Activation of protein kinase Cα inhibits growth of pancreatic cancer cells via p21cip-mediated G1 arrest

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SUMMARY

We have analyzed human pancreatic cancer cells to explore the growth regulatory function of protein kinase C (PKC)α. PKCα subcellular redistribution, activation kinetics and downregulation were examined in detail and correlated to immediate and delayed effects on cell-cycle regulatory pathways. TPA treatment resulted in transient PKCα activation accompanied by translocation of the enzyme into membrane and nuclear compartments, and was followed by subsequent downregulation. TPA-induced inhibition of DNA synthesis was prevented by a PKC-antagonist and was reproduced by microinjection of recombinant PKCα, indicating that activation of this isoenzyme was required and sufficient for growth inhibitory effects. PKCα activation arrested cells in the G1 phase of the cell cycle as a consequence of selective inhibition of cyclin dependent kinase (CDK)2 activity with concomitant hypophosphorylation of Rb. The inhibition of CDK2 activity resulted from induction of p21cip cyclin-dependent kinase inhibitors. Levels of p21cip remained elevated and CDK2 activity repressed in spite of PKCα downregulation, indicating that downstream effectors of PKCα are the primary determinants for the duration of PKCα-mediated growth inhibition. The PKCα-induced block in cell proliferation persisted even though cells were kept in the presence of growth factors, suggesting that induction of PKCα results in a permanent withdrawal of pancreatic cancer cells from the cell cycle.

Key words: Protein kinase C, Cell cycle, Pancreatic cancer cell, Cyclin dependent kinase, p21cip

INTRODUCTION

Protein kinase C (PKC) represents a family of phospholipid-dependent serine-threonine kinases that participate in multiple aspects of cellular signal transduction (Mellor and Parker, 1998). Prolonged activation of PKC has been implicated in the regulation of cellular growth, differentiation, transformation and survival. While initial interest focused on the role of PKC in mitogenic signal transduction and cellular transformation, subsequent work provided evidence that PKC functions more generally as a flexible and dynamic signaling interface between extracellular stimuli and cell-cycle control (reviewed in Livneh and Fishman, 1997). In mammalian cells, PKC has been demonstrated to either promote or inhibit G1-phase progression and/or induce G2/M arrest, depending on the cell model. Even within a cell model, bidirectional growth regulation by PKC was seen, depending on the timing of PKC activation relative to Go/G1-S cell-cycle progression (Zhou et al., 1993).

Much progress has been made in elucidating cell-cycle regulatory targets of PKC. Effects of PKC on the G1/S cell-cycle transition are invariably reflected by the phosphorylation status of the retinoblastoma-associated tumor suppressor gene product Rb. In its hypophosphorylated, active form, Rb controls G1 progression by repression of gene products required for DNA synthesis. As cells progress through G1, Rb is inactivated via sequential phosphorylation by the G1-specific cyclin-dependent kinases (CDKs) CDK4/CDK6 and CDK2. The activity of G1-CDKs in turn is tightly regulated by their association with G1-cyclins, i.e. cyclin D and E, as well as interactions with cyclin-dependent kinase inhibitors (CKIs) (reviewed in Sherr, 1996). In addition, modifying events such as dephosphorylation by dual specificity CDC25 phosphatases are required for full enzymatic activity.

PKC appears capable of modulating the function of CDKs by various mechanisms. Phorbol esters induce cyclin D in quiescent cells, which may facilitate exit from G0 in mitogenic models (Huang et al., 1995). In contrast, the inhibitory effects of phorbol esters at the G1/S cell-cycle transition were linked predominantly to CDK2 complexes. In some models, PKC-mediated reduction of CDK2 activity was attributed to cyclin E repression (Sasaguri et al., 1996; Zhou et al., 1994), whereas in other models an inhibition of CDK2 activity in the presence of cyclin E was linked to induction of the p21cip/p27kip family of CDK inhibitors (CKIs) (Livneh et al., 1996; Huang et al., 1995). Effects of PKC on CDK2 phosphorylation may also be part of cell-cycle regulatory functions. The reduced CDK2
activity in phorbolester-treated IMR-90 human fibroblasts corresponded to their failure to induce the subunits of CAK, cyclin H and CDK 7 (Hamada et al., 1996). Also, regulation of cdc25 phosphatase expression (Kosaka et al., 1996; Hass et al., 1993) or activity (Barth et al., 1996) appears to contribute to CDK2 inhibition in cell models that arrest at the G2/M transition.

In addition to these cell-cycle regulatory effects, PKC activation was demonstrated to initiate apoptosis in epithelial (Zhao et al., 1997; de Vente et al., 1995) and hematopoetic cell lines (Macfarlane and O’Donnell, 1993; Haggerty and Monroe, 1994).

The great variability in PKC-mediated growth control, with respect to both biological outcome and underlying mechanisms, has been attributed to differences in the cell type-specific pattern of PKC-isoenzyme expression. Multiple isoforms have been identified in humans (reviewed in Mellor and Parker, 1998; Hug and Sarre, 1993), and are divided in three subfamilies based on their structural homology and mode of activation. The conventional isoenzymes (α, βI, βII and γ) require diacylglycerol (DAG) and calcium as coactivators in addition to phosphatidylserine, whereas activation of the novel isoforms (δ, ε, η and θ) is calcium-independent. Both conventional and novel PKCs function as major intracellular receptors for phorbol esters. In contrast, the atypical PKCs (ζ, λ) are neither DAG- nor calcium-dependent. Most cells express more than one PKC isoenzyme and these are thought to serve nonredundant functions. In vitro, the different PKC isoforms display only limited substrate specificity; however, in vivo they are localized to specific subcellular compartments and translocate to new, distinct intracellular sites upon activation (Kiley et al., 1995).

In consequence, an important step in understanding the function of PKC in growth control is the identification of the growth regulatory pathways utilized by individual isoenzymes. In most cell systems, differentiation between the specific effects of individual isoenzymes is hampered by the expression of multiple isoforms, which may not be dissected pharmacologically (Hofmann, 1997). The human pancreatic cancer cell lines utilized in the current study provide particularly suitable cell models, because they allow for selective activation of endogenous PKCα by phorbol esters on the basis of their extremely restricted PKC isoenzyme expression pattern (Rosewicz et al., 1996a,b). We therefore utilized DanG pancreatic cancer cells to specifically explore the growth regulatory function of PKCα. The results presented here characterize PKCα as a potent inhibitor of the G1/S transition in proliferating human pancreatic cancer cells and identify the selective induction of p21cip1 as the underlying molecular mechanism.

MATERIALS AND METHODS

Materials

Human pancreatic carcinoma cell lines were obtained from ‘Deutsches Krebsforschungszentrum’, Heidelberg (AsPc 1, Capan 2 and DanG) or from the American Type Tissue Culture Collection, ATCC (Panc 1). Dulbecco’s Modified Eagle’s Medium (DMEM), RPMI 1640 medium and phosphate-buffered saline (PBS) were supplied by Gibco BRL (Berlin, Germany), fetal calf serum (FCS), trypsin/EDTA, penicillin and streptomycin were from Biochrom (Berlin, Germany). Antibodies were purchased from the following manufacturers: BRL, Bethesda, MD, USA (PKCα and PKCζ); Santa Cruz Biochemicals, Santa Cruz, CA, USA (CDK4, CDK2, CDK7, CDc25A); Transduction laboratories, Inc., Lexington, KY, USA (p27kip1); Pharmingen, San Diego, CA, USA (Rb, cyclin E, cyclin D1, cyclin A); Calbiochem-Novabiochem GmbH, Bad Soden, Germany (p21cip1); DAKO Diagnostica GmbH, Hamburg, Germany (BrdU) and Dianova GmbH, Hamburg, Germany (all secondary antibodies). [3H]Thymidine and (γ-32P)dATP were from Amersham, Braunschweig, Germany, GF 109203X and myelin basic protein (MBP) were obtained from Calbiochem-Novabiochem GmbH. Recombinant rabbit PKCα was supplied by Upstate Biotechnology (Lake Placid, NY, USA). Calf histone H1, Proteinase K and ATP were from Boehringer Mannheim (Mannheim, Germany). Western blot supplies were purchased from BioRad Laboratories GmbH (München, Germany). TPA, protein A-Sepharose beads and all other reagents were from Sigma Chemical Co. (Deisenhofen, Germany).

Cell lines and tissue culture

Cell lines were grown as subconfluent monolayer cultures under 95% air and 5% CO2 at 37°C. DanG and AsPc 1 cell lines were cultured in RPMI 1640 medium supplemented with 10% and 15% (v/v) FCS, respectively; Panc 1 and Capan 2 cells were cultured in DMEM supplemented with 10% (v/v) FCS. Penicillin (100 IU/ml) and streptomycin (100 µg/ml) were added to all media. Experiments were routinely carried out in the log phase of growth after cells had been plated for 16-24 hours. 12-O-tetradecanoylphorbol-13-acetate (TPA) was dissolved in dimethylsulfoxide (DMSO) to obtain a 1 mM stock solution and further diluted prior to use.

Cell growth assays

Cells were plated at 5000 cells/well in 96-well tissue culture plates and counted using a hemocytometer. Viability of cells as confirmed by Trypan Blue exclusion was >90%. For measurement of [3H]thymidine incorporation, cells were plated at 20,000/well in 96-well tissue culture plates and treated for 1-12 hours with 1 µM TPA. During the final 6 hours, 1 µCi/ml [3H]thymidine was added. Cells were lysed by freezing, harvested and transferred to glass-fiber membranes (LKB Wallac). Following extensive washing with PBS, the radioactivity retained on the membranes was determined by scintillation counting.

Analysis of DNA fragmentation

To evaluate apoptosis, 1x106 cells were submitted to the treatments indicated and culture supernatants as well as adherent cells were harvested, centrifuged and resuspended in 300 µl lysis buffer (10 mM Tris, pH 8.2, 400 mM NaCl, 2 mM EDTA, 1% sodium dodecyl sulfate (SDS), 0.2 mg/ml Proteinase K). Following a 4 hour incubation at 37°C, an equal volume of 6 M NaCl was added and samples were vigorously vortexed. After brief centrifugation, nucleic acids were precipitated from the supernatant by addition of 2 volumes of ethanol. Pellets were resuspended in TE-RNase buffer (10 mM Tris, pH 8.0, 0.01 mM EDTA, 100 µg/ml RNase A (Quiagen) and analyzed on a 1.5% agarose gel.

Flow cytometric analysis

1x106 cells were fixed in ethanol (70%) for 30 minutes at –20°C, then washed with PBS and incubated for 30 minutes at room temperature in PBS containing 100 µg/ml RNase A, 0.1% Triton X-100, 1 µM EDTA and 1.5 µg/ml propidium iodide. Cell-cycle analysis was performed on a FACScan utilizing Cellquest and Modfit software (Becton Dickinson).

Western blotting

Cultures were rinsed twice with ice-cold PBS containing 1 mM sodium orthovanadate (Na3VO4) and extracted in cold lysis buffer (20 mM Tris, pH 7.8, 150 mM NaCl, 2 mM EDTA, 50 mM}
β-glycerophosphate, 0.5% Nonidet P-40 (NP-40), 1% glycerine, 10 mM NaF; 1 mM Na3VO4, 1 mM dithiothreitol (DTT), 2 μM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 2 μM leupeptin). Extracts were boiled in Laemmli sample buffer and samples were separated by SDS-PAGE, electroblotted to polyvinyl difluoride (PVDF) membranes (NEN) and blocked in PBS/0.1% Tween (PBST) containing 5% nonfat dry milk (cyclins D1, A, E, p21cip1, p27kip1, Rb, CDK4, Cdc25A) or 5% BSA (CDK2, CDK7) for 2 hours at room temperature. Incubation with primary antibodies was carried out overnight at 4°C. Bands were visualized using the enhanced chemiluminescence (ECL™) system (Amersham).

For repeated use, membranes were stripped in 62.5 mM Tris-HCl, pH 6.8, 2% SDS and 100 mM 2-mercaptoethanol for 30 minutes at 55°C followed by extensive washing in PBST. Results were quantitated by laser densitometry utilizing the Scanpak 2 software (Bioimage).

To evaluate PKC translocation, nuclear, cytosolic and membrane fractions were prepared according to Simboli-Campbell et al. (1994) and then processed as described above, except that alkaline phosphatase-based colorimetric visualization of bands was performed.

**Immunoprecipitation and histone H1-kinase assays**

For immunoprecipitation of CDK4, cells were scraped in ice-cold IP-buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM DTT, 0.1% Tween 20, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na3VO4, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM PMSF) (Matsushima et al., 1994), quickly dipped in liquid nitrogen and subsequently incubated for 1 hour on ice with occasional vortexing. After brief centrifugation, lysates (1 mg/sample) were precleared for 1 hour with protein A-Sepharose beads. CDK4 complexes were then precipitated at 4°C for 4 hours by addition of protein A-Sepharose beads precleared with 10 μg of CDK4 antibody. Immunocomplexes were repeatedly washed in ice-cold IP-buffer and twice in 50 mM Hepes, pH 7.5, containing 1 mM DTT. The kinase reaction was then started by addition of 30 μl kinase buffer (50 mM Hepes, pH 7.5, 1 mM DTT, 2.5 mM EGTA, 10 mM MgCl2, 10 mM β-glycerophosphate, 50 μM ATP, 1 μg calf histone H1/sample) and 10 μCi of [γ-32P]ATP/sample, allowed to proceed for 30 minutes and terminated by boiling the samples in Laemmli buffer. The reaction was subjected to 10% SDS-PAGE and CDK4 activity was determined by autoradiography of the dried gels.

For CDK2 immunoprecipitation, cells were lysed by mild sonication in ice-cold ELB-buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 mM NaF, 0.1 mM Na3VO4, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM PMSF) followed by a 30 minute incubation on ice with occasional vortexing. Portions (500 μg) were precleared, precipitated with CDK2-coated protein A-Sepharose beads and washed 5x in ELB-buffer. For subsequent western blot analysis, immunocomplexes were boiled in Laemmli sample buffer, separated on 12% SDS-polyacrylamide gels and processed as described above. For CDK2 activity assays, immunocomplexes were washed twice with 50 mM Hepes, pH 7.5, containing 1 mM DTT and then submitted to the kinase reaction by addition of 50 mM Hepes, pH 7.5, 1 mM DTT, 10 mM MgCl2, 1 μg calf histone H1/sample, 50 μM ATP and 5 μCi of [γ-32P]ATP/sample. Reactions were terminated after 5 minutes and processed as described above.

**MAPK activity assays**

Mitogen activated protein kinase (MAPK) activity in-gel assays were conducted essentially as described (Kameshita and Fujisawa, 1989). Portions (10 μg) of cell lysates prepared as described for western blotting were separated on 10% SDS-polyacrylamide gels containing 0.5 mg/ml MBP. Following removal of SDS and denaturation-renaturation, the kinase reaction was allowed to proceed in kinase buffer (40 mM Hepes-HCl, pH 8.0, 2.0 mM DTT, 0.1 mM EGTA, 5 mM MgCl2, 20 μM ATP and 90 μCi [γ-32P]ATP) for 1 hour at room temperature. The reaction was terminated by repeated washes in 5% (w/v) trichloroacetic acid and 1% sodium pyrophosphate over a period of 2 hours. Autoradiographs of dried gels were then quantitated by densitometry.

**Immunofluorescence and microinjection studies**

To determine subcellular localization of PKC, cells grown on coverslips were quickly rinsed in PBS and immediately fixed for 10 minutes in 3% paraformaldehyde, pH 7.2. Cells were submitted to further fixation in 80% methanol (20 minutes, −20°C), dried and blocked for 30 minutes in PBS containing 3% BSA. Cells were incubated for 1 hour with 2.5 μg/ml isoenzyme-specific PKC antibodies. A fluorescein-conjugated secondary antibody was then used for detection of immunocomplexes by confocal microscopy, using the ‘MRC600 confocal imaging system’ (BioRad Laboratories).

For microinjection, approximately 200 cells/condition were injected on gridded coverslips, allowed to recover for 16 hours and then pulse-labeled with 0.2 mM bromodeoxyuridine (BrdU) for 2 hours. Coverslips were processed for both PKCα and BrdU immunostaining following the protocol given above. BrdU incorporation and PKCα staining were then evaluated by confocal microscopy. BrdU-labeling indices were then calculated from at least 100 injected cells/condition. Statistical differences between control and treatment groups were calculated by ANOVA (Newman-Keuls) and considered significant at P < 0.05.

**RESULTS**

**Protein kinase C inhibits growth of human pancreatic cancer cell lines**

To examine PKC-mediated growth regulation in human DanG pancreatic cancer cells, growth curves in the presence or absence of TPA were obtained over a period of 4 days (Fig. 1A). TPA treatment (1 μM) resulted in an almost complete inhibition of proliferation with cell doubling times prolonged to 5.4 days in TPA-treated cultures as compared to 1.3 days in control cultures. Since reduced cell numbers were already evident at 24 hours, the early effects of TPA were studied at the level of DNA synthesis (Fig. 1B). [3H]thymidine incorporation was significantly inhibited at 3 hours of TPA treatment (73±8.6% of control; P<0.05) and further decreased to 29±3.5% of control at 12 hours. Both the proliferation decrease and inhibition of DNA synthesis were concentration-dependent (Fig. 1C,D), with 45.3±6% of the maximal effect on proliferation occurring at 1 nM TPA. Growth-inhibitory effects of TPA were consistently reproduced in AsPc 1, Capan 2 and Panc pancreatic cancer cell lines (Fig. 1E).

Since phorbol esters may induce apoptosis rather than growth inhibition, DNA integrity was examined (Fig. 2). Control cultures, stimulated to undergo apoptosis by Interferon-γ and engagement of Fas receptors, readily demonstrated DNA fragmentation. In contrast, no DNA fragmentation was detected in cells treated with 1 μM TPA for up to 72 hours.
Thus, TPA-mediated growth inhibition of DanG pancreatic cancer cells was not due to apoptotic cell death.

**TPA stimulation of DanG pancreatic cancer cells results in selective translocation of PKCα**

Since distinct biological responses are linked to the differential activation of individual PKC isoforms, we next attempted to identify the PKC isoenzyme(s) responsible for TPA induced growth inhibition.

We have previously shown that the expression of conventional PKC isoenzymes in the human pancreatic cancer cell lines utilized in the current study is restricted to PKCα and thus differs from the more diverse set reported in the HPAC human pancreatic cancer cell line (Franz et al., 1996). Similar to HPAC cells, however, the cell lines utilized here consistently express PKCα and PKCζ, but lack the δ- and ε-PKC isoforms (Rosewicz et al., 1996a). Thus, PKCα appears most likely to mediate phorbolester effects in pancreatic cancer cells. PKCζ translocation in response to phorbolesters has been reported (Borner et al., 1992), however, suggesting that indirect activation may occur. Therefore, effects of TPA on the subcellular localization of both PKCα and PKCζ were examined in detail.

Utilizing confocal laser microscopy, we observed PKCα translocation from the cytosol into the nucleus within 30 seconds of TPA stimulation (Fig. 3A). Surprisingly, only weak immunofluorescence was detected in association with cell membranes at either 30 or 60 seconds, suggesting that activated PKC is redistributed predominantly into the nuclear compartment. In contrast, the localization of PKCζ, which presented a preferentially nucleolar staining pattern, remained unchanged by TPA (Fig. 3A). As an alternative approach, subcellular fractions were analyzed for their PKC content by immunoblotting. The quantitative evaluation of these experiments confirmed the nuclear translocation but also provided evidence for translocation of PKCα into the membrane compartment (Fig. 3B).

To confirm that translocation corresponded to increased PKC activity in the nuclear compartment, direct measurement of kinase activity in nuclear extracts were performed (Fig. 3C). A transient increase in nuclear PKC activity was observed with TPA (Fig. 3C).

**Fig. 1.** TPA inhibits growth of human pancreatic cancer cells in a time- and dose-dependent manner. Cells were treated with 1 μM TPA (closed circles) or vehicle (open circles) for the indicated times (A,B) or with the indicated doses for 72 hours (C) and 6 hours (D), respectively. For evaluation of long-term effects, cell numbers were determined (A,C). Short-term effects were examined by [3H]thymidine incorporation (B,D). Values are means ± s.e.m. from at least three separate experiments, each conducted in triplicate.

(E) Pancreatic cancer cell lines were treated with vehicle (open bars) or 1 μM TPA (closed bars) for 3 days and cell numbers were determined. Values are means ± s.e.m. from triplicate determinations.

**Fig. 2.** TPA treatment does not induce apoptosis. DanG cells were incubated with 1 μM TPA for the indicated times and genomic DNA was subsequently analyzed for oligonucleosomal fragmentation (lanes 4-6 from the left). DNA from cells induced to initiate apoptosis by costimulation with interferon γ and IgM-Fas antibody (48 hours) and untreated controls are shown in lanes 2 and 3. For size determination a 100 bp DNA standard was used (lane 1).
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Maximal stimulation to 2.5±0.3-fold of control levels obtained at 30 minutes of phorbol ester treatment.

While no growth regulatory substrates for PKCα have been identified in the nucleus, the MAP-kinase cascade represents a cytosolic downstream effector pathway with a pre-eminent role in cellular growth control. Performing in-gel kinase assays on TPA-stimulated DanG cells, we observed a significant increase in p42-MAP-kinase activity at 15 minutes followed by moderately elevated levels up to 12 hours (Fig. 3D).

Taken together, these results demonstrate that selective activation of PKCα by phorbol ester treatment results in transiently increased activity of the enzyme that precedes the onset of growth inhibition (compare Figs 3 and 1B).

Fig. 3. TPA stimulation results in selective translocation and activation of PKCα.

(A) Immunofluorescence staining of control and TPA-treated (1 µM) DanG cells for PKCα (upper panel) and PKCζ (lower panel). Fluorescence intensity is given on a pseudocolor scale (red, highest). (B) Time course of TPA-dependent redistribution of PKCα determined by immunoblotting of subcellular fractions.

(C) Determination of PKC activity in nuclear extracts of TPA-stimulated DanG utilizing Ac-MBP(4-14) as substrate. Values are means ± s.e.m. of three independent experiments.

(D) Time course of MAPK activity in TPA-stimulated (1 µM) DanG cells. Shown are the densitometric results of a representative in-gel assays.

TPA-mediated growth inhibition is based on specific and selective activation of PKCα

To directly evaluate the hypothesis that phorbol ester-induced growth inhibition is mediated by activation of PKCα, two complementary experimental approaches were pursued.

First, we examined the ability of the specific PKC inhibitor GF109203X (Toullec et al., 1991) to prevent TPA-mediated inhibition of DNA synthesis (Fig. 4A). DanG cells were preincubated for 30 minutes with increasing concentrations of GF109203X and then subjected to 100 nM TPA for 6 hours. GF109203X was capable of completely preventing growth inhibition by TPA when given at a maximal concentration of 1 µM, and was partially effective at 0.1 µM. Incubation with GF109203X alone did not affect DNA synthesis.

To directly demonstrate that growth inhibition can be specifically mediated by PKCα, DanG cells were microinjected with recombinant PKCα and effects on proliferation were determined without further addition of TPA (Fig. 4B,C). To control for unspecific effects, parallel cultures were injected either with buffer or with heat-inactivated recombinant PKCα. Injected cells were allowed to recover for 16 hours and subsequently submitted to BrdU pulse labeling. Both PKCα content and BrdU incorporation were then evaluated by immunofluorescence (Fig. 4B), and the percentage of BrdU-labeled nuclei was determined (Fig. 4C). Buffer-injected control cells presented moderate, predominantly cytosolic PKCα immunofluorescence (Fig. 4B, left panel). In these cells, the majority of nuclei stained positive for BrdU (Fig. 4B, right panel), indicating that the injection procedure per se did not impair proliferation. Similar immunostaining was obtained in cultures injected with heat-inactivated PKCα. In contrast, BrdU incorporation was nearly
abolished in cells injected with intact enzyme. These cells also displayed enhanced PKCα immunofluorescence, confirming the efficiency of microinjection.

Thus, PKCα activation was required and sufficient to inhibit DNA synthesis in DanG cells.

**PKCα-mediated inhibition of G1/S cell-cycle transition in DanG cells involves hypophosphorylation of Rb**

To determine cell-cycle events specifically regulated by PKCα in pancreatic cancer cells, effects of TPA on cell-cycle distribution were examined (Fig. 5A,B). TPA-treatment time-dependently reduced the proportion of cells in the S phase, while simultaneously increasing the G1 complement (~17% and +12%, respectively, at 24 hours of TPA treatment; Fig. 5B). The percentage of cells in the G2/M phase was low at all time points and varied by less than 4%. Thus PKCα activation primarily inhibited the G1/S progression of DanG cells.

Since this major cell-cycle transition is restricted by Rb and requires its functional inactivation via phosphorylation by G1-CDKs, effects of PKCα activation on Rb phosphorylation were studied by immunoblotting (Fig. 6). In control cultures Rb was detected as a single band representative of its hyperphosphorylated form (ppRb). Starting at 3 hours of TPA treatment, a faster migrating band indicative of the hypophosphorylated form of Rb (pRb) appeared and became more prominent over time (Fig. 6A). Pretreatment with GF109203X prevented the shift in Rb phosphorylation, indicating a requirement for PKC activation (Fig. 6B).

**PKCα inhibits predominantly CDK2 activity**

Since hyperphosphorylation of Rb at the G1-S transition is effected by the sequential activation of G1-CDK/cyclin complexes, we determined the pattern of G1/S cyclin and CDK expression in response to TPA (Fig. 7). Immunoblots probed with a cyclin D1-specific antibody revealed a transient reduction in cyclin D1 expression, which was decreased by 50% at 1 hour of TPA stimulation. However, cyclin D1 levels recovered subsequently and exceeded control levels by twofold at 24 hours. In contrast to cyclin D, cyclin E remained
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Thr 160 gradually disappeared in TPA-treated cells, suggesting an inhibition of CDK2 activity in response to PKCα activation.

To directly investigate regulation of G1-CDK activity by PKCα in DanG cells, CDK4 and CDK2 were immunoprecipitated as active kinase complexes from control and TPA-treated cultures and kinase activity was determined. The specificity of the kinase reactions was confirmed by immunoprecipitations with matched IgG control antibodies. (Fig. 8). Surprisingly, CDK4 activity remained unchanged for up to 6 hours of TPA treatment and was only moderately inhibited at 12 and 24 hours of treatment.

In sharp contrast to the results obtained in CDK4-kinase assays, phorbolester treatment profoundly diminished CDK2-kinase activity. The inhibition started as early as 1 hour of TPA incubation, thereby preceding the onset of Rb hypophosphorylation. Further TPA treatment resulted in progressive loss of CDK2 activity, which became barely detectable at 24 hours. The kinetics of CDK2 inhibition place this event between activation of PKCα and the initiation of growth inhibition, suggesting that inhibition of CDK2

Fig. 7. TPA-induced changes in G1-cyclin and CDK expression are consistent with reduced CDK2 activity. Time course of changes of G1-cyclin and CDK expression in response to TPA treatment (1 μM). Whole cell lysates were resolved by 15% (for cyclin D, CDK2, CDK4) or 12% (for cyclin A, cyclin E) SDS-PAGE and then analyzed by immunoblotting. Shown are representative blots of three independent experiments yielding similar results.
represents the mechanism underlying the antiproliferative action of PKCα in pancreatic cancer cells.

The inhibitory action of PKCα on CDK2 activity is mediated by p21cip1

In proliferating cells, CDK2 is found in quaternary complexes containing members of the p21cip1 and/or p27kip1 family of cyclin dependent kinase inhibitors (CKIs) in addition to cyclins and the essential DNA replication factor proliferating-cell nuclear antigen (PCNA). Further control on CDK2 activity is imposed by the requirement for phosphorylation by CAK and CDC25-mediated dephosphorylation. Both CAK and CDC25 are subject to downregulation by PKC in specific cellular settings, providing alternative regulatory targets for inhibition of CDK2 activity.

In order to identify potential mechanisms for PKC-dependent CDK regulation in pancreatic cancer cells, whole cell lysates were analyzed for the expression of CDK2-regulatory molecules by immunoblotting (Fig. 9A). TPA-treated DanG cells displayed an early and striking increase in p21cip1 levels (17.4±4.5-fold control at 12 hours) that exactly matched the time course of CDK2 inhibition. In addition, p27kip1 concentrations were found to be consistently increased at 24 hours of TPA treatment, albeit moderate induction at 12 hours was occasionally observed. In contrast, neither the expression of Cdc25a phosphatase nor the levels of Cdk7 were influenced by PKCα activity. Also the cellular content of PCNA remained unchanged.

Based on the observed induction of p21cip1, the composition of immunoprecipitated CDK2 complexes was examined next (Fig. 9B). Comparable amounts of CDK2 were detected, confirming that equal quantities of CDK2 complexes were analyzed. As expected, cyclin A and cyclin E could be readily detected in control and TPA-treated cells. Similar to whole cell lysates, CDK2 immunoprecipitates contained strongly elevated levels of p21cip1; again, the time course exactly paralleled the inhibition of CDK2-kinase activity. Finally, the p27kip1 content was increased at later time points (12 and 24 hours) of TPA treatment. Taken together, these observations support the hypothesis that p21cip1 mediates the inhibitory action of PKCα on CDK2 activity in DanG cells.

To confirm that this proposed mechanism of PKCα-mediated growth inhibition is not restricted to the DanG cell line, the crucial results were reproduced in three more pancreatic cancer cell lines, i.e. AsPC1, Capan2 and Panc.

First, effects of PKC activation on cell-cycle distribution were analyzed. TPA-treated cultures all presented an increased fraction of cells in the G1 phase of the cell cycle, which could be accounted for by a reduction of S phase cells (Fig. 10A). Second, induction of p21cip1 following phorbol ester treatment was examined by immunoblotting of whole cell lysates (Fig. 10B). Consistently, increased levels of p21cip1 were detected at 3 hours of TPA stimulation. In all of the four cell lines examined, the cellular p21cip1 content remained elevated at 24 hours after TPA stimulation, although in three of the cell lines
(particularly in Capan 2 cells) levels at 24 hours were substantially reduced compared to the strong initial induction.

**DISCUSSION**

Negative growth regulatory effects mediated by PKC have been described in various cell models (Livneh and Fishman, 1997) and, moreover, have been linked to cellular differentiation in vivo (Saxon et al., 1994) and in vitro (Hass et al., 1991, 1993; Chen et al., 1989; Melloni et al., 1989; Scaglione-Sewell et al., 1998; Abraham et al., 1998). Considerable knowledge has accumulated on the cell-cycle inhibition by PKC, suggesting that both p21cip1 and p27kip1 participated in cell-cycle inhibitory effects (Frey et al., 1997).

In the current study, the cellular p27kip1 content remained at control levels until CDK2 activity had significantly decreased. In parallel, the p21cip1 complement of CDK2 complexes increased and CDK2 activity declined. Recently, p21cip1 induction has been linked to phorbolester-mediated growth inhibition in diverse cell models such as fibroblasts, endothelial cells and intestinal epithelial cells, as well as epithelial and hematopoietic tumor cell lines (Livneh et al., 1996; Kosaka et al., 1996; Frey et al., 1997; Abraham et al., 1998; Coppock et al., 1995).

In intestinal epithelial cells, the induction of p21cip1 in response to PKC activation was paralleled by increased levels of the related CDK inhibitor p27kip1. Although p27kip1 increases were moderate, the time course paralleled growth inhibition by PKC, suggesting that both p21cip1 and p27kip1 participate in cell-cycle inhibitory effects (Frey et al., 1997). In the current study, the cellular p27kip1 content remained at control levels until CDK2 activity had significantly decreased. Apparently, effects of PKCα on p21cip1 and p27kip1 levels in pancreatic cancer cells rely on different mechanisms. Phorbolesters induce p21cip1 at the transcriptional level in a p53-independent manner (Jiang et al., 1994; Zeng et al., 1996), which is consistent with the TPA-induced increase of p21cip1 in p53-deficient AsPc 1, Panc 1 and Capan 2 pancreatic cancer cells. The mechanisms underlying the regulation of cellular p27kip1 content by PKC have not yet been determined, but the delayed elevation of p27kip1 levels in TPA-treated DanG cells may reflect accumulation as a result of decreased CDK2 activity (Sheaﬀ et al., 1997). While p27kip1 accumulation in pancreatic cancer cells appeared delayed compared to the time course of CDK2 inhibition, it coincided with the moderate reduction of CDK4 activity. Therefore, the increased overall content of cellular CKIs may account for the late inhibition of CDK4 activity observed in the current study.

PKCα-mediated inhibition of G1/S cell-cycle progression has previously been explored in nontransformed IEC-18 intestinal epithelial cells (Frey et al., 1997), where inhibition of cell-cycle progression, hypophosphorylation of Rb and induction of p27kip1 were linked to PKCα activation, based on their parallel kinetics. Specifically, cell-cycle effects in IEC-18...
cells desensitized with PKCα downregulation. In contrast, PKCα-dependent growth inhibition of DanG cells persisted in spite of PKC depletion, suggesting that PKCα initiated rather than sustained the cell-cycle regulatory effects.

In contrast to the persistently high levels in DanG cells, the p21cip1 increase in AsPc 1, Capan 2 and Panc cells was transient, with high levels at 3 hours and only moderately elevated levels at 24 hours of TPA stimulation. Nonetheless, these cell lines remained growth inhibited during our 3-day observation period, suggesting that either the moderately elevated p21 levels were sufficient to maintain CDK2 inhibition or, alternatively, that additional mechanisms such as increases in p27kip1 may contribute to the sustained effect.

Similar to p21cip1 induction, MAP-kinase activity remained elevated in DanG cells after downregulation of PKCα, suggesting that dissociation of activation kinetics can occur at the level of early downstream effectors. The duration of MAP-kinase activity has been implicated as a major determinant in alternate cellular responses to growth factors, such that sustained activation favors differentiation and growth inhibition, whereas mitogenic effects are supported by short-term activation (Traverse et al., 1992; Marshall, 1995). Specifically, the prolonged MAP-kinase activation (>6 hours) induced by TPA in MCF7 breast cancer cells correlated with growth inhibition, while the transient increase elicited by insulin (<15 minutes) stimulated DNA synthesis. Furthermore, a mitogen-activated protein kinase kinase 1 (MEK1) inhibitor blocked the elevation of p21cip1 levels by phorbol esters in human endothelial cells and A431 cells, identifying the MAP-kinase cascade as an upstream signaling pathway linking PKC to CKI induction (Zezula et al., 1997). Thus, the sustained activation of the MAP-kinase cascade in DanG pancreatic cancer cells represents a likely pathway linking PKCα activation to p21cip1-mediated growth arrest.

The growth-inhibitory action of PKCα reported here is in apparent contrast to effects elicited by modulation of PKCα expression in HPAC human pancreatic cancer cells, where growth inhibition as well as reduced tumorigenicity were correlated to lowered PKCα expression, and overexpression resulted in enhanced tumorigenicity and increased proliferation (Franz et al., 1996; Denham et al., 1998). However, the conclusions from experimental approaches that rely on long-term changes in the expression level of PKC isoenzymes might not be applicable to their role in cellular growth control upon acute stimulation. We have previously demonstrated decreased PKCα levels in pancreatic cancer cell lines induced to differentiate by retinoic acid (Rosewicz et al., 1996a) as well as in pancreatic cancer cells that were growth-inhibited by Interferon-α (Rosewicz et al., 1996b). Based on these previous results, particular care was taken in the current study to convincingly establish that regulation of growth inhibitory effectors preceded phorbol ester induced downregulation of PKCα. Both p21cip1 induction and CDK2 inhibition were manifest at 1 hour after TPA exposure, whereas downregulation of PKCα did not occur until 3 and 6 hours in the membrane and nuclear compartments, respectively.

It is also important to bear in mind that our experiments were carried out on exponentially growing, serum-stimulated cells and therefore do not reflect the effects of PKC activation on cell-cycle reentry of quiescent pancreatic cells. This would potentially reconcile the data presented in the current study with results from other studies that focussed on the role of PKCα in pancreatic transformation, which observed elevated PKCα activity in human pancreatic cancer samples when compared to normal pancreatic tissue and documented enhanced tumorigenicity of human pancreatic cancer cells overexpressing PKCα (Denham et al., 1998). Inversely, the application of antisense oligonucleotides to PKCα reduced the tumorigenicity of injected HPAC cells in an orthotopic model of pancreatic cancer. Both of these phenomena likely reflect the capability of PKC to promote G0/G1 to S phase progression in quiescent cell populations. Thus, PKC activation may have opposite growth regulatory functions in pancreatic cancer cells, as has previously been documented by the bidirectional modulation of DNA synthesis in vascular endothelial cells (Zhou et al., 1993).

Addressing the function of PKCα, our study revealed important insights into growth control of human pancreatic cancer cells. In pancreatic cancer, loss or inactivation of p16ink4a appears to be the predominant mechanism underlying the unrestrained progress from quiescence to proliferation in the process of transformation (Schütte et al., 1997; Naumann et al., 1996). The current study demonstrates, however, that physiological regulators of CDK2 are still capable of imposing an effective functional control over G1/S progression in pancreatic cancer cells, as indicated by the effects of p21cip1 induction on cell-cycle progression. The sensitivity of pancreatic cancer cells to growth inhibition by p21cip1 differs markedly from the observations on a panel of varied tumor cell lines reported by Blagosklonny et al. (1997), who classified 5 out of 7 cell lines as resistant to phorbolester-mediated growth inhibition despite induction of p21cip1. Obviously, the susceptibility to p21cip1 imposed cell-cycle control varies between tumor entities. Since the representative panel of human pancreatic cancer cell lines examined here consistently demonstrated growth arrest in G1, the action of p21cip1 might be further explored for therapeutic potential in pancreatic cancer.

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REFERENCES


PKCα inhibits CDK2 activity via p21cip1