Differential Downstream Functions of Protein Kinase C\(\eta\) and -\(\theta\) in EL4 Mouse Thymoma Cells

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Sensitive EL4 mouse thymoma cells (s-EL4) respond to phorbol esters with growth inhibition, adherence to substrate, and production of cytokines including interleukin 2. Since these cells express several of the phorbol ester-sensitive protein kinase C (PKC) isozymes, the function of each isozyme remains unclear. Previous studies demonstrated that s-EL4 cells expressed substantially more PKC\(\eta\) and PKC\(\theta\) than did EL4 cells resistant to phorbol esters (r-EL4). To examine potential roles for PKC\(\eta\) and PKC\(\theta\) in EL4 cells, wild type and constitutively active versions of the isozymes were transiently expressed using a Sindbis virus system. Expression of constitutively active PKC\(\eta\), but not PKC\(\theta\), in s- and r-EL4 cells altered cell morphology and cytoskeletal structure in a manner similar to that of phorbol ester treatment, suggesting a role for PKC\(\eta\) in cytoskeletal organization. Prolonged treatment of s-EL4 cells with phorbol esters results in inhibition of cell cycling along with a decreased expression of most of the PKC isozymes, including PKC\(\theta\). Introduction of virally expressed PKC\(\theta\), but not PKC\(\eta\), overcame the inhibitory effects of the prolonged phorbol ester treatment on cell cycle progression, suggesting a possible involvement of PKC\(\theta\) in cell cycle regulation. These results support differential functions for PKC\(\eta\) and PKC\(\theta\) in T cell activation.

Protein kinase C (PKC), a family of phospholipid-dependent serine/threonine-specific kinases, has been implicated in numerous signaling pathways in lymphocytes and other cells (reviewed in Refs. 1–3). At least 12 isozymes are recognized as members of the PKC family, and although all of these proteins share some structural similarities and rely on phospholipids for activation, many differences exist among them (reviewed in Refs. 2–4). The isozymes exhibit diverse tissue distribution, subcellular localization, and requirements for diacylglycerol (DAG) and Ca\(^{2+}\) as cofactors (reviewed in Refs. 2–4). The conventional isozymes, PKCa-, -\(\beta\)-, -\(\beta\)I-, and -\(\gamma\), require both Ca\(^{2+}\) and DAG; novel isozymes PKC\(\delta\), -\(\epsilon\), -\(\eta\), and -\(\theta\) are Ca\(^{2+}\) -independent; atypical isozymes PKC\(\chi\) and -\(\lambda\) are Ca\(^{2+}\) -independent and DAG- or phorbol ester-resistant. These differences argue for distinct functions of the isozymes.

Altered expression or activity of an individual PKC isozenzyme can lead to specific changes in biological function. In the human Jurkat T lymphocyte line, antisense constructs (5), PKC down-regulation (6), and co-expression with AP1 and nuclear factor of activated T cell transcription element reporter constructs (7) implicate PKC\(\alpha\) in interleukin 2 (IL2) production. Microinjection of isozyme-specific antibodies has implicated a PKC\(\beta\) isozenzyme in down-regulation of elevated intracellular Ca\(^{2+}\) in these cells (8). Ca\(^{2+}\) -independent PKC isozenzymes also have been implicated in activation of Jurkat cells. Genot et al. (7) showed that PKCe, but not PKCa, could induce expression of an NFkB reporter construct as well as expression of AP1 and nuclear factor of activated T cell transcription element reporter constructs, which PKCa also induced. However, different PKC isozenzymes have been implicated in some of these functions in other lymphocyte systems. Reasons for conflicting results may include cell-specific differences, redundancy in isozenzyme function, or incomplete inhibition, down-regulation, or activation of specific isozenzymes with the various reagents or methods used.

To investigate roles for individual PKC isozenzymes in various T cell functions, we have compared phorbol ester-sensitive (s) and -resistant (r) lines of EL4 mouse thymoma cells. s-EL4 cells, unlike r-EL4 cells, produce cytokines, adhere to plastic substrates, and become growth-inhibited when stimulated with phorbol esters (9, 10). An explanation for these differences may be a divergence in the PKC expression profile of the two cell lines. Northern and Western analysis revealed that s-EL4 and r-EL4 cell lines expressed comparable amounts of PKC\(\alpha\), -\(\beta\), and -\(\delta\) but that the r-EL4 cells produced substantially less PKCe (11), PKC\(\eta\), and PKC\(\theta\) (12). Long term phorbol ester treatment of s-EL4 cells resulted in the down-regulation of all of the PKC isozenzymes examined except for PKC\(\eta\), which exhibited a 5-fold increase in expression in comparison with control cells (12). These observations suggest that PKCe, PKC\(\eta\), and/or PKC\(\theta\) may contribute to the phorbol ester-induced responses in s-EL4 cells. In support of a role for PKCe, Baier et al. (13) noted that the overexpression of PKC\(\theta\) in s-EL4 cells resulted in an increase in transcription of an IL2 reporter construct when cells were treated with phorbol ester. That group also showed that expression of a constitutively active PKC\(\theta\) construct activated an AP1 reporter construct and that expression of a dom-
inent negative PKCθ construct blocked it (14). Consistent with a role for this isozyme in T cell activation, Monks et al. (15) observed that antigen stimulation of T cell clones led to the selective activation and translocation of PKCγ concomitant with proliferation of the T cells, and similar induction of IL2 and c-jun reporter constructs with expression of PKCθ was reported recently in Jurkat cells (16).

To elucidate further potential functions of PKCθ and the uniquely up-regulated PKCγ in EL4 cell activation, a virus-based transient expression system was used to introduce these PKC isoforms into the cells. Expression of constitutively active PKCγ in EL4 cells resulted in dramatic changes in the cell morphology as well as cytoskeletal organization that were similar to those observed in s-EL4 cells stimulated with phorbol ester. In contrast, expression of constitutively active PKCθ, but not PKCγ, counteracted the inhibitory effects of prolonged phorbol ester treatment on cell cycle progression. Taken together, these results suggest that PKCγ and PKCθ play distinct roles in cellular signaling, with PKCγ involved in cytoskeletal organization and PKCθ implicated in cell cycle progression.

**EXPERIMENTAL PROCEDURES**

**Materials—**DMEM, RPMI 1640, phosphate-buffered saline (PBS), trypsin/EDTA, and other tissue culture materials were purchased from Mediatech Inc. (Herndon, VA). Heat-inactivated fetal bovine serum was obtained from either Sigma or Summit Biotechnology (Fort Collins, CO). Penicillin/streptomycin was acquired from Life Technologies, Inc. and used between passages 6 and 14 after acquisition. s- and r-EL4 cells and used as follows: 1) addition of medium alone (mock infection), and 2) infection with dsSIN recombinants. Medium was replaced every 2–3 days. The supernatant was removed by centrifugation and the cells were treated with 0.2% trypsin/0.5 mM EDTA to detach them.

**Cell Culture—**Baby hamster kidney-21 clone 13 cells (BHK) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in DMEM supplemented with 10% fetal bovine serum, 10 μg/ml penicillin, 10 μg/ml streptomycin, and 2 ml glutamine and used between passages 6 and 14 after acquisition. s- and r-EL4 cells were obtained from ATCC and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10 μg/ml penicillin, 10 μg/ml streptomycin, and 2 ml glutamine. L929 fibroblasts were obtained from ATCC and were maintained in DMEM supplemented with 10% fetal bovine serum, 10 μg/ml penicillin, and 10 μg/ml streptomycin and were used between passages 8 and 17.

**Generation of Sindbis Recombinants—**The generation of double subgenomic Sindbis recombinants (dsSIN) capable of expressing either PKCγ or PKCθ was accomplished by exciting cDNAs encoding PKCγ and PKCθ from plasmid vectors kindly provided by H. Mishack and then cloning these cDNAs into the Sindbis plasmid pTE2JC1 (18). The fidelity of cloning was examined by analysis of restriction enzyme digestion. Those pTE2JC1 plasmids with the appropriate PKCγ or PKCθ inserts were amplified in Escherichia coli, pTE2JC1:CAT (chloramphenicol acetyltransferase) was described previously (18). Purified pTE2JC1:CAT, pTE2JC1:PKCγ, and pTE2JC1:PKCθ, linearized with the XhoI restriction enzyme, were employed as templates for in vitro transcription using SP6 RNA polymerase as described previously (19). 4 × 10⁶ BHK cells (10⁶/ml) were transfected with 5–10 μg of the RNA transcripts by square pulse electroporation using a BTX820 square pulse generator (BTX, San Diego) at 860 V in 50 μl (5 pulses with 1-s interval between pulses). Approximately 24 h post-transfection, the medium was collected and assayed for infectious virus titer in L929 cells. The resulting recombinant viruses were called dsSIN:CAT, dsSIN:PKCγ, and dsSIN:PKCθ for their ability to express CAT, PKCγ, and PKCθ, respectively.

The constitutively active clones of PKCγ and PKCθ were made by site-directed mutagenesis of their pseudosubstrate regions. Alanine 161 in PKCγ was replaced by a glutamate using oligonucleotides (5'-GC-CAAAGGAGAATCGGAA-3' and 5'-CTCCTGCACTCCCTTGGC-3') and alanine 148 in PKCθ was replaced by glutamate using oligonucleotides (5'-GCCAGAGAGATCACC-3' and 5'-AGTTTGATCTCTTCTCGG-3'). The mutant PKCγ and PKCθ fragments were inserted into the pTE2JC1 plasmid, and recombinant viruses capable of expressing constitutively active PKCγ (dsSIN:PKCγCAT) and constitutively active PKCθ (dsSIN:PKCθCAT) were generated as described above. The kinase-dead PKCθ mutant (dsSIN:PKCθK276) was generated by replacing lysine 384 with an alanine in the background of the A161E mutant. The PKCθ fragments were generated by polymerase chain reaction using 5'-TCAGGAGGCGACCAGCGCT-3' and 5'-TCAGACCCGGCAACGGAT-3'.

Catalytic domains of PKCγ and PKCθ were amplified by polymerase chain reaction using 5'-CATATGCTGCTAGAATCCTTACGACGAA-3' and 5'-CATATGCTGCTAGAATCCTTACGACGAACTCC-3', respectively, as 5' primers and the SP6 primer in the vector as the 3' primer. Resulting DNA fragments were separated and cloned into pTE plasmid, and recombinant viruses capable of expressing the catalytic domains of PKCγ (dsSIN:PKCγCAT) or PKCθ (dsSIN:PKCθCAT) were generated as described above. dsSIN:PKCγ starts with engineered Met as an initiation codon and is followed by Asn-345 to the carboxyl terminus of the protein (residue 684). PKCΔγ starts with Met-355 as an initiation Met and continues to the end of the protein (residue 708). Appropriate protein expression was confirmed by detection of labeled polyepitides following in vitro translation followed by translation in rabbit reticulocyte lysate and in the BHK cells by immunoblotting.

At least two clones were isolated from each independent plasmid construct. All experiments involving virus manipulation and handling were performed in a BL2 facility under the protocol approved by the Institutional Biosafety and Recombinant DNA Committee of the University of Virginia.

**Infection of Cells with dsSIN Recombinants—**Cells in monolayer or suspension culture were maintained in late logarithmic phase for infection with dsSIN recombinants. Medium was replaced every 2–3 days. The supernatant was removed by centrifugation and the cells were treated with 0.2% trypsin/0.5 mM EDTA to detach them.

**Western Blot Analysis—**1–2 × 10⁶ cells were washed with cold PBS and then lysed with either 200 μl of boiling Laemmli sample buffer (19) or RIPA buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM 4-(2-aminoethyl)-benzenesulfonic acid, 0.3 μM aprotinin, and 1 μM leupeptin). The RIPA buffer lysates were incubated for 15–30 min at 0 °C, centrifuged at 15,500 g for 2 min, and the protein concentrations of the supernatants were measured using a bicinchoninic acid (BCA)-based protein assay (Pierce). 4% formaldehyde for 20 min and then washed with PBS. Permeabilization was accomplished by incubating the cells for 30 min in washing solution (PBS, 1% bovine serum, 0.25% Nonidet P-40 and 0.02% sodium azide). Cells were incubated for 30 min in washing solution containing 100 μM fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma). Excess fluorescein was removed by washing the cells at least twice with the washing solution. The coverslips were mounted onto glass slides, and the cells were visualized by both phase contrast and fluorescence microscopy.
RESULTS

Expression of Viral PKC\(\eta\) and PKC\(\theta\) in EL4 Cells—To examine potential roles for PKC\(\eta\) and PKC\(\theta\) in T cells, logarithmic phase s-EL4 and r-EL4 cells were infected with Sindbis virus capable of expressing either PKC\(\eta\) (dsSIN:PKC\(\eta\)), PKC\(\theta\) (dsSIN:PKC\(\theta\)), or CAT (dsSIN:CAT) as an infection control. The infection times ranged from 4 to 24 h and employed an m.o.i. of 20 or 24 h post-infection, cells were collected and lysed in boiling Laemmli sample buffer. Proteins from 2.5 x 10^6 cells were separated by 8% SDS-PAGE and subjected to Western analysis. Immunoblots were probed with 0.2 \(\mu\)g/ml rabbit polyclonal antibody directed against the carboxyl terminus of PKC\(\eta\) and fluorescent microscopy (at 515 nm) using a 40 x Planaporph objective lens.

Cell Cycle Analysis—Logarithmic phase s-EL4 cells were treated with 100 \(\mu\)M PDB or 0.01% ethanol vehicle and then incubated for 6, 12, or 18 h at 37 \(^\circ\)C. Cells were then permeabilized in an isotonic solution, and the DNA was stained using propidium iodide (50 \(\mu\)g/ml in 0.3% Nonidet P-40, 100 \(\mu\)g/ml boiled RNAse A, and 0.1% sodium citrate). Stained cells were incubated at 4 \(^\circ\)C for at least 30 min. DNA content of the cells was examined using fluorescence activated cell sorting (FACS) at the FL2 channel wavelength on a FACScan instrument (Becton Dickinson), and results were analyzed by the CellQuest program (Becton Dickinson).

Effect of PKC\(\eta\) on the Cytoskeleton—To address whether PKC\(\eta\) is involved in the regulation of cell morphology or adherence, logarithmic phase s-EL4 and r-EL4 cells were either mock-infected or infected with dsSIN:CAT, dsSIN:PKC\(\eta\), dsSIN:PKC\(\eta\)\_ca, dsSIN:PKC\(\theta\), dsSIN:PKC\(\theta\)\_ca, or dsSIN:PKC\(\theta\)\_cd for 5 h and then fixed onto glass coverslips using a solution of 4% formaldehyde in PBS. Fixed cells were permeabilized and stained with FITC-conjugated phallolidin that binds to F-actin. Inspection of EL4 cells by phase contrast and fluorescence microscopy revealed that phorbol ester stimulation or expression of constitutively active or catalytic domain PKC\(\eta\) induced cytoskeletal changes. dsSIN:CAT-infected s-EL4 cells (Fig. 3, A and B), essentially identical to mock-infected cells (data not shown), were refractory indicating a rounded morphology. PDB treat-
PKC\(\eta\) and PKC\(\theta\) Function in T Cells

Prolonged treatment of r-EL4 cells led to an increase in production of filopodia and reorganization of the cytoskeleton as demonstrated by the formation of new F-actin containing structures (Fig. 4, C and D). Infection of these cells with dsSIN:PKC\(\eta_{CD}\) (Fig. 4, E and F) caused the appearance of membrane ruffling somewhat like that observed in s-EL4 cells with PDB treatment or the expression of PKC\(\eta_{CD}\). Expression of dsSIN:PKC\(\eta_{CD}\) in r-EL4 (Fig. 4, G and H) caused the appearance of one or two prominent actin-containing protrusions as it did in s-EL4 cells. However, expression of these active PKC\(\eta\) constructs did not result in adhesion of the s- or r-EL4 cells to plastic (data not shown). Expression of constitutively active PKC\(\theta\) (Fig. 4, I and J), the catalytic domain of PKC\(\theta\), or kinase dead PKC\(\eta\) in r-EL4 cells did not alter morphology or induce cytoskeletal reorganization (data not shown).

**FIG. 3.** Morphology of s-EL4 cells either treated with phorbol ester or infected with the constitutively active PKC\(\eta\) or PKC\(\theta\) virus. Logarithmic phase s-EL4 cells infected with the dsSIN:CAT (A and B), mock-infected and treated with 100 nM PDB for 30 min (C and D), infected with dsSIN:PKC\(\eta_{CA}\) (E and F), infected with dsSIN:PKC\(\eta_{CA}\) (G and H), or infected with dsSIN:PKC\(\eta_{CA}\) (I and J) were incubated at 37°C for 5 h. Cells were fixed with 4% formaldehyde, permeabilized with 0.025% Nonidet P-40 in PBS, and stained with 100 nM FITC-conjugated phalloidin in PBS with 0.025% Nonidet P-40. Cells were examined by phase contrast microscopy (A, C, E, G, and I) or by fluorescence microscopy at 515 nm (B, D, F, H, and J) using a 40\(\times\) Planarphor objective. Results shown are representative of nine independent experiments.

Involvement of PKC\(\theta\) in Cell Cycle Progression—Prolonged treatment of EL4 cells with phorbol esters inhibits cell cycle progression (10) with cell cycle blocks in G\(1\) and G\(2\)/M (22). This cell cycle inhibition may be due to the down-regulation of the majority of the PKC isozymes (12). Antigen-stimulated T cell clones show selective activation and translocation of PKC\(\theta\) concomitant with the proliferation of those T cells (15), suggesting the involvement of PKC\(\theta\) in the regulation of cell cycle progression. A potential connection between the activity of PKC\(\theta\) and cell cycle progression in s-EL4 cells was examined by analysis of DNA content. Following treatment with 100 nM PDB or 0.01% ethanol vehicle control, s-EL4 cells were collected at 6, 12, and 18 h, permeabilized, and stained with propidium iodide. DNA histograms obtained by FACS analysis revealed a dramatic decrease in cell populations corresponding to S phase (the area between the diploid (2N) and tetraploid (4N) DNA peaks) as early as 6 h after treatment (Fig. 5). The proportion of cells in S phase was only 7% of the total population at 6 h after PDB treatment in comparison with 32% of the ethanol-treated control cells. [\(^{3}\)H]Thymidine incorporation experiments also showed greater than a 3-fold decrease in DNA synthesis after 6 h of PDB treatment and 1 h of pulse labeling (data not shown), consistent with earlier studies (10, 22).

Potential roles for PKC\(\theta\) and PKC\(\eta\) in cell cycle progression were examined using a combination of prolonged PDB treatment, which down-regulates the majority of PKC isozyme activities but up-regulates PKC\(\eta\) (12), and transient expression of PKC\(\theta\), PKC\(\eta\), or both. Infection of s-EL4 cells with dsSIN:PKC\(\theta\) at an m.o.i. of 2 (versus an m.o.i. of 20 in Fig. 2) showed an increased expression of a PKC\(\theta\)-reactive band at approximately 80 kDa (Fig. 6) and some increase in lower molecular weight PKC\(\theta\)-reactive bands upon overexpression. A lower m.o.i. of 2 was used in the experiment of Fig. 6 so that the low endogenous level of PKC\(\theta\) could be visualized on the same blot without overexpression of the lanes from cells overexpressing PKC\(\theta\). PDB treatment for 8 h resulted in the down-regulation of PKC\(\theta\) expression in control cells, whereas expression of PKC\(\theta\) in cells infected with dsSIN:PKC\(\theta\) remained high, with 80 kDa and smaller PKC\(\theta\)-reactive bands readily detectable (Fig. 6).

To compare effects of PKC\(\eta\) and -\(\theta\) expression, logarithmic phase s-EL4 cells were mock-infected or infected with dsSIN:CAT, dsSIN:CAT and dsSIN:PKC\(\eta\), dsSIN:CAT and dsSIN:PKC\(\theta\), or dsSIN:PKC\(\theta\) and dsSIN:PKC\(\eta\) at a combined multi-
Multiplicity of 20 infectious particles per cell. Two h post-infection, cells were divided into two equal groups, and one group was treated with 100 nM PDB. After 6 h at 37 °C, cellular DNA was stained with propidium iodide and then analyzed using FACS. Fig. 7 shows that expression of PKC*_u can overcome the inhibition of cell cycle progression induced by phorbol ester treatment. Cells infected with dsSIN:PKC*_u or dsSIN:PKC*_h and dsSIN:PKC*_u progressed through the cell cycle in the presence of PDB, whereas cells infected with dsSIN:CAT or dsSIN:PKC*_h did not. Similar results were obtained after 12 and 18 h of PDB treatment.

Expression of dsSIN:PKC*_CA also rescued PDB-treated cells from growth inhibition; however, expression of the catalytic domain of PKC*_u in the cells did not (data not shown). Given the rapid degradation of the expressed catalytic fragment (Fig. 2), a potential function for the PKC*_u catalytic domain in cell cycle progression cannot be ruled out. r-EL4 cells are not growth-inhibited by phorbol ester treatment, and expression of PKC*_u or -h did not alter the cell cycle profiles (data not shown).

**DISCUSSION**

EL4 mouse thymoma cells have been used to help identify steps in phorbol ester-induced lymphocyte responses. s-EL4 cells respond with growth inhibition, adherence to plastic, and production of cytokines including IL-2 (9–14). A resistant line, r-EL4, lacks these responses (9, 10), and overexpression of PKC isozymes or constitutively active constructs in sEL4 cells has supported a role for PKC*_u in IL-2 production (13, 14). Previous studies revealed greatly diminished expression of PKCs -e, -h, and -u in the resistant cells (11, 12). In attempt to determine whether PKCs -h or -u contribute to phorbol ester responses of EL4 thymoma cells, s-EL4 and r-EL4 cells were transiently infected with recombinant Sindbis viruses capable of expressing CAT (dsSIN:CAT), the wild type isozymes (dsSIN:PKC*_h or -u), constitutively active isozymes generated by mutation in the pseudosubstrate sites (dsSIN:PKC*_CA or -CA), or catalytic domain constructs (dsSIN:PKC*_CD or -CD). All recombinants were expressed in all of the cells as determined by Western blot analysis (Figs. 1, 2, and 6).

PKC*_h immunoblots often revealed a doublet at approximately 80 kDa with an increase in dsSIN:PKC*_h and dsSIN: PKC*_CA predominantly in the lower band. The upper band may represent a nonspecific cross-reacting protein or, given that PKCs are subject to phosphorylation at multiple sites (reviewed in Ref. 3), it may represent a more phosphorylated
form of PKC\(\eta\), and increased expression of the smaller band in virus-infected cells may suggest incomplete phosphorylation of the virally expressed protein, perhaps because the high expression overwhems available kinases.

In addition, expression of PKC\(\eta\)-reactive bands at \(~45-50\) kDa was detected in the overexpressing cells. In the case of the dsSIN:PKC\(\eta\)RCA-infected cells, greatly increased expression of the \(~50\)-kDa band was observed, and this band seemed to increase at the expense of the 80-kDa bands at long times (24 h) of infection (Fig. 2). Since the antibody is specific for a carboxyl-terminal peptide, it is likely that these bands represent degradation products containing catalytic domains of PKC\(\eta\).

Detection of a 50-kDa fragment is suggestive of accumulation of a catalytically active carboxyl-terminal fragment (PKM). PKCs of the classical type (\(\alpha\), \(\beta\), and \(\gamma\)) are more susceptible to degradation by membrane-associated calpains when they are in their active conformation with the hinge region exposed (23, 24). However, in EL4 cells the isozymes typically are further degraded (12). It would seem that PKC\(\eta\) is not susceptible to this form of degradation since no degradation is observed with long term phorbol ester stimulation of s-EL4, and in fact, the cells up-regulate expression of this isozyme when other isoforms are degraded (12). It is possible that overexpressed PKC\(\eta\) is present at sufficient concentration to experience some degradation via calpains. Another possibility is that a less phosphorylated form of the enzyme may be targeted for proteolysis. Finally, PKC\(\beta\) (25–27) and human PKC\(\theta\) (28) are susceptible to caspase 3-mediated proteolysis at a DEVD site present in the hinge region of those isoforms. Resultant generation of stable PKC\(\delta\) or PKC\(\theta\) catalytic fragments in U937 myeloid leukemia cells has been implicated in apoptosis (26, 28). PKC\(\gamma\) lacks a DEVD site in the V3 region so it is not clear whether this member of the Ca\(^{2+}\)-independent PKCs also is susceptible to another form of proteolysis.

PKC\(\theta\) immunoblots also showed lower molecular weight forms of overexpressed enzyme (Figs. 2 and 6), especially in the case of the constitutively active construct. Again, these are consistent with the size of catalytically active PKM. Human PKC\(\theta\) is susceptible to caspase 3-mediated proteolysis at the V3 hinge region to generate a 42-kDa active fragment (28); however, murine PKC\(\theta\) lacks the relevant DEVD site so it is not clear how the fragment is generated in the EL4 cells.

Expression of the constitutively active PKC\(\eta\) caused a significant morphological change in both s- and r-EL4 cell lines. s-EL4 cells with dsSIN:PKC\(\eta\)RCA exhibited a flatter, less refractory appearance with membrane ruffling very similar to that of s-EL4 cells treated with 100 \(\mu\)M PDB (B, D, F, H, and J) and allowed to incubate an additional 6 h at 37 °C. Cells were permeabilized in isotonic solution, and their DNA was stained with propidium iodide. The DNA contents were examined by FACS analysis using the FL2 channel for at least 10,000 cells for each sample, and the data were analyzed using a CellQuest analysis program. A representative of three independent experiments is shown, and the mean ± S.D. of % of cells in S phase is indicated in each panel.

Fig. 7. Complementation of PDB-mediated inhibition of cell cycle progression by PKC\(\theta\) expression. Logarithmic phase s-EL4 cells were mock-infected (A and B), infected with dsSIN:CAT at an m.o.i. of 20 (C and D), infected with dsSIN:CAT and dsSIN:PKC\(\eta\) virus at an m.o.i. of 10 each (E and F), infected with dsSIN:CAT and dsSIN: PKC\(\theta\) at an m.o.i. of 10 each (G and H), or infected with dsSIN:PKC\(\eta\) and dsSIN:PKC\(\theta\) at an m.o.i. of 10 each (I and J). 2 h post-infection, cells were either treated with 0.01% ethanol (A, C, E, G, and I) or with 100 \(\mu\)M PDB (B, D, F, H, and J) and allowed to incubate an additional 6 h at 37 °C. Cells were permeabilized in isotonic solution, and their DNA was stained with propidium iodide. The DNA contents were examined by FACS analysis using the FL2 channel for at least 10,000 cells for each sample, and the data were analyzed using a CellQuest analysis program. A representative of three independent experiments is shown, and the mean ± S.D. of % of cells in S phase is indicated in each panel.
In r-EL4 cells phorbol ester treatment caused production of numerous small filipodia rather than the large membrane ruffles observed in phorbol-ester-treated s-EL4 cells (Fig. 4). Overexpression of PKC \( \eta \) caused membrane ruffling with enhanced F-actin staining in the ruffles similar to that in s-EL4 cells (Fig. 4). This result is consistent with a requirement for PKC \( \eta \) for these morphological changes since the control r-EL4 cells lack significant expression of PKC \( \eta \). Expression of the catalytic domain construct of PKC \( \eta \) in r-EL4 cells resulted in the appearance of some longer processes as it did in s-EL4 cells. This different morphology of cells expressing catalytic domain PKC \( \eta \) versus constitutively active PKC \( \eta \) may reflect some difference in subcellular localization and/or substrate recognition of the two PKC \( \eta \) constructs. Differences in subcellular localization might be expected since the catalytic domain constructs lack the membrane binding domains, and these domains have been observed to alter substrate specificity for PKC \( \eta \) (29).

Alteration of cytoskeletal organization in response to PKC activation is not limited to EL4 cells but is a rather universal response in cells of different lineage and is characterized by changes in F-actin content and formation of new actin structures (30–33). Cytoskeletal reorganization modulates many cellular functions such as adhesion (34), motility (35), and polarity (36, 37). However, the roles of individual isoforms in regulation of the cytoskeleton are not well established. In several adherent cell lines including BHK, chicken embryo fibroblasts, and L929 fibroblasts, as well as in primary rat aortic smooth muscle cells, expression of constitutively active PKC \( \eta \) but not constitutively active PKC \( \theta \) resulted in fewer stress fibers and more extensions of plasma membrane as well as stronger staining of F-actin near the plasma membrane. Goodnight et al. (38) also observed flattening of NIH3T3 fibroblasts and cytoplasmic blebbing upon phorbol ester treatment of NIH3T3 cells overexpressing PKC \( \eta \). Thus differences in expression of PKC \( \eta \) correlate with morphological changes in a variety of cell types.

We have observed association of PKC \( \eta \) with particulate fractions of s-EL4 cells in both control and phorbol ester-treated cells as have Sansbury et al. (39). Basu (40) observed similar particulate localization in two breast cancer cell lines. Goodnight et al. (38) noted localization of PKC \( \eta \) in a juxtanuclear area consistent with Golgi in untreated cells. Phorbol ester treatment caused translocation of a portion of PKC \( \eta \) to the outer cell membrane as well as transient punctate nuclear staining potentially consistent with nuclear pores (38). Chida et al. (41) had observed expression of PKC \( \eta \) in association with rough endoplasmic reticulum in keratinocytes and overexpressing COS cells, and Grief et al. (42) has reported nuclear staining of human keratinocytes with PKC \( \eta \) antibody. Immunofluorescent localization of overexpressed PKC \( \eta \) was not pursued here because of the inability to distinguish intact versus proteolyzed PKC \( \eta \) with the available antibody.

Regulation of PKC \( \eta \) appears to be quite distinct from that of other PKC isoforms. We had observed up-regulation of PKC \( \eta \) in response to phorbol ester treatment of s-EL4 cells under conditions where other isoforms were significantly down-regulated (12), and Basu (40) observed similar up-regulation of PKC \( \eta \) in breast cancer lines in correlation with protection of the cells against tumor necrosis factor-induced cytolysis. Although the functions of PKC \( \eta \) in various cells are not yet well elucidated, the data available support distinct regulation of this isozyme and its unique involvement in cellular signaling. Evidence is consistent with a role in morphological changes that may contribute to a variety of functional alterations depending on the cell type.

Although expression of neither wild type nor constitutively active PKC \( \theta \) affected morphology or cytoskeletal organization of EL4 cells (Figs. 3 and 4), overexpression of PKC \( \theta \), but not PKC \( \eta \), did have effects on cell cycle progression in EL4 cells (Fig. 7). Treatment of many hematopoietic cells including HL60 (43), s-EL4 (9, 10, 22), and T cells (44) with phorbol esters results in growth inhibition. A dramatic decrease in the number of s-EL4 cells entering S phase occurs after phorbol ester treatment (Fig. 5). This block was overcome by overexpression of PKC \( \theta \) but not PKC \( \eta \) (Fig. 7) implying a specific role for PKC \( \theta \) in cell cycle progression. Sansbury et al. (39) overexpressed PKC \( \eta \) in a different phorbol ester-resistant line of EL4 cells and observed a similar lack of effect on growth of the cells. The conclusion that PKC \( \theta \) contributes to cell cycle progression is consistent with the report from Monks et al. (15) that T cell proliferation correlated with PKC \( \theta \) translocation to the membrane. Phorbol ester treatment is expected to activate PKC \( \eta \) in EL4 cells, but it is possible that other activated isoforms account for the growth inhibition and/or that down-regulation of PKC \( \theta \) contributes to the cell cycle block. If PKC \( \theta \) is sufficient for inducing cells to transit the cell cycle, one would have to conclude that it is not essential since r-EL4 cells lack significant PKC \( \theta \) expression and continue to proliferate in the presence of phorbol ester. Indeed, expression of SIN:PKC \( \theta \) in r-EL4 cells failed to exhibit an effect, probably because the cells continue to cycle in the presence of phorbol ester (data not shown). It is possible that other isoforms may substitute for a cell cycle progression function of PKC \( \eta \) in those cells or that PKC \( \theta \) is important for counteracting the effects of other PKC isoforms that r-EL4 cells also lack. Alternatively, cell cycle progression in r-EL4 cells may be completely independent of PKC activation.

Again, it is interesting that caspase-3-mediated proteolysis of PKC \( \theta \) to generate an active catalytic fragment has been implicated in apoptosis in human U937 cells. Although the murine PKC \( \theta \) lacks the DEVD site for this proteolysis, it is possible that the PKC \( \theta \) fragment that is generated carries out a significant function distinct from that of the intact active isoform. This, in fact, seems likely since the two forms of the enzyme would be expected to exhibit distinct subcellular localization and thus to encounter different substrates.

In summary, evidence has been presented that PKC \( \theta \) and PKC \( \eta \) mediate distinct downstream functions in EL4 cells with PKC \( \eta \) contributing to actin cytoskeletal reorganization and morphological changes and PKC \( \theta \) contributing to cell cycle progression as well as to IL2 production (13, 14, 16). More detailed analysis of the subcellular localization of these isoforms, their constitutively active forms, and potentially active fragments of the enzymes should help elucidate their respective functions as will identification of downstream substrates.

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