using a Zeiss Axioskop epifluorescence microscope (x63 Plan Aplanachromat (1.4 NA) objective lens) and Hamamatsu C7780 digital camera.

Results

Alterations in PKC α expression in neoplastic intestinal cells occur independently of perturbations in APC/ β -catenin signaling

To explore further the role of alterations in PKC α signaling in CRC, PKC α protein expression was profiled in a panel of mouse intestinal tumors and human CRC cell lines that reflect the range of genetic changes associated with colon cancer development (Supplementary Table 1) [24]. Immunohistochemical analysis of PKC α was performed in murine intestinal tumors expressing mutant *Apc* (from *Apc*^{Min/+} and *Apc*¹⁶³⁸ mice [25, 26]) or enhanced β -catenin signaling (from azoxymethane-treated KK/HIJ and A/J mice [27]), as well as in tumors which are wild-type for APC and β -catenin but mutant for K-Ras (from pVillin-*KRas*^{V12G} mice [28]) or deficient in *Muc2* (from *Muc2*^{-/-} mice [29]). Comparison of PKC α immunostaining in tumors and “normal” adjacent mucosa revealed loss of the enzyme in all neoplastic tissues examined, irrespective of genetic background (Figure 1A, A/J mice not shown), thus demonstrating for the first time that PKC α deficiency can occur independently of aberrations in APC/ β -catenin signaling.

Analysis was then extended to a panel of 18 human CRC cell lines. Consistent with expression patterns in mouse intestinal tumors (Figure 1A), a majority of human CRC cell lines express significantly reduced levels of PKC α protein compared with non-transformed IEC-18 cells and FHC human colonic crypt cells (Figure 1B). APC mutant (e.g., FET/DNR, GEO, DLD-1), β -catenin mutant (HCT-116, LS180), and APC/ β -catenin wild-type cells (RKO) exhibit decreased abundance of this isozyme, confirming that loss of PKC α occurs independently of APC/ β -catenin signaling status and providing further support for the importance of PKC α deficiency in intestinal tumor development.

CRC cell lines were also profiled for other members of the PKC family that have been linked to intestinal carcinogenesis, including PKC δ , β II, and ϵ . PKC δ levels were also generally decreased in CRC cell lines, irrespective of APC/ β -catenin status (Figure 1B). This expression pattern is consistent with its downregulation in murine and human neoplastic intestinal tissues (Figure 1C and [4]). Interestingly, anti-PKC δ antibody detected several specific bands in tumor cells (confirmed using blocking peptide; Figure 1B, *lower right panel*), but not in non-transformed cells, indicating that this isozyme may undergo differential post-translational modification as well as downregulation in colon tumors. In contrast to PKC α and δ , PKC β II levels were consistently higher in CRC cells, paralleling findings in experimental models of colon cancer (for reviews, see [2, 4]). PKC ϵ protein showed variability in total levels and in the relative proportion of the two major species detected in CRC cells, consistent with discrepancies in the literature regarding its expression in primary colon tumors [30-32]. Thus, CRC cell lines are similar to neoplastic intestinal tissues in that they generally express reduced levels of PKC α and δ , increased levels of PKC β II, and variable levels of PKC ϵ . These studies, therefore, identify a panel of human CRC cell lines that can be used to address the role of alterations in the PKC enzyme system in intestinal neoplasia.

Expression of PKC α decreases cyclin D1 steady-state levels in CRC cells

Based on evidence that cyclin D1 is a key target of PKC/PKC α signaling in non-transformed IECs [3,12,33], we explored the effect of restoring PKC α expression on cyclin D1 levels in CRC cells. Effects of PKC δ restoration were determined for comparison. Expression of PKC α , but not PKC δ , promoted a marked reduction in steady-state levels of cyclin D1 protein in all cell lines tested, with the exception of RKO cells (Figure 2A; RCA and HCT-15 data not shown). Expression of comparable levels of kdPKC α did not affect cyclin D1 abundance, pointing to a kinase-dependent effect (Figure 2B). Specificity for cyclin D1 was indicated by the failure of PKC α expression to promote consistent alterations in levels of cyclin E (Figure 2A, lower panel). PKC α did not alter cell cycle distribution in the majority of CRC cell lines (Supplementary Figure 1), confirming that cyclin D1 downregulation was not simply a reflection of PKC-induced cell cycle perturbation. Interestingly, increased expression of PKC α led to a reduction in cyclin D1 levels in SW620, FET, HCT116, and LS180 cells (Figure 2A, Supplementary Figure 4B), arguing that levels of PKC α are limiting even in CRC cells that retain expression of the protein (see Figure 1A). The effect appears to be specific to transformed cells since PKC α expression failed to affect steady-state cyclin D1 levels in non-transformed IEC-18 cells (Figure 2C). Together, the data point to PKC α as an important suppressor of cyclin D1 protein accumulation in CRC cells.

PKC α -mediated repression of cyclin D1 in CRC cells does not appear to be dependent on enhanced APC/ β -catenin signaling

The inability of PKC α to suppress cyclin D1 steady-state levels in APC/ β -catenin wild-type intestinal cells (RKO and IEC-18) suggested that the effect may be dependent on aberrant APC/ β -catenin signaling, a characteristic of a majority of CRC cells. To investigate this possibility, β -catenin activity was abrogated in DLD-1 (APC mutant) and HCT116 (β -catenin mutant) cells using siRNA, prior to expression of exogenous PKC α . Despite near-complete silencing of β -catenin expression, PKC α maintained the ability to promote marked downregulation of cyclin D1 steady-state protein levels in both cell lines (Figure 3A), thus excluding a direct link between aberrant APC/ β -catenin signaling and PKC α -induced effects. We next compared the subcellular distribution of PKC α in non-responsive RKO and IEC-18 cells with that in responsive HCT116 and DLD-1 cells. Immunofluorescence analysis detected PKC α in the cytoplasm of all PKC α -transduced cells (Figure 3B; IEC-18 and DLD-1 data not shown). Notably, a pool of the enzyme was also detected at the plasma membrane in responsive HCT116 and DLD-1 cells, but not in non-responsive RKO and IEC-18 cells (Figure 3B, arrows). Since membrane association of PKC can reflect enzyme activation [5], these data indicate that repression of cyclin D1 steady-state levels correlates with the presence of an intrinsic mechanism for PKC α stimulation. Responsive cell lines, therefore, appear to have at least two pools of transduced PKC α : a cytoplasmic pool that is available for future activation by exogenous PKC agonists, and a membrane-associated active pool that mediates repression of steady-state levels of cyclin D1. The latter appears to be absent in non-responsive cell lines.

PKC α expression decreases cyclin D1 transcription in CRC cells

To investigate mechanism(s) underlying PKC α -induced downregulation of cyclin D1 steady-state levels in CRC cells, cyclin D1 protein stability and mRNA expression were examined. Comparison of cyclin D1 protein decay rate in CHX-treated PKC α - and LacZ-expressing FET, FET/DNR, or DLD-1 cells excluded changes in cyclin D1 protein turnover (Supplementary Figure 2A and not shown). In contrast, Northern blot analysis detected a reduction in cyclin D1 mRNA levels in PKC α -transduced CRC cells (Figure 4A). The extent of inhibition varied among cell lines, ranging from 0.3- to 0.6-fold. Notably, PKC α expression failed to affect cyclin D1 message levels in RKO (Figure 4A) or IEC-18 cells (not shown), consistent with the failure to alter cyclin D1 steady-state protein expression. Northern analysis of kdPKC α -

expressing DLD-1 cells confirmed that catalytic activity is required for PKC α -mediated repression of cyclin D1 mRNA (Figure 4A, *lower panel*).

Analysis of ActD-treated cells demonstrated that PKC α does not alter the stability of cyclin D1 message in CRC cells (Supplementary Figure 2B), pointing to a transcriptional effect. To explore this possibility, LacZ- and PKC α -expressing DLD-1 cells were transfected with a construct in which luciferase expression is driven by 1745 bp of the cyclin D1 promoter (-1745CD1Luc) or by a promoter truncated at -163 bp (-163CD1Luc) [23]. PKC α expression reduced the activity of the -1745CD1 promoter by ~50%, relative to the LacZ control. In contrast, PKC α had no significant effect on the -163CD1 promoter, excluding non-specific effects of PKC α expression on transfection efficiency or general transcription, and pointing to the involvement of promoter elements between -1745 and -163. The extent of PKC α -induced repression of cyclin D1 promoter activity was comparable with the decrease in cyclin D1 mRNA accumulation, arguing that transcriptional modulation is the predominant mechanism underlying the reduction in cyclin D1 mRNA levels. Thus, these studies identify a novel mechanism of cyclin D1 regulation by PKC α in colon cancer cells.

Exogenous PKC α restores the ability of PKC agonists to downregulate cyclin D1 and inhibit cap-dependent translation

The data presented above demonstrate that PKC α expression reduces cyclin D1 steady-state levels in CRC cells by kinase-dependent transcriptional repression. Based on evidence that full activation of PKC/PKC α in non-transformed IEC-18 cells promotes near depletion of cyclin D1 via translational suppression ([12,33], Figure 5A, *left panel*), we examined the ability of exogenous PKC agonists to further suppress cyclin D1 expression in CRC cells. PMA failed to affect cyclin D1 levels in all untransduced (not shown) or LacZ-transduced CRC cell lines tested, including CRC cells that retain PKC α expression (e.g., HCT116, LS180), further supporting the notion that PKC α levels are limiting in these cells (Figure 5A, *right panel*; similar results were seen in FET, FET/DNR, HCT-15, and RCA cells). Notably, however, expression of exogenous PKC α , but not kdPKC α or PKC δ , restored the ability of PMA to promote near depletion of cyclin D1 protein in all CRC cells tested (Figure 5B,D).

PMA-induced downregulation of cyclin D1 in IEC-18 cells involves inhibition of cap-dependent translation initiation via PKC α -dependent dephosphorylation/activation of 4E-BP1 [12,33]. To determine if activation of exogenous PKC α affects translation in CRC cells, LacZ- and PKC α -transduced cells were treated with PMA for 2 h and analyzed for the presence of translation-repressive hypophosphorylated α/β forms of 4E-BP1, which migrate faster than the inactive hyperphosphorylated γ form in SDS-PAGE gels (Figure 5C, HCT116 and RKO data not shown). Samples were also directly examined for alterations in Thr⁴⁶ phosphorylation, using an antibody specific for the unphosphorylated residue. PKC activation did not significantly affect 4E-BP1 phosphorylation/activity in untransduced or LacZ-transduced cells (Figure 5C). In contrast, PMA promoted marked accumulation of translation-inhibitory α/β forms of 4E-BP1 in PKC α -expressing cells, but not in kdPKC α -expressing cells (Figure 5C,D). Thus, consistent with the mechanism of cyclin D1 downregulation identified in non-transformed IECs [12,33], expression of exogenous PKC α in CRC cells restores the ability of PKC agonists to regulate cyclin D1 protein expression via translational repression.

PKC α broadly inhibits anchorage-independent growth of CRC cells via a mechanism that is partially dependent on reduced cyclin D1 expression

PKC α reduces the growth and *in vitro* tumorigenicity of APC mutant CaCo-2 and HT-29 CRC cells [10,11,34]. To determine if PKC α is broadly tumor suppressive in CRC cells with different genetic alterations and degrees of differentiation, the ability of PKC α to inhibit soft agarose colony formation was tested in a panel of CRC cell lines. Effects of PKC α were compared with

those of PKC δ . PKC α expression generally promoted near complete inhibition of anchorage-independent growth in CRC cell lines (Figure 6A, Supplementary Figure 3), consistent with a strong tumor suppressive activity of this isozyme in colon cancer [10,11,34]. The failure of kdPKC α to significantly affect colony formation pointed to a kinase-dependent effect (Figure 6B). Interestingly, restoration of PKC α was unable to affect anchorage-independent growth of RKO cells (Figure 6A), which are also resistant to the suppressive effects of the enzyme on cyclin D1 steady-state levels (Figure 2A). To further explore the contribution of cyclin D1 deficiency to the growth inhibitory actions of PKC α , DLD-1 and HCT116 cells were co-transduced with cyclin D1 or LacZ adenovirus and PKC α adenovirus, and plated in soft agar. Notably, co-transduction with cyclin D1 led to a ~10-fold increase in colony number in PKC α expressing cells, while having no effect in LacZ control cells (Figure 6C). This partial rescue indicates that cyclin D1 is limiting for anchorage-independent growth of PKC α -expressing CRC cells, while pointing to the existence of additional mediators of the powerful tumor suppressive effects of PKC α in CRC cells (e.g., p21^{waf1}) [10].

PKC δ expression also reduced anchorage-independent growth of CRC cells (Figure 6A, Supplementary Figure 3), although this suppression was more modest and variable (ranging from little effect in RKO and GEO cells to near complete inhibition in HCT116 and LS180 cells). Thus, consistent with its effects on cyclin D1 regulation, PKC α appears to be more effective than PKC δ in suppressing the transformed phenotype of CRC cells.

Inverse relationship between PKC α and cyclin D1 expression in neoplastic intestinal tissues and cells with and without perturbations in APC/ β -catenin signaling

To determine if there is a relationship between expression of PKC α and cyclin D1 in intestinal tumors with different genetic backgrounds, immunohistochemical analysis was performed in murine intestinal tumors that are *Apc* mutant, β -catenin-mutant, or wild-type for APC/ β -catenin but mutant for K-Ras or deficient in Muc2. Markedly increased levels of cyclin D1 were consistently observed in neoplasms arising in all of these mice (Figure 7A and not shown). In contrast, levels of PKC α expression were consistently reduced in these neoplastic tissues (Figure 7A). Thus, reciprocal changes in the expression of cyclin D1 and PKC α are seen at early stages of tumor development in a range of mouse models of intestinal cancer, consistent with a potential link between these events.

Cyclin D1 and PKC α protein levels were also surveyed in a panel of 12 human CRC cell lines used in this study. Notably, all of these cell lines (except for SW620) express increased levels of cyclin D1 relative to non-transformed IEC-18 cells (Figure 7B). While a majority of these cell lines showed a concomitant reduction in PKC α levels, some lines (e.g., HCT116 and LS180) showed upregulation of cyclin D1 in the context of significant levels of PKC α . Our findings (Figure 5A) indicate that this expression pattern may reflect limiting levels of PKC α signaling in these cells, a defect that can be overcome by increased expression of wild-type enzyme.

Discussion

PKC α is growth inhibitory in non-transformed IECs and has been implicated in tumor suppression in APC/ β -catenin mutant intestinal neoplasms [3,5-11,30,34,35]. This study extends previous findings by providing the first demonstration that the tumor suppressive effects of PKC α signaling, as well as early loss of PKC α protein, occur independently of specific genetic alterations or degree of differentiation. The study further demonstrates that an important target of PKC α regulation in CRC cells is the potent mitogen cyclin D1. PKC α is shown to suppress cyclin D1 accumulation in CRC cells independently of β -catenin signaling, and cyclin D1 deficiency is implicated in PKC α -induced inhibition of anchorage-independent growth. Two distinct mechanisms of PKC α -mediated cyclin D1 control are identified:

transcriptional repression and blockade of cap-dependent translation. Finally, to our knowledge, this study is the first to report a comprehensive analysis of PKC isozyme expression in a panel of CRC cells, and to validate these cells as models for studies on the PKC enzyme system in intestinal tumors.

PKC α is downregulated in murine intestinal tumors and in human CRC cells that are APC mutant (e.g., *Apc*^{Min/+}, *Apc*¹⁶³⁸ and *KRas/Apc*^{+/1638N} mice, and FET/DNR, DLD-1, GEO, HCT-15, and RCA cells) or β -catenin-mutant (e.g., azoxymethane-treated mice and HCT116 and LS180 cells), as well as in neoplastic intestinal tissues and cells that are APC/ β -catenin wild-type (i.e., pVillin-*KRas*^{V12G} and *Muc2*^{-/-} mice, and RKO cells). PKC α signaling appears to be limiting even in CRC cells that retain expression of the enzyme (e.g., FET, HCT116 and LS180), as indicated by their resistance to the effects of PKC agonists on cyclin D1 expression, a deficiency that can be overcome by exogenous expression of the enzyme. Although the underlying mechanisms remain to be delineated, defective PKC α signaling appears to be a general characteristic of early intestinal tumorigenesis, and alterations in this pathway are likely related to its negative growth effects rather than a secondary consequence of specific alterations in individual tumors.

Previous studies in CaCo-2 [10,11] and HT-29 [34] cells, which are capable of enterocytic differentiation *in vitro* and form moderately well- or well-differentiated tumors in mice [36], demonstrated the ability of PKC α signaling to inhibit anchorage-independent growth. The finding that PKC α expression inhibits colony formation in soft agarose of highly aggressive, poorly differentiated cells (DLD-1, HCT116, and FET/DNR), intermediately aggressive, moderately differentiated cells (RCA), as well as non-aggressive, well-differentiated cells (GEO), argues that PKC α retains its tumor suppressive effects even in advanced tumors. This notion is consistent with evidence that PKC α deficiency not only increases the number of early lesions (i.e., aberrant crypt foci and adenomas) in the murine intestine, but also promotes the development of aggressive intestinal adenocarcinomas in *Apc*^{Min/+} mice [8].

Loss of PKC α and upregulation of cyclin D1 are both early events in intestinal tumorigenesis (Figure 7 and [6,7,37]). Evidence in support of a direct relationship between these events includes (a) reciprocal changes in the abundance of PKC α and cyclin D1 in diverse mouse models of intestinal neoplasia and human CRC cell lines; (b) rapid downregulation of cyclin D1 protein in non-transformed IECs in response to PKC α activation [12,33]; (c) the failure of PKC agonists to downregulate cyclin D1 in colon tumor cells, which have limiting levels of PKC α signaling; (d) the ability of PKC α re-expression to reduce steady-state levels of cyclin D1 in CRC cells and to re-establish PKC agonist-mediated downregulation of the protein; and (e) the involvement of cyclin D1 deficiency in PKC α -induced suppression of CRC cell growth in soft agar.

Analysis of the effects of PKC α re-expression in CRC cells has identified two distinct mechanisms of PKC α -induced cyclin D1 repression. In addition to agonist-induced translational effects mediated by activation of 4E-BP1, PKC α re-expression inhibits steady state levels of cyclin D1 by a novel transcriptional mechanism not previously identified in non-transformed IECs. Notably, both of these mechanisms appear to be defective in tumor cells, even in those that retain appreciable levels of PKC α . The fact that the defect could be rescued by expression of exogenous PKC α likely reflects reduced sensitivity of downstream signaling components to levels of PKC α activation or defects in activation of the endogenous enzyme. In regard to the latter possibility, it is interesting to note that the migration of PKC α in SDS-PAGE gels often differed between CRC cells and non-transformed IECs (Figure 1A), pointing to a potential difference in post-translational modification. The precise defects in CRC cells are currently under investigation. Our data also indicate that the transcriptional and translational effects on cyclin D1 can occur independently: while endogenous levels of

PKC α agonist activity in CRC cells were sufficient to elicit transcriptional effects following PKC α re-expression, further activation with pharmacological agonists was required for the translational effects. Whether this reflects differential sensitivity to levels of PKC activation or unique effects of different stimuli remains to be determined.

The ability of PKC α to downregulate cyclin D1 in CRC cell lines did not require activated β -catenin signaling and was independent of known genetic changes. While our studies in non-transformed IECs point to a role for PP2A in the translational effects of PKC α [33], downstream effectors that mediate the reduced steady-state levels of cyclin D1 mRNA are not immediately apparent. A potential target is the epidermal growth factor receptor (EGFR) which can promote cyclin D1 expression through various signaling intermediates [38]. Inhibition of EGFR activation by PKC α has been noted in a number of systems (e.g., [39-41]) and was also seen following re-expression of the isozyme in CRC cells (Supplementary Figure 4A). However, while this receptor is likely to play a role in the regulation of cyclin D1 under some circumstances, our data point to the involvement of additional factors. PKC α repressed cyclin D1 (both at the steady state level and in response to PKC agonist treatment) in SW620 cells which lack EGFR expression [42,43]. Indeed, EGFR-independent mechanisms appear to play a significant role even in cells that express the receptor since (a) PKC α is downregulated in tumors and CRC cell lines with activating mutations in proteins downstream of EGFR (e.g., KRas mutant mice and KRas/PI3K mutant DLD1, HCT116 and HCT-15 cells [44]), mutations that correlate with insensitivity to EGFR inhibition [44,45], and (b) effects of PKC α on cyclin D1 mRNA levels did not correlate with sensitivity of CRC cells to EGFR inhibitors [44] (e.g., the effect seen in resistant HCT116 and HCT-15 cells was comparable with that in sensitive GEO cells (Figure 4A and data not shown). These EGFR-independent mechanisms are currently under investigation.

A role for downregulation of cyclin D1 in the tumor suppressive effects of PKC α is consistent with known functions of this cyclin in tumorigenesis [13,14,16,17]. Overexpression of cyclin D1 mRNA and protein is a common characteristic of various cancers, including those of the intestine, where it appears to be caused by *trans*-acting influences as opposed to genetic mutation/rearrangement [46]. A direct role in intestinal tumorigenesis is supported by evidence that cyclin D1-deficiency (a) reduces the number and size of tumors in *Apc*^{Min/+} mice [14], (b) inhibits intestinal tumor progression following conditional loss of APC *in vivo* [17], and (c) reverses the transformed phenotype of SW480 CRC cells [15]. The finding that PKC α -induced downregulation of cyclin D1 in CRC cells does not affect the cell cycle (Supplementary Figure 2) suggests that levels of this cyclin are not limiting for growth of tumor cells under 'optimal' conditions (as seen in SW480 cells [15]). The high levels of cyclin D1 in CRC cells may, thus, be required to maintain cell growth in the less favorable environments found in tumors.

Previous studies also support a role for PKC δ in tumor suppression in the intestine [47]. Expression of this isozyme is also reduced/lost in rodent and human intestinal neoplasms and CRC cell lines (this study and [4,7,30,32,48]). Our comparative analysis demonstrates that, although PKC δ variably affects CRC cells of different genetic background, PKC α is generally more potent in blocking soft agar colony formation, suppressing cyclin D1 steady-state levels, re-establishing downregulation of cyclin D1 in response to PKC agonists, and mediating translational inhibition in response to PMA. Thus, these two isozymes are likely to mediate their tumor suppressive effects through different mechanisms.

Taken together, our findings point to cyclin D1 repression as an important component of PKC α tumor suppression in intestinal cells. Since PKC α has been shown to have tumor suppressive properties in other tissues (e.g., epidermis [49,50], pituitary and thyroid [51]), these findings may be relevant to multiple tumor types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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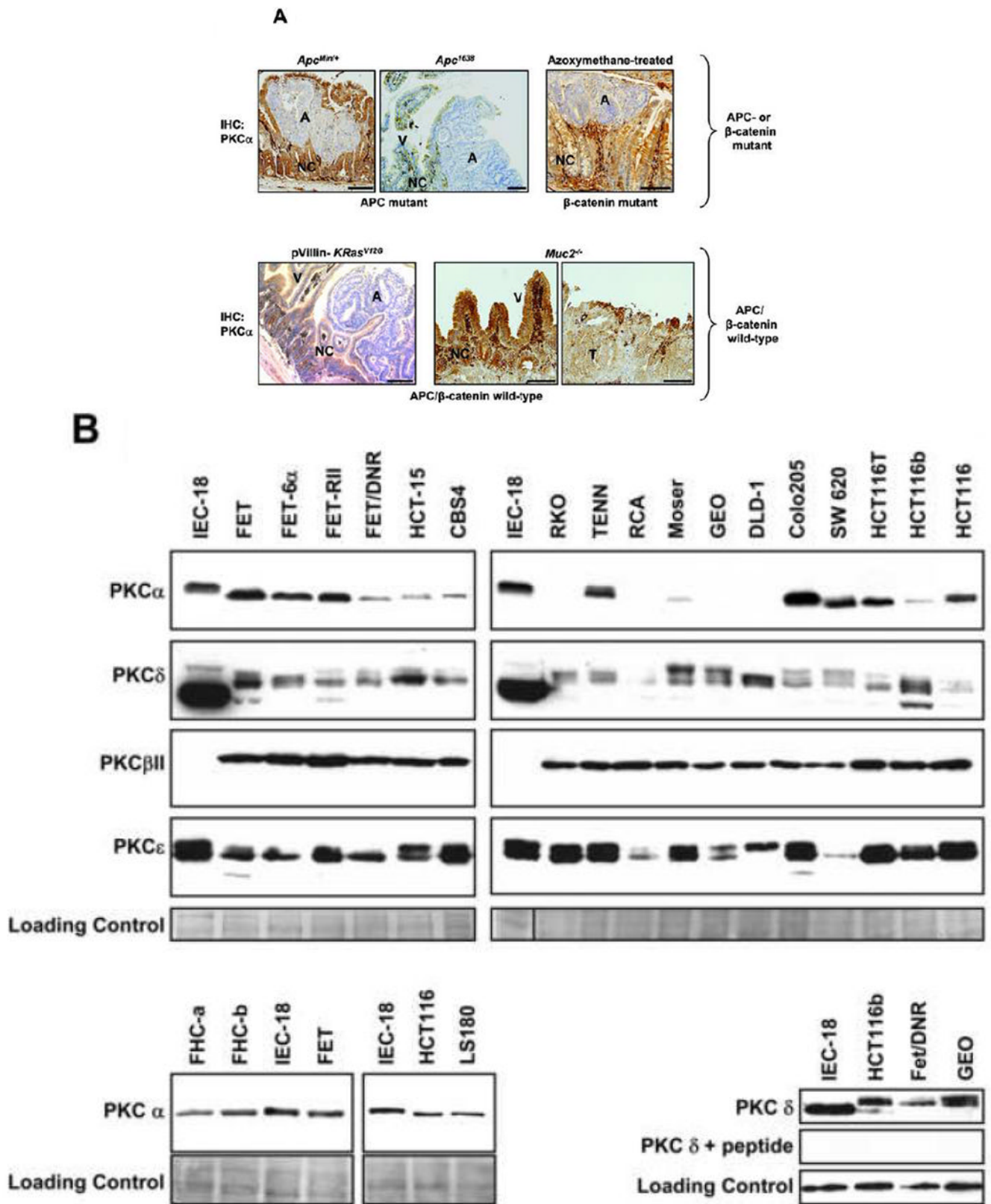
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Abbreviations

ActD	actinomycin D
APC	adenomatous polyposis coli
CHX	

	cycloheximide
CRC	colorectal cancer
4E-BP1	eIF4E-binding protein 1
FBS	fetal bovine serum
FHC	fetal human colon
IEC	intestinal epithelial cell
ifu	infection units
kdPKCα	kinase-dead PKC α
moi	multiplicity of infection
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
RPCI	Roswell Park Cancer Institute
TGF	transforming growth factor



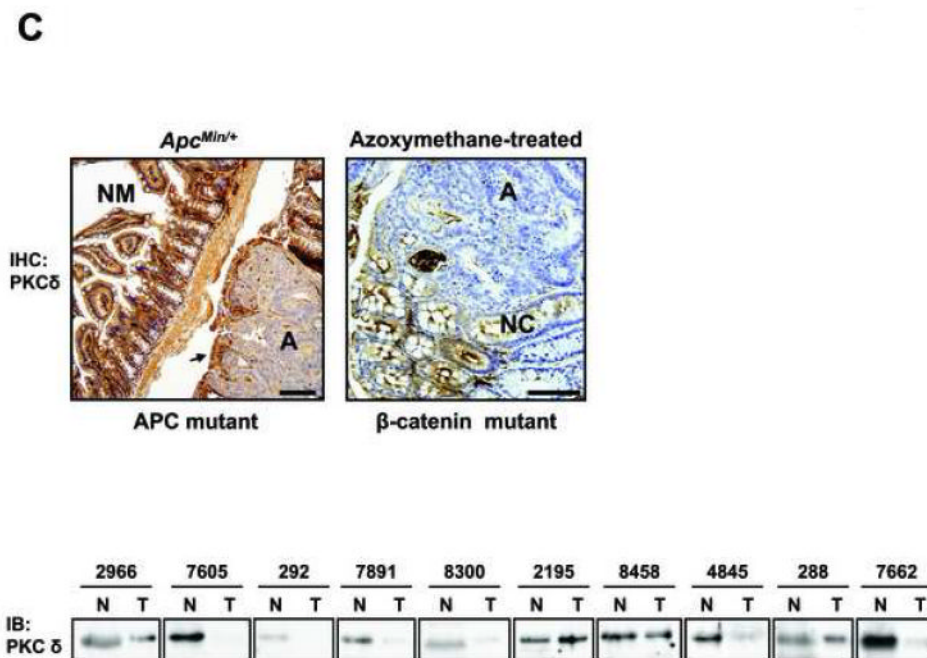


Figure 1. PKC isozyme expression is altered in neoplastic intestinal tissues and cell lines
(A) Immunohistochemical analysis of PKC α expression (brown staining) in adenomas (A) or tumors (T) and adjacent normal crypt (NC) and villus (V)/surface mucosa cells from *Apc^{Min/+}* and *Apc¹⁶³⁸* mice (APC mutant), [25], [26] azoxymethane-treated KK/HIJ mice (β -catenin mutant), [27] and *Apc*/ β -catenin wild-type pVillin-*KRas^{V12G}* mice [28] and *Muc2^{-/-}* mice [29]. Magnification Bar: 100 μ m. PKC α is diffusely distributed throughout the cytoplasm of normal, proliferating intestinal crypt cells (NC) (likely in an inactive conformation), largely at the plasma membrane/active in normal post-mitotic intestinal cells (V), and absent from neoplastic cells.
(B) Immunoblot analysis of PKC isozymes in human CRC cells and non-transformed IEC-18 and FHC cells [cultured in the same medium as CRC cells (FHC-a) or in ATCC Complete Culture Medium (FHC-b)]. The faster mobility PKC α species seen in CRC cells likely lacks phosphorylation at Ser⁶⁵⁷, one of the priming sites on the enzyme (Leontieva and Black, unpublished data). PKC δ consistently showed differential migration patterns in CRC cells. *Lower right panel:* Peptide competition analysis of the specificity of PKC δ immunoblotting. Samples were probed with anti-PKC δ antibody or anti-PKC δ antibody pre-incubated with blocking peptide. Data represent ≥ 3 independent experiments.
(C) *Top panel:* Immunohistochemical analysis of PKC δ expression in adenomas (A) and adjacent normal mucosa (NM)/normal crypt (NC) cells from *Apc^{Min/+}* and azoxymethane-treated KK/HIJ mice. Magnification Bar: 100 μ m. *Lower panel:* Immunoblot analysis of PKC δ expression in extracts of colon tumor tissue (T) and adjacent normal colonic mucosa (N) from 10 patients. Equal loading was confirmed by fast green staining (not shown).

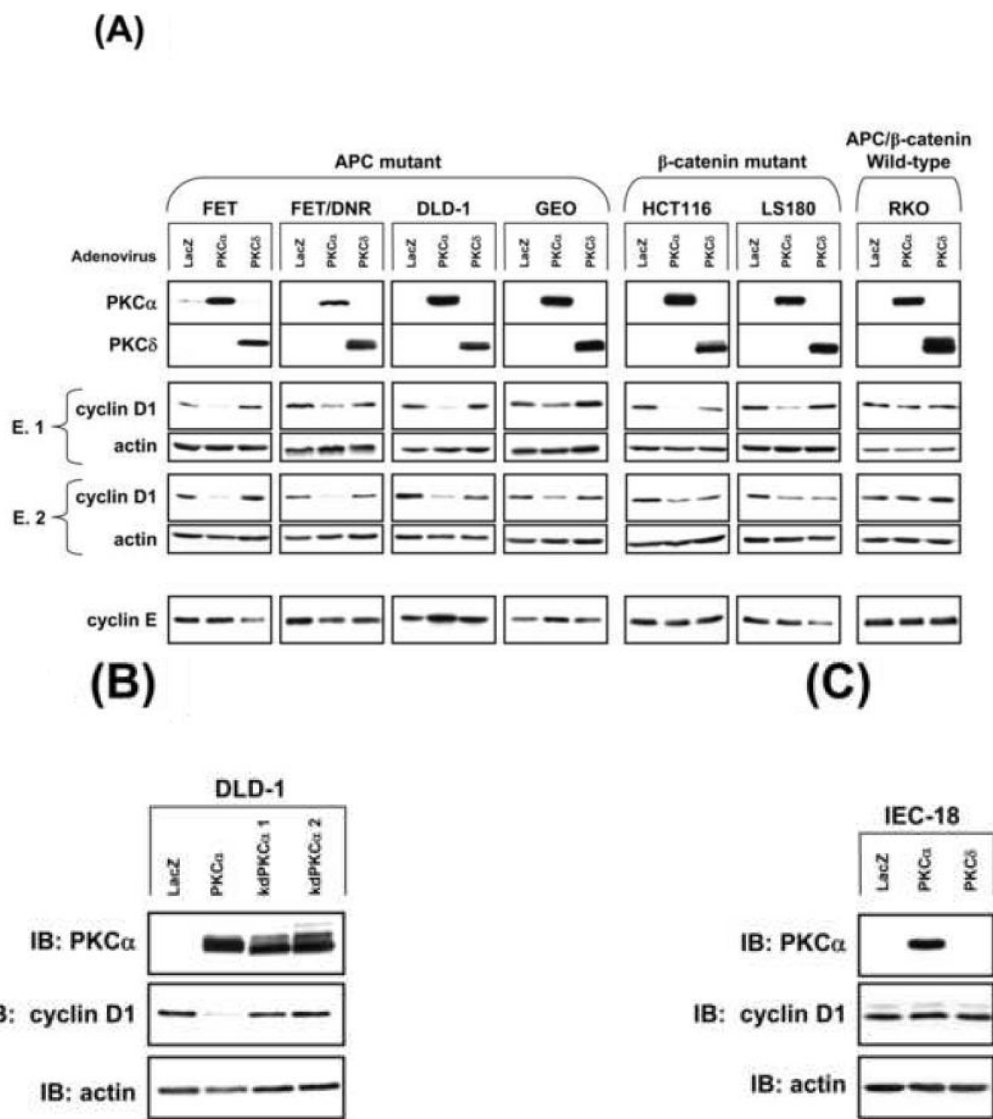


Figure 2. PKCα represses cyclin D1 protein expression in CRC cells
(A) CRC cells infected with 20 moi LacZ, PKCα, or PKCδ adenovirus (150 moi for RKO) were subjected to immunoblot analysis as indicated. Data represent >3 independent experiments; two experiments (E.1 and E.2) are shown to demonstrate consistency of results.
(B) Immunoblot analysis of DLD-1 cells infected with 20 moi LacZ, 20 moi PKCα, 500 moi (kdPKCα1) or 700 moi (kdPKCα2) kdPKCα adenovirus (increased moi compensated for instability of the kinase dead enzyme[21]).
(C) Immunoblot analysis of transduced (20 moi) IEC-18 cells.

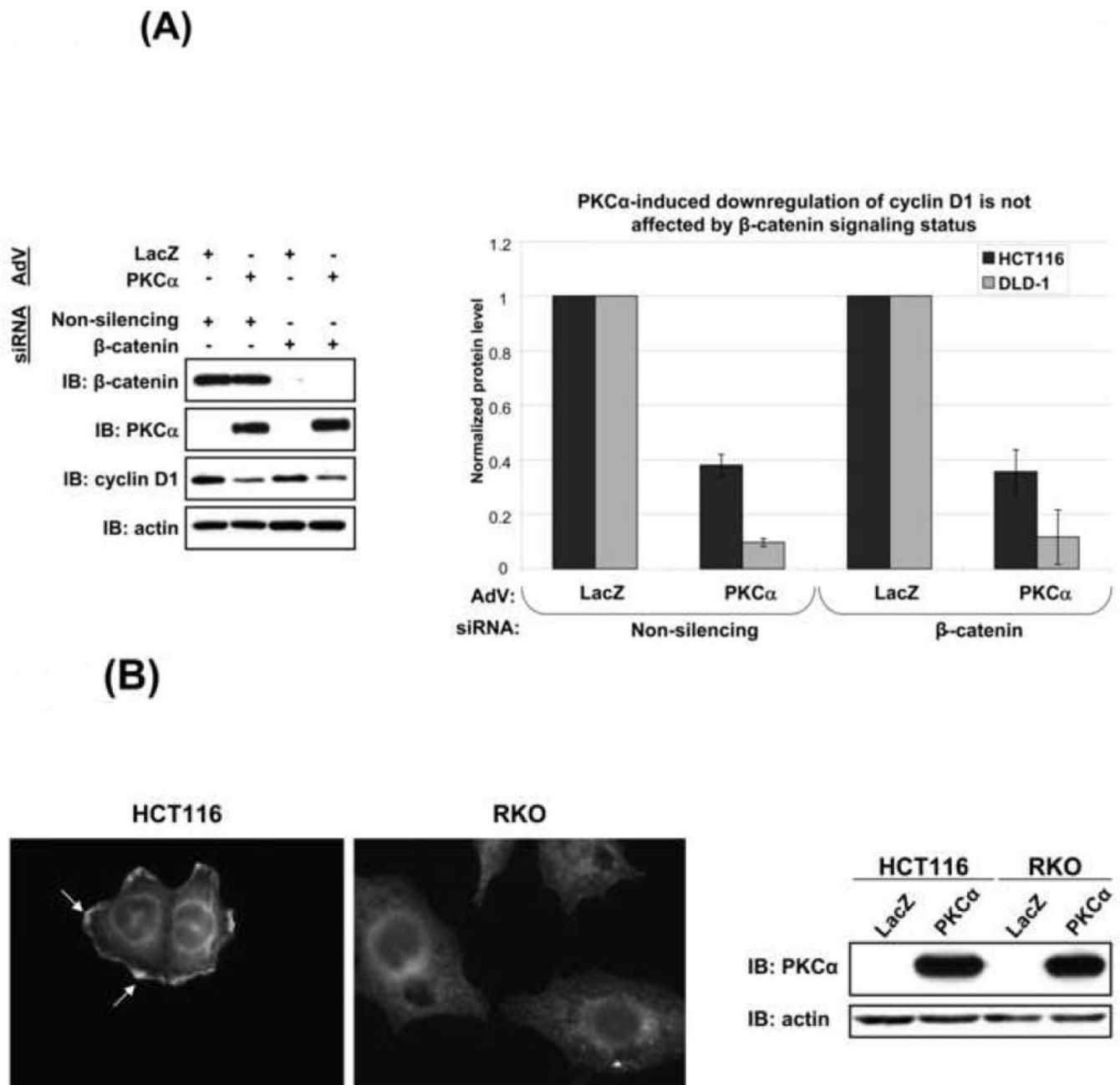
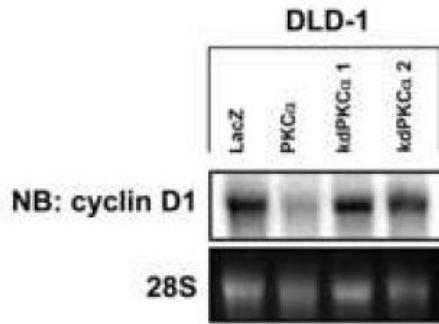
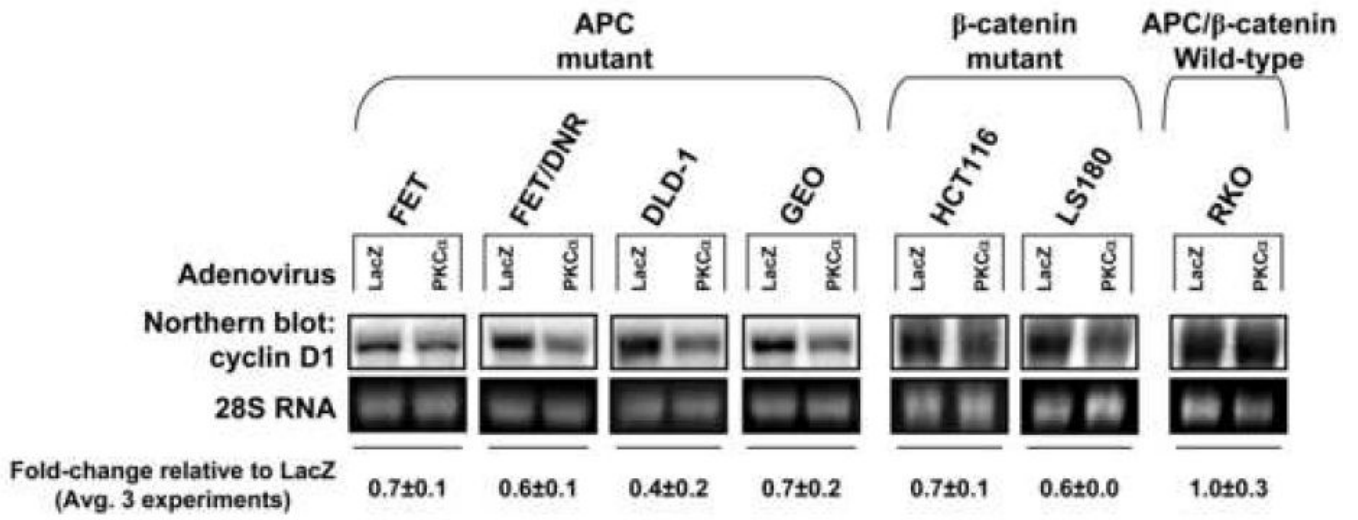


Figure 3. PKC α -induced repression of cyclin D1 occurs independently of aberrant β -catenin signaling and may be regulated by PKC α subcellular distribution

(A) DLD-1 and HCT116 cells were transfected with 100 nM non-silencing or β -catenin siRNA and infected with 20 moi LacZ or PKC α adenovirus 72 h later. *Left panel*: Immunoblot analysis of HCT116 cells 48 h after adenoviral infection. *Right panel*: Quantification of cyclin D1 expression (normalized to actin) for 2 (DLD-1) or 3 (HCT116) experiments (average \pm s.e.). (B) Immunofluorescence detection of PKC α in PKC α -transduced HCT116 (20 moi) and RKO cells (150 moi). *Arrows*: membrane staining. *Right panel*: Immunoblot analysis confirmed similar PKC α expression levels in these cells following adenoviral infection. Data represent ≥ 2 independent experiments.

(A)



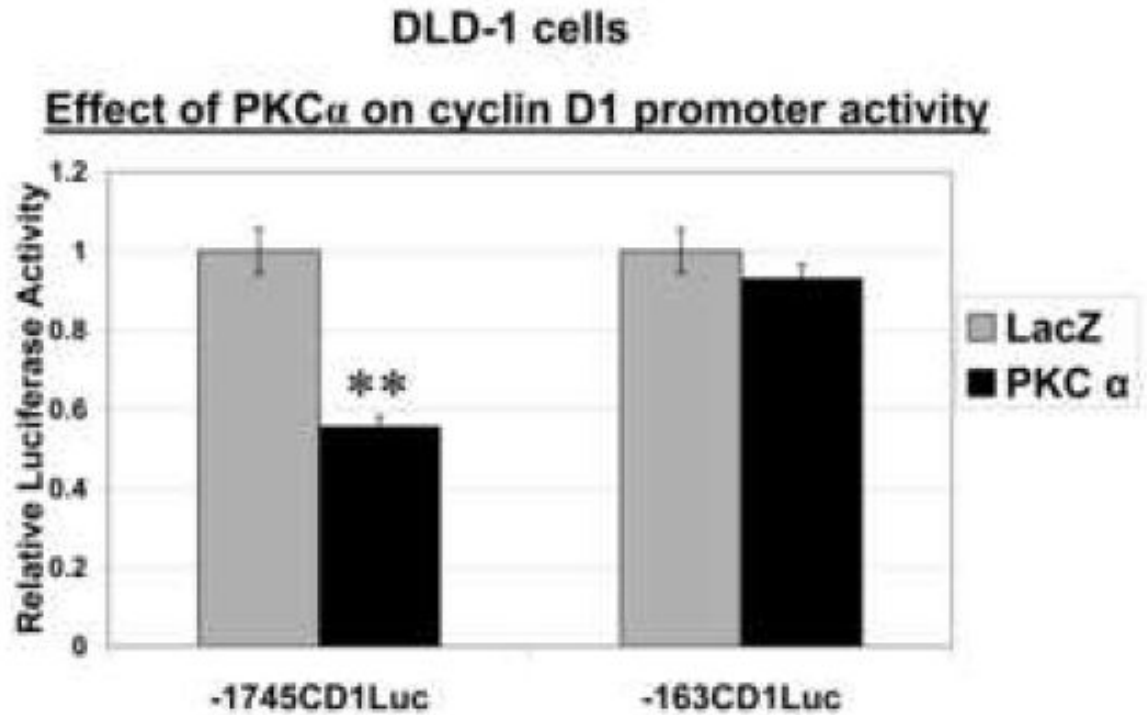
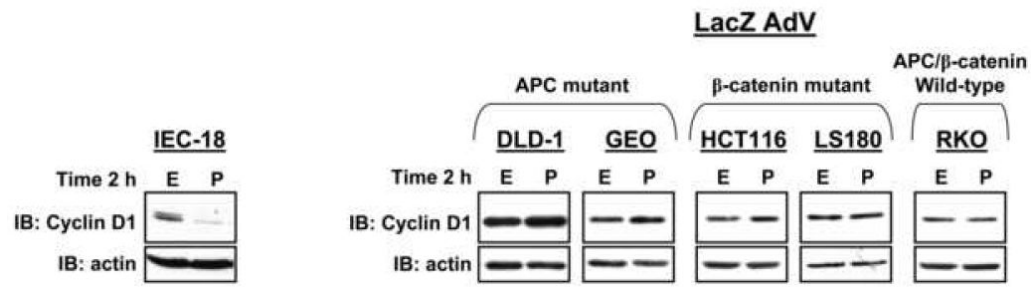
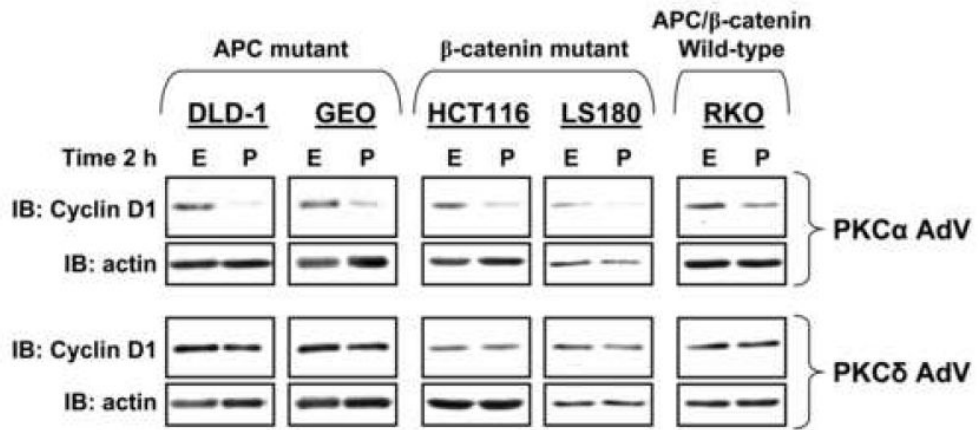
(B)

Figure 4. PKC α reduces cyclin D1 steady-state levels through transcriptional repression
(A) PKC α suppresses cyclin D1 mRNA accumulation. *Upper panel:* Northern blot analysis of cyclin D1 mRNA in adenovirus-transduced cells. Numbers show levels of cyclin D1 mRNA (normalized to 28S rRNA) in PKC α -expressing cells relative to LacZ-transduced cells (averages \pm s.e.). *Lower panel:* Northern blot analysis of DLD-1 cells infected as in Figure 2B. **(B)** LacZ- or PKC α -transduced DLD-1 cells were transfected with the indicated cyclin D1 promoter-luciferase reporter constructs, and luciferase activity (expressed relative to LacZ-transduced cells) was determined 24 h later. ** Significantly different from LacZ control ($P<0.005$).
 Data represent ≥ 3 independent experiments.

(A)



(B)



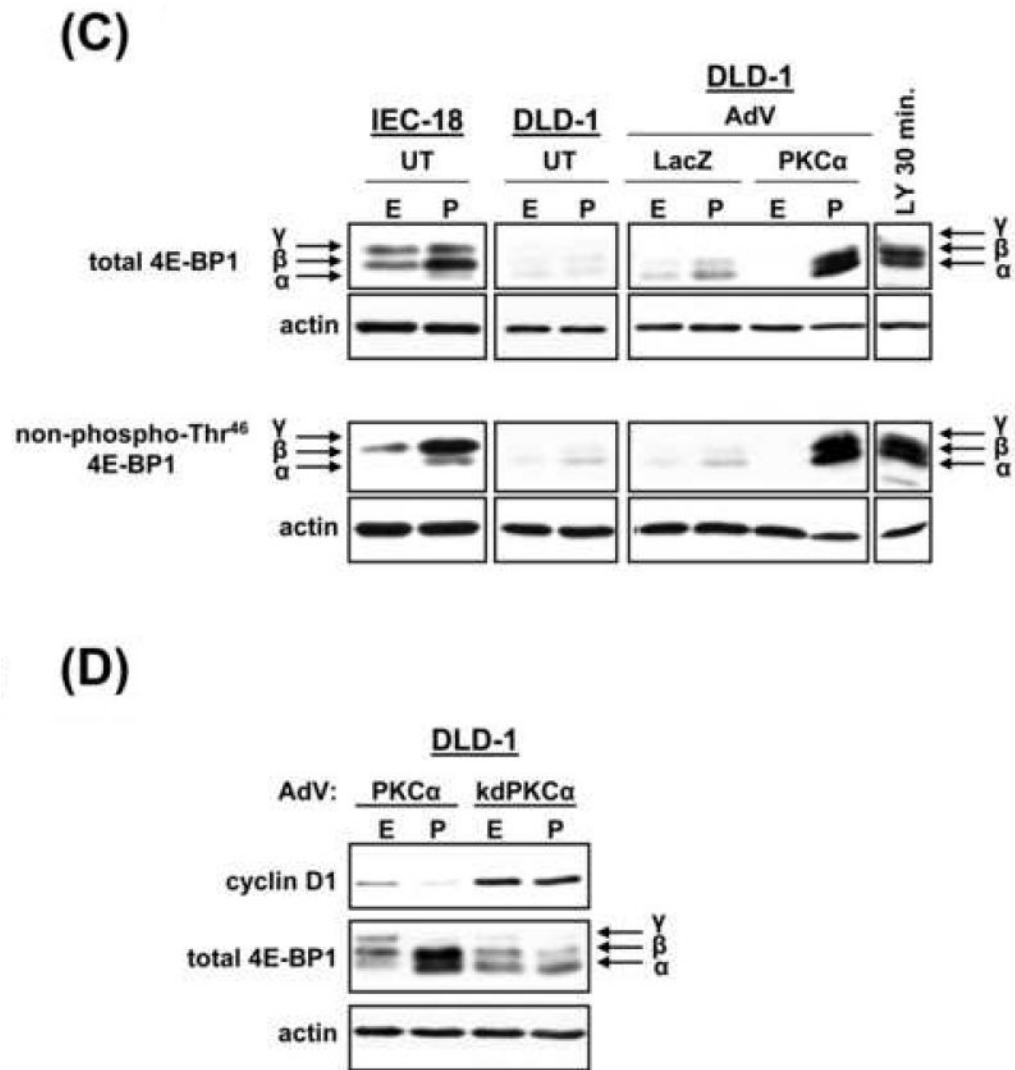


Figure 5. PKC α is required for PMA-induced downregulation of cyclin D1 and inhibition of cap-dependent translation in CRC cells

(A) Immunoblot analysis of cyclin D1 in IEC-18 cells (*left panel*) or LacZ-transduced CRC cells (*right panel*) treated with 100 nmol/L PMA (P) or vehicle (EtOH, E).

(B) Immunoblot analysis of CRC cells transduced with PKC α or PKC δ adenovirus and treated with PMA or ethanol for 2 h.

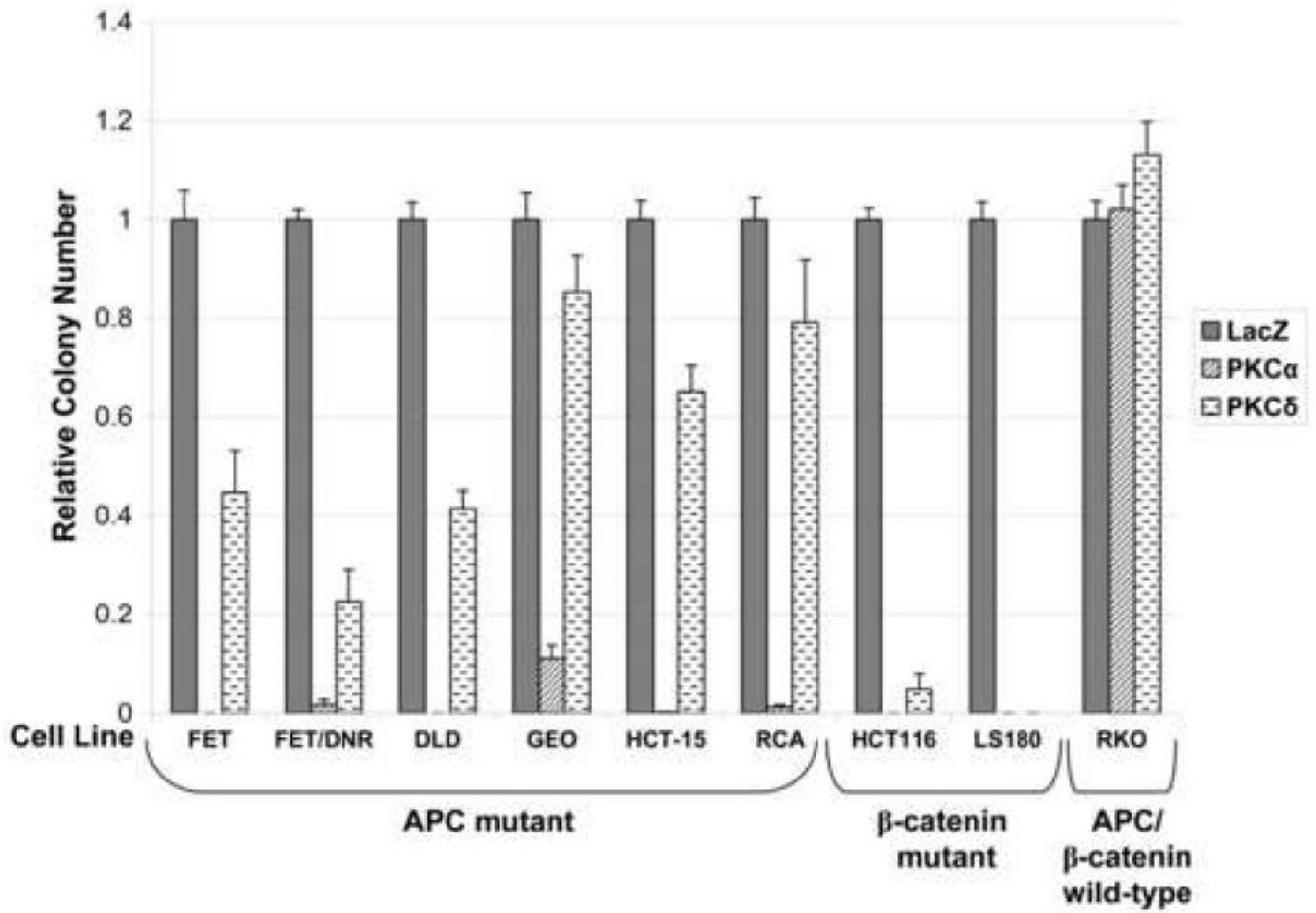
(C) Untransduced (UT) IEC-18 and DLD-1 cells, and LacZ- or PKC α -transduced DLD-1 cells were treated with PMA or vehicle for 2 h and subjected to immunoblotting for total and non-phospho-Thr⁴⁶ 4E-BP1. DLD-1 cells treated with LY294002 (LY) (50 μ mol/L, 30 min) are included as a positive control for 4E-BP1 activation. Arrows indicate 4E-BP1 phosphoforms.

(D) Immunoblot analysis of cyclin D1 and total 4E-BP1 in PKC α (20 moi)- or kdPKC α (500 moi)-transduced DLD-1 cells treated with PMA or vehicle for 2 h.

Data represent ≥ 3 independent experiments.

(A)

Quantitation of Soft Agarose Colony Formation



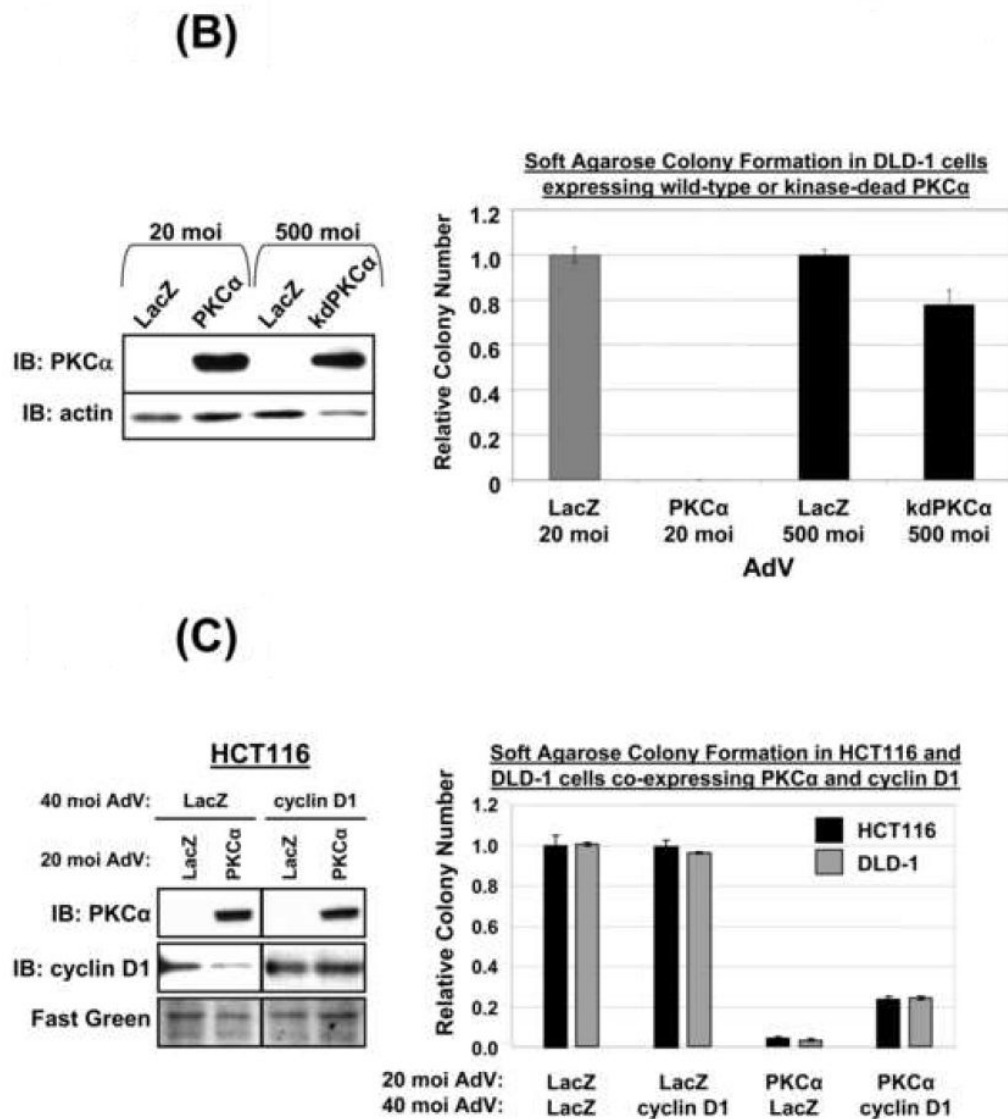


Figure 6. PKC α and PKC δ differentially suppress anchorage-independent growth of CRC cells (A) CRC cells were infected with LacZ, PKC α , or PKC δ adenovirus as indicated and plated in soft agarose. Quantification (average of ≥ 2 independent experiments \pm s.e.) of colony formation is presented relative to LacZ control. All samples transduced with PKC α were significantly different from LacZ-transduced controls ($P < 0.05$) except for RKO cells. (B) DLD-1 cells were infected with LacZ, PKC α or kdPKC α adenovirus as indicated and subjected to immunoblot analysis (*left panel*) or soft agarose colony formation assays (*right panel*). Data represent the average of two independent experiments (\pm s.e.). (C) DLD-1 and HCT116 cells were infected with indicated combinations of PKC α , LacZ and cyclin D1 adenovirus and analyzed by immunoblotting (*Left panel*) or colony formation in soft agarose (quantified as in A, *Right panel*). Data represent the average of 3 independent experiments \pm s.e. Similar results were obtained using 20 moi cyclin D1 adenovirus.

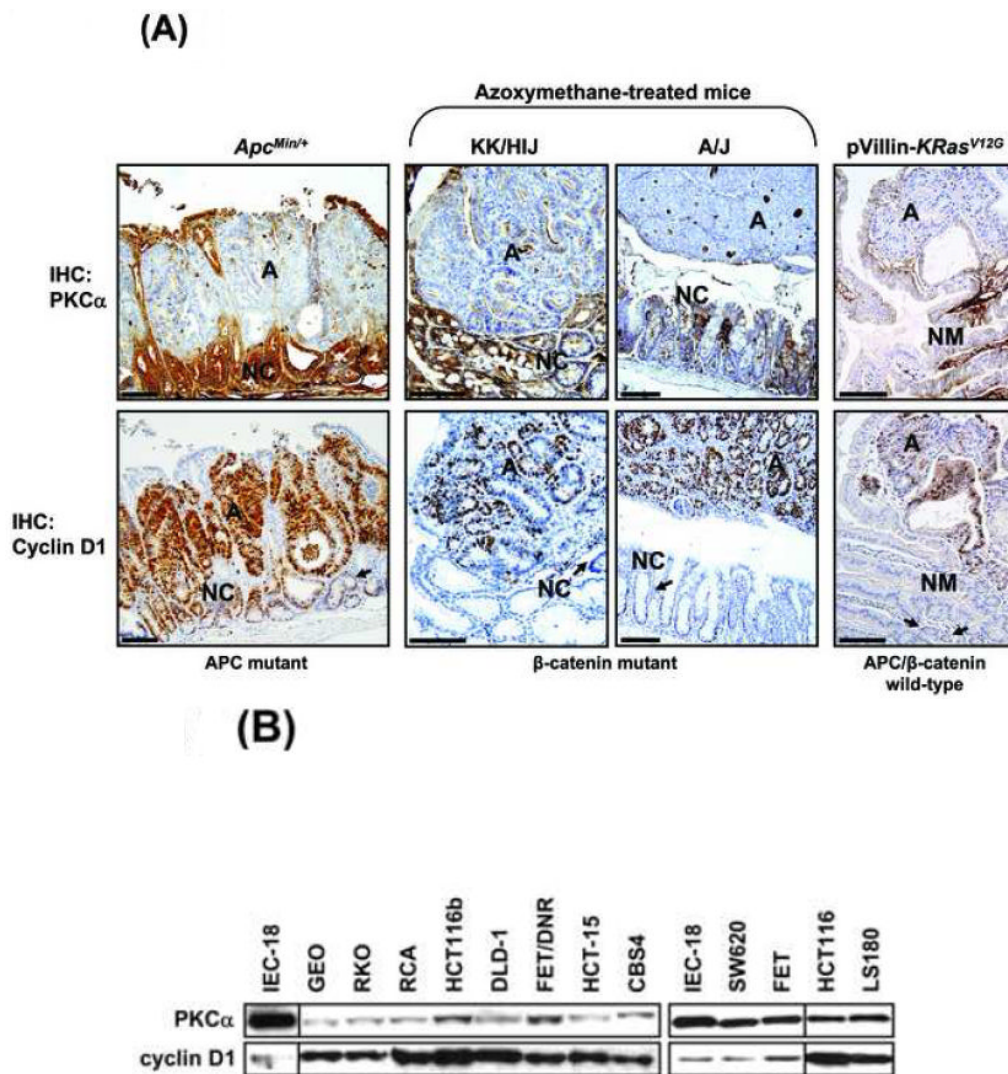


Figure 7. PKC α deficiency and increased cyclin D1 expression are characteristics of intestinal tumors and CRC cell lines independent of APC/ β -catenin mutation status
(A) Immunohistochemical analysis of PKC α and cyclin D1 in adenomas (A) and adjacent "normal" mucosa/crypts (NM; NC) from *Apc*^{Min/+} mice, azoxymethane-treated KK/HIJ and A/J mice, and pVillin-*KRas*^{V12G} mice. Arrows: cyclin D1 staining in "normal" crypt cells. Magnification Bar: 100 μ m.
(B) Western blot analysis of PKC α and cyclin D1 in CRC cells and non-transformed IEC-18 cells. Lines indicate regrouping of lanes from the same gel.