Roles of Specific Isoforms of Protein Kinase C in the Transcriptional Control of Cyclin D1 and Related Genes*

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Although protein kinase C (PKC) has been implicated in cell cycle progression, cell proliferation, and tumor promotion, the precise roles of specific isoforms in these processes is not clear. Therefore, we constructed and analyzed a series of expression vectors that encode hemagglutinin-tagged wild type (WT), constitutively active mutants (ΔNPS and CAT), and dominant negative mutants of PKCs α, β1, β2, γ, δ, ε, η, θ, and ι. Cyclin D1 promoter reporter assays done in serum-starved NIH3T3 cells indicated that the constitutively active mutants of PKC-α and PKC-ε were the most potent activators of this reporter, whereas the constitutively active mutant of PKC-δ inhibited its activity. Transient transfection studies with a series of 5′-deleted cyclin D1 promoter constructs showed that the proximal 964-base region contained AP-1, SP1, and CRE enhancer elements, is required for activation of the cyclin D1 promoter by PKC-α. Deletion of the AP-1 enhancer element located at position −954 upstream from the initiation site abolished PKC-α-dependent activation of cyclin D1 expression. Deletion of the SP1 or CRE enhancer elements did not have any effect. A dominant negative mutant of c-Jun inhibited activation of the cyclin D1 promoter in a concentration-dependent manner, providing further evidence that AP-1 activity is required for activation of the cyclin D1 promoter by PKC-α and PKC-ε. The constitutively active mutants of PKC-α and PKC-ε also activated c-fos, c-jun, and cyclin E promoter activity. Furthermore, NIH3T3 cells that stably express the constitutively active mutants of PKC-α or PKC-ε displayed increased expression of endogenous cyclins D1 and E and faster growth rates. These results provide evidence that the activation of PKC-α or PKC-ε in mouse fibroblasts can play an important role in enhancing cell cycle progression and cell proliferation.

Protein kinase C (PKC) is a multigene family that encodes at least 11 distinct isoforms of lipid-regulated serine/threonine kinases (1, 2). Specific isoforms play pivotal roles in several signal transduction pathways that regulate cellular growth, transformation, and differentiation (3, 4). The isoforms are classified into three groups, based on their structure and cofactor requirement: (i) classic PKCs (α, β, βII, and γ), which are activated by diacylglycerol (DAG) or calcium, (ii) novel PKCs (δ, ε, η, θ, and μ), which are activated by DAG but not by calcium, and (iii) atypical PKCs (ζ and ξ), which are not responsive to either DAG or calcium. Each of these isoforms contains an N-terminal regulatory domain and a C-terminal catalytic kinase domain. The regulatory domains contain a pseudosubstrate domain, an autoinhibitory domain with substrate-like sequences that maintain the enzyme in an inactive state presumably by interacting with the substrate binding site in the catalytic domain. PKC activators like DAG, phorbol esters, and calcium are thought to relieve this intramolecular inhibition, resulting in a conformational change that liberates the substrate binding domain from the pseudosubstrate domain, thereby activating the enzyme.

In previous studies we obtained evidence that in NIH3T3 fibroblasts PKC-α and PKC-ε can enhance the activities of at least three signaling pathways that converge on the serum response element (SRE): c-Raf-MEK1-ERK-TCF, MEKK1-SEK1-JNK-TCF, and rhoA-SRF (5). The SRE is a transcriptional control element that plays an important role in the transcription of c-fos and other genes involved in cell proliferation. These findings suggest that specific isoforms of PKC integrate complex networks of signal transduction pathways that control gene expression. Cyclin D1 plays a critical role in the progression of mammalian cells through the G1 phase of the cell cycle. Amplification and/or overexpression of the cyclin D1 gene is often seen in several types of human cancer (6). The cyclin D1 promoter is one of the major targets for several growth stimulatory signaling pathways (7, 8). Therefore, in the present study we examined the possible roles of specific isoforms of PKC in the transcriptional control of cyclin D1, using serum-starved NIH3T3 mouse fibroblasts as a model system. We present evidence that, among the nine isoforms of PKC we examined, constitutively active mutants of PKC-α and PKC-ε were the most potent activators of the cyclin D1 promoter. We found that the AP-1 enhancer element in the cyclin D1 promoter is required for activation of the cyclin D1 promoter by PKC-α and PKC-ε, because activation of the cyclin D1 promoter by PKC-α or PKC-ε was abolished by either deletion of the AP-1 site or expression of a dominant negative c-Jun. Constitutively active mutants of PKC-α and PKC-ε also activated the promoters for c-fos, c-jun, and cyclin E and when stably expressed in

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§ The abbreviations used are: PKC, protein kinase C; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase/ERK kinase; JNK, c-Jun N-terminal kinase; SRF, serum response factor; SRE, serum response element; TCF, ternary complex factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; CAT, catalytic domains; DAG, diacylglycerol; WT, wild type; DN, dominant negative; DMEM, Dulbecco’s modified Eagle’s medium; CMV, cytomegavirus; IP, immunoprecipitation; GST, glutathione S-transferase; MARCKS, myristoylated alanine-rich C kinase substrate; ΔNPS, N-terminal pseudosubstrate deleted mutant.

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NIH3T3 cells stimulated cell growth. Thus, these findings provide evidence that in murine fibroblasts PKC-α and PKC-ε play important roles in enhancing cell cycle progression and cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The expression vector pHACE (5) was used to generate plasmids that encode WT or PKC mutants with a C-terminal HA tag (see Fig. 1A and Table 1). pHACE-PKC-WT expression plasmids were generated by ligating full-length open reading frames of different PKC isoforms into pHACE digested with EcoRI. pHACE-PKC-DN expression plasmids were generated by ligating full-length open reading frames of PKC isoforms with a dominant negative (DN) (R → R or K → M) point mutation at the ATP binding site into pHACE digested with EcoRI. pHA-PEC-KC-ΔNPS expression plasmids were generated by ligating cDNA fragments encoding pseudosubstrate deletion (ΔNPS) mutants of PKC isoforms into pHACE digested with EcoRI. pHACE-PKC-CAT expression plasmids were generated by ligating cDNA fragments encoding only the catalytic domains (CAT) of PKC isoforms into pHACE digested with EcoRI. All of the cDNA fragments of these PKC mutants were generated by PCR and were analyzed to confirm their sequences, using an automated DNA sequencing service (Applied Biosystems). The expression vectors encoding the WT and mutant forms of PKC-α, δ, ε, or η have been previously described (5). The cDNA for rat PKC-β1 was described previously (9). The cDNA for mouse PKC-β2 was a gift from Dr. C. L. Ashendel (10). The cDNA for mouse PKC-γ was a gift from Dr. R. M. Bell (11). The cDNA for mouse PKC-η was a gift from Dr. S. Ohno (12). The cDNA for human PKC-γ was a gift from Dr. T. Biden (13). The cyclin D1 promoter-luciferase plasmids and pSVE-c-Jun-N138 were gifts from Dr. R. G. Pestell (7), and the c-fos promoter-luciferase and c-jun promoter-luciferase plasmids were gifts from Dr. R. Prywes (14). The cyclin E promoter-luciferase plasmid was a gift from Dr. J. T. Parsons (15).

**Cell Culture, Transfection, and Reporter Assays—**NIH3T3 mouse fibroblasts were grown in Dulbecco’s minimal essential medium (DMEM) containing 10% calf serum. For reporter assays, triplicate samples of 1 × 10^5 cells in 35-mm plates were transfected using Lipofectin (Invitrogen) with 1 μg of the reporter plasmid, 0.05–5 μg of various expression vectors, and 1 μg of the control plasmid pCMV-β-gal. The pcDNA plasmid DNA was added to the transfections to achieve the same total amount of plasmid DNA per transfection. Twenty-four hours after transfection, cell extracts were prepared and luciferase assays were done using the Luciferase Assay System (Promega). Luciferase activities were normalized with respect to parallel β-galactosidase activities, to correct for differences in transfection efficiency. β-Galactosidase assays were performed using the β-Galactosidase Assay System (Promega).

**Western Blot Analysis—**NIH3T3 cells were grown in DMEM containing 10% calf serum, and COS-7 cells were grown in DMEM containing 10% fetal bovine serum. With both cell types, 2 × 10^5 cells in 60-mm plates were transfected using Lipofectin (Invitrogen) with 5 μg of the indicated expression vectors or the control vector pcDNA3. Six hours after transfection, the cells were fed with DMEM containing 10% fetal bovine serum and incubated overnight. The cells were then trypsinized and transferred to 10-cm plates and grown for 24 h before protein extraction. Cellular proteins were extracted by cell lysis in radioimmunoprecipitation assay buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate). HA-tagged PKC proteins were immunoprecipitated from 500 μg of cell extract protein using 3 μg of the anti-HA antibody and 30 μl of protein G-Sepharose, after a 3-h incubation at 4°C. The immunoprecipitates were washed twice with PKC extraction buffer and then twice with IP kinase buffer (50 mM HEPES, pH 7.5, 1.0 mM MgCl_2, 2.5 mM EGTA, 1 mM NaF, 0.1 mM Na_3VO_4, 10 mM β-glycerophosphate) and resuspended in 20 μl of IP kinase buffer. The kinase assay was initiated by adding 40 μl of IP kinase buffer containing 10 μg of a GST-MARKS substrate and 5 μCi of [γ-32P]ATP. The reactions were performed at 30°C for 30 min. The reactions were terminated by adding SDS sample buffer and boiled for 5 min. The reaction products were then analyzed by SDS-PAGE and autoradiography. Recombinant GST-MARKS proteins were expressed in Escherichia coli strain BL21(DE3)LysS and purified to homogeneity using glutathione S-Sepharose beads (Amersham Biosciences). The experiments were repeated three times and gave similar results.

**RESULTS**

**Generation and Characterization of NIH3T3 Cell Lines That Stably Express the ΔNPS Mutants of PKC-α and PKC-ε—**NIH3T3 cells were transfected with the control vector pcDNA3, pHACE-PKC-α-ΔNPS, or pHACE-PKC-ε-ΔNPS, using Lipofectin (Invitrogen). Twenty-four hours after transfection, the cells were transferred to DMEM containing 10% calf serum and neomycin (600 μg/ml, Invitrogen) to select for cells that stably expressed the transfected plasmids. Neomycin-resistant clones were pooled and passaged in DMEM containing 10% calf serum and neomycin (200 μg/ml).

For growth curve analysis, cells were plated in triplicate at a density of 2 × 10^4 cells per well in 6-well (35 mm) plates with 2 ml of DMEM medium containing 10% calf serum. The cells were re-fed with fresh medium every 3 days. The number of cells per well was counted using a Coulter counter, every day for the subsequent 7 days.

**Generation of Constitutively Active and Dominant Negative Mutants of Specific Isoforms of PKC—**The presence of multiple PKC isoforms in mammalian cells and the difficulty of generating molecular weight isoform-specific inhibitors of PKC, or a comprehensive series of isoform-specific PKC mutants, have made it difficult to determine the specific roles of individual isoforms in cell cycle progression and cell proliferation. Therefore, as described under “Experimental Procedures” (Fig. 1A and Table 1), we developed a series of expression vectors that encode HA-tagged wild type (WT), dominant negative (DN), constitutively active pseudosubstrate deleted (ΔNPS) and constitutively active catalytic domain fragments (CAT) of PKCs α, β1, β2, γ, δ, ε, η, ζ, and ι. Some of the mutants of PKCs α, δ, ε, and ζ were developed and described in one of our previous publications (5). This series of expression vectors was transfected into COS-7 cells to characterize the proteins encoded by each of these constructs. Western blot analysis (Fig. 1B) indicated that all but one of these constructs (PKC-ζ-ΔNPS) expressed significant amounts of the related HA-tagged proteins and that all of the major bands for these proteins were of the expected sizes. PKC-δ-ΔNPS was expressed at a much lower level than the other proteins (Fig. 1B), perhaps due to its instability or toxicity (as discussed below). It was also of interest to examine the in vitro kinase activities of these proteins. Therefore, immunoprecipitates of the same COS-7 cell extracts were prepared using an anti-HA antibody, and these immunoprecipitates were added to an in vitro kinase assay that contained a GST-MARKS-96–184 fusion protein (5) as the substrate. Western blot analysis showed that equal amounts of HA-tagged PKC proteins were immunoprecipitated (data not shown). We found that all of the PKC-WT, PKC-ΔNPS, and PKC-CAT constructs but none of the PKC-DN constructs displayed kinase activities with this substrate (Fig. 1C). Most of the ΔNPS mutants had slightly higher kinase activities than the corresponding WT proteins, and the CAT mutants had much higher kinase activities than the corresponding WT or ΔNPS proteins, except for PKC-ι-ΔNPS, which had higher kinase activity than
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PKC-ε-CAT. The fact that for most of the isoforms the CAT mutants had higher activity than the ΔNPS mutants suggests that the N-terminal regulatory domains of PKCs may contain kinase inhibitory sequences in addition to the pseudosubstrate domain. We found that the PKC-δ-ΔNPS mutant had a relatively high level of kinase activity (Fig. 1C) even though this protein was only expressed at a low level (Fig. 1D).

Activation of Cyclin D1 Promoter by Specific Isoforms of PKC—The ability of specific isoforms of PKC to activate the cyclin D1 promoter, in the absence of exogenous growth factors, was studied by using transient transfection reporter assays. NIH3T3 mouse fibroblasts were transfected with the control plasmid, PKC-WT, PKC-ΔNPS, or PKC-CAT constructs together with the −1745CD1-luciferase reporter plasmid, which contains the full-length cyclin D1 promoter. The cells were then serum-starved for 24 h and assayed for luciferase activity (Fig. 2A). Among the nine PKC-WT constructs tested, only PKC-ε-WT was able to cause a statistically significant activation of the cyclin D1 promoter (about 3-fold). However, when we transfected the PKC-ΔNPS constructs, which lack the pseudosubstrate sequences, the PKC-ΔNPS mutants of PKC-α and -ε caused significant activation of the cyclin D1 promoter (6- to 7-fold) and the PKC-ΔNPS mutants of PKCs β1, β2, γ, δ, and ε caused moderate activation of the cyclin D1 promoter (2- to 4-fold). When we transfected the constitutively active PKC-CAT constructs, the CAT mutants of PKCs α, β1, β2, γ, δ, ε, δ, and ε caused significant activation of the cyclin D1 promoter (3- to 12-fold). Of these constructs, PKC-α-CAT and PKC-ε-CAT were the most potent, because they caused about a 12-fold activation of the cyclin D1 promoter. In contrast to the stimulation seen with other CAT mutants, the PKC-δ-CAT mutant inhibited cyclin D1 promoter activity (by about 40%), which is consistent with evidence that PKC-δ can inhibit cell growth and induce apoptosis (17, 18). Down-regulation of endogenous cyclin D1 expression by PKC-δ has been reported in rat fat pad epididymal endothelial cells and rat smooth muscle cells (19–21). The v-src expression vector was used as a positive control and showed strong activation of the cyclin D1 promoter, as described previously (22). In general the CAT mutants gave higher activity than the corresponding ΔNPS mutants (Fig. 2A). These results are consistent with our in vitro kinase assays (Fig. 1C) and provide further evidence that there are inhibitory sequences in the N-terminal regions of these isoforms of PKC in addition to the pseudosubstrate region. The fact that the CAT mutants for these nine isoforms of PKC differed considerably in their activities (Fig. 2A) provides evidence that even though they lack the N-terminal regulatory domain they retain specificity. This is especially evident with PKC-ε, because both the WT and CAT constructs of this isoform had high activity and with PKC-δ, because both the WT and CAT constructs of this isoform had no or actually an inhibitory effect on cyclin D1 promoter activity (Fig. 2A). Furthermore, the relatively high activities of the CAT mutants of PKC-α and PKC-ε are not simply a result of their expression at higher levels than the other CAT mutants (Fig. 1B). We found that the

![Diagram of PKC structures](image)
Table I

| Coding sequences of the PKC mutants |
|------------------------------------|
| WT | DN | ΔNPS | CAT |
| PKC-α | 2–672 | 2–672 (K368R) | 30–672 | 326–672 |
| PKC-β | 2–671 | 2–671 (K371R) | 30–671 | 329–671 |
| PKC-β | 2–673 | 2–673 (K371R) | 30–673 | 329–673 |
| PKC-γ | 2–697 | 2–697 (K380R) | 29–697 | 338–697 |
| PKC-δ | 2–674 | 2–674 (K376R) | 152–674 | 334–674 |
| PKC-ε | 2–737 | 2–737 (K497R) | 164–737 | 395–737 |
| PKC-η | 2–683 | 2–683 (K384R) | 166–683 | 342–683 |
| PKC-ζ | 2–592 | 2–592 (K281M) | 124–592 | 239–592 |
| PKC-ε | 2–587 | 2–587 (K273M) | 125–587 | 232–587 |

ΔNPS and CAT mutants of PKC-α and PKC-ε also activate the cyclin D1 promoter in human epithelial cells, including HeLa, MCF-7, and SW480 cells. Therefore, these findings are not confined to mouse fibroblasts.

To confirm the roles of PKC isoforms in the transcriptional control of cyclin D1, we examined the effects of dominant negative mutants (DN) of the same PKC isoforms on serum-induced cyclin D1 promoter activity. NIH3T3 mouse fibroblasts were transfected with either the control plasmid or PKC-DN constructs together with the cyclin D1-luciferase reporter plasmid. The cells were then serum-starved for 24 h, treated with or without 20% serum for 24 h to induce cyclin D1 promoter activity, and assayed for luciferase activity. Fig. 2B shows that activation of the cyclin D1 promoter by serum was strongly inhibited by the DN mutants of PKC-α and PKC-ε (by about 80%), and partially by the DN mutants of PKCs β1, β2, γ, η, ι, or ε (by 10–30%). The DN mutants of PKC-δ did not cause significant inhibition of the activation of the cyclin D1 promoter by serum, which is consistent with our finding that the activated mutants of PKC-δ did not activate the cyclin D1 promoter (Fig. 2A). Taken together, these experiments provide evidence that PKC-α and PKC-ε are the two major PKC isoforms among the nine PKC isoforms examined in our studies that activate signal transduction pathways that lead to activation of the cyclin D1 promoter.

Involvement of AP-1 in Activation of the Cyclin D1 Promoter by Specific Isoforms of PKC—To map the region of the cyclin D1 promoter required for activation by specific isoforms of PKC, a series of cyclin D1 promoter truncation mutants (Fig. 3A) were transfected in the presence or absence of the constitutively active mutant of PKC-α (PKC-α-NPS). The cyclin D1 promoter mutants, which lack the AP-1, SP1, and CRE transcriptional response elements, were also used to map the response elements required for activation by specific isoforms of PKC. As shown in Fig. 3B, transient transfection studies with a series of 5′-deleted cyclin D1 promoter constructs showed that the proximal 964-base region, which contains AP-1, SP1, and CRE enhancer elements, is required for activation of the cyclin D1 promoter by PKC-α. Deletion of the AP-1 enhancer element located at the −954 position upstream of the initiation site completely abolished PKC-α-dependent activation of cyclin D1 expression. However, deletion of the SP1 or CRE enhancer elements did not have any effect on PKC-α-dependent activation of the cyclin D1 promoter. Similar results were obtained with the constitutively active mutant of PKC-ε (PKC-ε-NPS) (data not shown). To confirm the important role of the AP-1 site in activation of the cyclin D1 promoter by PKC-α, an increasing amount (0, 0.5, 1, or 2 μg) of a plasmid containing a dominant negative mutant of c-Jun (c-Jun-N138), which lacks the N-terminal transcriptional activation domain (Δ2–138) (7, 23), was transfected together with the activated mutant of PKC-α (PKC-α-NPS) and the −1745CD1-luciferase reporter plasmid. The dominant negative mutant of c-Jun (c-Jun-N138) inhibited activation of cyclin D1 promoter by PKC-α-NPS in a dose-dependent manner (Fig. 3C). The dominant negative mutant of c-Jun (c-Jun-N138) also inhibited activation of the cyclin D1 promoter by PKC-ε-NPS in a dose-dependent manner (data not shown). Taken together with the studies described in Fig. 3B, these results indicate that AP-1 activity is required for activation of the cyclin D1 promoter by PKC-α and PKC-ε in these cells.

Activation of c-fos, c-jun, and Cyclin E Promoters by Specific Isoforms of PKC—we also examined the effects of these isoforms of PKC on the expression of other genes involved in cell proliferation and cell cycle progression using transient transfection assays with promoter-luciferase reporter constructs of c-fos, c-jun, and cyclin E. c-fos and c-jun are immediate early response genes and are targets of several growth factors and oncogenes. Cyclin E is an important regulator of the G1/S transition of the cell cycle, together with cyclin D1. These studies focused on PKC-α and PKC-ε, because, as described above, they were the most potent activators of the cyclin D1 promoter. NIH3T3 cells were transfected with either the c-fos, c-jun, or cyclin E promoter reporter plasmid together with either the control plasmid or PKC-α-WT, PKC-α-NPS, PKC-ε-WT, PKC-ε-NPS, PKC-ε-CAT, PKC-ε-DN, PKC-ε-WT, PKC-ε-NPS, PKC-ε-CAT, or PKC-ε-DN constructs. Again, the v-scr expression vector was used as a positive control. Twenty-four hours after growing the transfected cells in serum-free medium, cell extracts were prepared and assayed for luciferase activity. We found that the ΔNPS and CAT mutants of both PKC-α and PKC-ε were able to activate the c-fos, c-jun, and cyclin E promoters in the absence of serum (Fig. 4, A–C). On the other hand, the DN mutants of PKC-α and -ε inhibited the background activities of all of these promoters, by about 30%. The ΔNPS and CAT mutants of PKC-α and PKC-ε were also able to activate the c-myc promoter in transient transfection assays (data not shown). These findings suggest that activation of PKC-α or PKC-ε alone is sufficient to activate signal transduction pathways that lead to transcriptional activation of both immediate early response genes (c-fos and c-jun) and G1 cyclins (cyclin D1 and cyclin E) in NIH3T3 mouse fibroblasts. It is likely that transcriptional activation of both c-fos and c-jun by PKC-α or PKC-ε can lead to enhanced activity of AP-1, because it is a dimeric complex between the c-Fos and c-Jun proteins and thus induce transcriptional activation of cyclin D1 through the AP-1 site in the cyclin D1 promoter.

Up-regulation of Endogenous Cyclin D1 and Cyclin E Expression by Stable Expression of ΔNPS Mutants of PKC-α and PKC-ε—NIH3T3 cell lines that stably overexpress the ΔNPS mutants of PKC-α or PKC-ε were generated and characterized to confirm the roles of PKC-α and PKC-ε as positive transcriptional regulators of G1 cyclins in mouse fibroblasts. We used pools of the transfected cells to eliminate possible effects of spontaneous clonal variations. Western blot analysis indicated that the protein levels of endogenous PKCs α, δ, ε, ζ, and ι in these derivatives were similar to those in the control NIH3T3 cells (data not shown). On the other hand, the NIH3T3 cells that stably overexpress ΔNPS mutants of PKC-α or PKC-ε (NIH3T3/PKC-α-NPS or NIH3T3/PKC-ε-NPS) expressed increased levels of endogenous cyclin D1 and cyclin E (4- to 7-fold) when compared with the control cells (NIH3T3/C) (Fig. 5A). Therefore, the results obtained in the above-described studies are not confined to transient expression reporter assays.

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2 J.-W. Soh and I. B. Weinstein, unpublished data.
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Discussion

The present studies provide evidence that among the nine isoforms of PKC examined PKC-α and PKC-ε are the most potent isoforms with respect to activating the expression of cyclin D1 (Fig. 2A). Furthermore, we obtained evidence that these two isoforms of PKC act mainly through the AP-1 element in the cyclin D1 promoter (Fig. 2B), although other elements in this promoter may also play important roles with respect to the effects of these two isoforms of PKC. These results are consistent with our finding that constitutively active mutants of PKC-α and PKC-ε also activate c-fos and c-jun promoter activity (Fig. 4), as well as our previous findings that in NIH3T3 cells PKC-α and PKC-ε activate MAP kinase pathways that would be expected to enhance AP-1 activity (5).

Our results are consistent with previous evidence that the phorbol ester tumor promoter TPA, a potent activator of several isoforms of PKC, induces cyclin D1 expression in mammalian cells (24) and that TPA triggers cell cycle progression at the G1 phase in NIH3T3 (25) and C3H 10T1/2 (24) mouse embryo fibroblast cells. In addition, Yan et al. (24) reported that activation of PKC-ε is required for activation of the MEK/mitogen-activated protein kinase signaling cascade during TPA-enhanced cell cycle progression in C3H 10T1/2 cells. On the other hand, Besson et al. (26) reported that PKC-α activity controls cell cycle progression in U215N human glioma cells by up-regulating the expression of p21Waf1/Cip1, presumably because the latter protein enhances the formation of cyclin-CDK complexes. However, none of these previous studies provided a comprehensive picture of the roles of a series of specific isoforms of PKC in controlling the expression of cyclin D1 and enhancing cell proliferation. This has been hampered by the lack of a series of low molecular weight compounds that are highly specific inhibitors of individual isoforms of PKC or of a series of expression vectors that encode mutant forms of different isoforms of PKC.

Therefore, to facilitate this type of analysis we have generated and characterized a series of expression vectors that encode the following: wild type forms of nine isoforms of PKC; mutants of these isoforms that lack the inhibitory N-terminal pseudosubstrate sequences (termed ΔNPS); mutants that lack the entire N-terminal regulatory domains (termed CAT); and mutants that lack kinase activity and, therefore, function as dominant negatives (termed DN) (Fig. 1A). When transfected into COS-7 cells all of these constructs expressed proteins of the expected sizes (Fig. 1B). Furthermore, both the ΔNPS and

To determine the effects of overexpression of the activated mutants of PKC-α or PKC-ε on the proliferation of NIH3T3 cells, the same number of NIH3T3/pcDNA3, NIH3T3/PKC-αΔNPS, or NIH3T3/PKC-εΔNPS cells were plated on 6-well plates, and cell proliferation was determined by counting cell numbers during the subsequent 7 days. When grown in monolayer culture with 10% calf serum, NIH3T3/PKC-αΔNPS or NIH3T3/PKC-εΔNPS cells displayed decreased exponential doubling times and increased saturation densities when compared with the control cells, as shown in Fig. 5B. The doubling times for NIH3T3/PKC-αΔNPS and NIH3T3/PKC-εΔNPS cells were shortened by ~25% (17.9 h) and 19% (19.3 h), respectively, in comparison with the doubling time of the control cells (23.8 h). The time required for NIH3T3/PKC-αΔNPS or NIH3T3/PKC-εΔNPS cells to enter S phase after serum starvation followed by serum stimulation was shorter by 2–4 h in these cells when compared with the control cells (data not shown). NIH3T3 cells that stably express the CAT mutants of PKC-α or PKC-ε also displayed increased expression of endogenous cyclins D1 and E and faster growth rates when compared with the control cells (data not shown). Taken together, these results show that PKC-α and PKC-ε positively regulate the expression of cyclin D1 in NIH3T3 cells and enhance cell proliferation. We are currently studying possible effects of stable expression of the ΔNPS mutants of PKC-α or PKC-ε on cell transformation and tumorigenesis.

Fig. 2. Activation of the cyclin D1 promoter by various isoforms of PKC. A, NIH3T3 cells were transfected with the −1745CD1-luciferase reporter plasmid, which has a full-length cyclin D1 promoter linked to the luciferase gene, together with a v-src plasmid (positive control), or the empty vector (C), or the indicated PKC constructs. The cells were then grown in serum-free medium for 24 h. Cell extracts were then prepared, and luciferase activities were measured and normalized with respect to parallel β-galactosidase activities. In this and the subsequent reporter assays, the data shown are representative of at least three independent experiments in which each assay was done in triplicate. The error bars indicate the standard deviations. Luciferase activities are expressed as -fold induction, after correction for β-galactosidase activities. B, NIH3T3 mouse fibroblasts were transfected with either the control plasmid (C) or PKC-DN constructs together with the −1745CD1-luciferase reporter plasmid. The cells were then serum-starved for 24 h, treated with or without 20% serum for 24 h to induce cyclin D1 promoter activity, and assayed for luciferase activity.

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the CAT mutants displayed considerably higher constitutive in vitro kinase activities than the corresponding wild type forms, and the DN mutants totally lacked in vitro kinase activity (Fig. 1C). It is of interest that the CAT mutants consistently displayed higher constitutive in vitro kinase activities (by 20–50%) than the corresponding NPS mutants, and the CAT mutants were also more active when assayed for their abilities the CAT mutants displayed considerably higher constitutive in vitro kinase activities than the corresponding wild type forms, and the DN mutants totally lacked in vitro kinase activity (Fig. 1C). It is of interest that the CAT mutants consistently displayed higher constitutive in vitro kinase activities (by 20–50%) than the corresponding NPS mutants, and the CAT mutants were also more active when assayed for their abilities percentage of this value. C. NIH3T3 cells were transfected with the −1745CD1-luciferase reporter plasmid together with either the empty vector (−), or the PKC-α-NPS construct and increasing amounts (0, 0.5, 1, or 2 μg) of the dominant negative c-Jun construct (pRSV-c-Jun-N138), as indicated. The cells were then grown in serum-free medium for 24 h. Cell extracts were then prepared, and luciferase activities were measured and normalized with respect to parallel β-galactosidase activities. The error bars indicate the standard deviations. Luciferase activities are expressed as -fold induction, after correction for β-galactosidase activities.
to activate the cyclin D1, c-Jun, and c-Fos proteins in NIH3T3 cells stably transfected with the ΔNPS mutants of PKC-α or PKC-ε. A, NIH3T3 cells were Lipofectin-transfected with the control vector pcDNA3, pHACE-PKC-α-ΔNPS, or pHACE-PKC-ε-ΔNPS. Twenty-four hours after transfection, the cells were transferred to DMEM containing 10% calf serum and neomycin (600 µg/ml) to select for stably transfected recipients. Neomycin-resistant clones were pooled and passaged in DMEM containing 10% fetal bovine serum and neomycin (200 µg/ml). Exponentially growing cells were collected, and cell lysates were subjected to Western blot analysis with an anti-cyclin D1 or anti-cyclin E antibody. Immunoblotting for β-actin served as a control for equal loading. B, growth curves of the control cells and PKC-α-ΔNPS- and PKC-ε-ΔNPS-expressing cells. The NIH3T3/pcDNA3 (C), NIH3T3/PKC-α-ΔNPS, and NIH3T3/PKC-ε-ΔNPS cell lines were plated in 6-well culture plates at the same density (2 × 10⁶ cells/well) in DMEM medium containing 10% calf serum. The number of adherent cells per well was then measured using a Coulter counter every day for 7 days.

Fig. 5. Increased expression of the cyclin D1 and cyclin E proteins in NIH3T3 cells stably transfected with the ΔNPS mutants of PKC-α or PKC-ε. A, NIH3T3 cells were Lipofectin-transfected with the control vector pcDNA3, pHACE-PKC-α-ΔNPS, or pHACE-PKC-ε-ΔNPS. Twenty-four hours after transfection, the cells were transferred to DMEM containing 10% calf serum and neomycin (600 µg/ml) to select for stably transfected recipients. Neomycin-resistant clones were pooled and passaged in DMEM containing 10% fetal bovine serum and neomycin (200 µg/ml). Exponentially growing cells were collected, and cell lysates were subjected to Western blot analysis with an anti-cyclin D1 or anti-cyclin E antibody. Immunoblotting for β-actin served as a control for equal loading. B, growth curves of the control cells and PKC-α-ΔNPS- and PKC-ε-ΔNPS-expressing cells. The NIH3T3/pcDNA3 (C), NIH3T3/PKC-α-ΔNPS, and NIH3T3/PKC-ε-ΔNPS cell lines were plated in 6-well culture plates at the same density (2 × 10⁶ cells/well) in DMEM medium containing 10% calf serum. The number of adherent cells per well was then measured using a Coulter counter every day for 7 days.

Fig. 6. Proposed roles of PKC isoforms in activation of cyclin D1 promoter. Various external stimuli such as growth factors and hormones can lead to activation of the PKC-α and PKC-ε. These, in turn, lead to activation of c-fos and c-jun gene expression, probably through the SRE promoter element of the c-fos gene and AP-1 promoter element of the c-jun gene. The newly synthesized c-Fos and c-Jun form AP-1 complexes, which activate the cyclin D1 promoter through the AP-1 promoter element located at the −954 position upstream from the transcription initiation site of cyclin D1. Expression of cyclin D1 then facilitates cell cycle progression from G1 to S phase and enhances cell proliferation. Activation of PKC-α and PKC-ε also leads to activation of the cyclin E promoter, through mechanisms that remain to be determined. This and other downstream effects probably also contribute to the enhancement of cell proliferation.

TPA is known to induce the expression of several immediate early response genes, including c-fos, c-jun, and c-myc, through the activation of PKC (29-31). These immediate early response genes are thought to be involved in the transition of cells from the G1 to G2 phases of the cell cycle. However, it was not known how specific isoforms of PKC regulate the progression of cells through the G1 to S phases of the cell cycle. In the present study we obtained evidence that PKC-α and PKC-ε are potent activators of the transcriptional activity of the cyclin D1 promoter and that this is mediated mainly through the AP-1 enhancer element present in the cyclin D1 promoter (Fig. 6). AP-1 sites are present in the promoters of the c-fos (32, 33) and c-jun (14, 34) genes. We found that in serum-starved NIH3T3 cells TPA induces expression of the c-Fos protein within 1 h, the c-Jun protein within 2 h, and the cyclin D1 protein within 6 h, when measured by Western blot analysis (data not shown). Maintenance of this chronological order of gene expression is probably essential for orderly transition of the cell cycle. It is not clear, however, how activation of AP-1 mediated by PKC-α or PKC-ε results in differences in the timing of when the corresponding promoters of these three genes, all of which contain the same AP-1 enhancer element, are transcriptionally activated. Angel et al. (34) showed that the transcriptional activation of c-jun by TPA requires both the post-translational activation of pre-existing AP-1 molecules and de novo gene expression of c-jun, thus leading to an increase in the total amount of AP-1. This mechanism could prolong the effect of transient signals generated by activation of PKC. It is possible that the AP-1 enhancer element in the cyclin D1 promoter requires a specific composition of AP-1 complexes that can be assembled only after the expression of c-fos and c-jun, or that activation of the cyclin D1
promoter is delayed until other enhancer elements are also activated. Sequential changes in chromatin structure might also play a role in controlling this temporal pattern of gene expression.

We found that there are three distinct groups of PKC isoforms with respect to their transcriptional activation of the cyclin D1 promoter: strong positive regulators (PKCs α and ε), weak positive regulators (PKCs β1, β2, γ, η, ζ, and ι), and a negative regulator (PKC-δ) (Fig. 2A). Even though our current studies using reporter assays indicate that PKC-α and PKC-ε exhibit similar activation patterns with the cyclin D1, c-fos, c-jun, and cyclin E promoters, it is possible that these isoforms and possibly other isoforms play more complex roles during the physiological regulation of expression of the corresponding endogenous genes because of their relative in vivo concentrations, cofactor requirements, substrate specificities, and tissue distributions (3, 35, 36).

A possible limitation of the present studies is that, because the N-terminal regulatory domain of PKC has been implicated in substrate specificity (37, 38) and intracellular localization (39–41), the ΔNPS and CAT mutants that we used might be altered with respect to their functions. However, these mutant forms still retain considerable specificity as shown in Fig. 2A. Thus, the ΔNPS and CAT mutants of PKC-α and PKC-ε were more active in stimulating cyclin D1 promoter activity than the corresponding mutants of the other seven isoforms of PKC examined. Furthermore, wild type PKC-δ generally exerts inhibitory effects on cell growth (17, 18), and wild type PKC-δ and the ΔNPS and CAT mutants of PKC-δ did not stimulate cyclin D1 promoter activity (Fig. 2A). Furthermore, the dominant negative (DN) mutants of PKC-α and PKC-ε markedly inhibited serum-stimulated activation of the cyclin D1 promoter, whereas the DN mutants of the other seven isoforms of PKC exerted no or only partial inhibition (Fig. 2B). These findings confirm our results obtained with the ΔNPS and CAT mutants of this series of PKCs. Furthermore, the ΔNPS and CAT mutants of PKC-α and PKC-ε were also strong activators of the promoters for c-fos, c-jun, and cyclin E (Fig. 4). The results obtained in the reporter assays with the mutants of PKC-ε are consistent with previous studies indicating that overexpression of PKC-ε stimulates growth and morphological transformation of rodent fibroblasts (27). Furthermore, the ability of the ΔNPS mutants of PKC-α and PKC-ε to stimulate transcription from the cyclin D1 promoter (Fig. 2A) is not confined to reporter assays, because we found that stable expression of these mutants in NIH3T3 cells enhanced cyclin D1 expression and cell proliferation (Fig. 5). Because we were concerned about the possible losses in the specificity of the ΔNPS mutants, in recent studies we introduced point mutations into PKCs α, δ, and ε that inactivate the pseudosubstrate domain. In these mutants a critical lysine or arginine residue in this domain was converted to glutamic acid. When these mutants were used in transient transfection assays with cyclin D1, c-fos, or c-jun promoter-luciferase reporters in NIH3T3 cells, we obtained results similar to those shown with the corresponding ΔNPS constructs in Fig. 2A (data not shown). Therefore, we believe that the effects we obtained with the ΔNPS mutants are physiologically relevant.

In summary, our results provide evidence that the activation of PKC-α or PKC-ε in murine fibroblasts is sufficient to induce the transcription of both immediate early response genes and G1 cyclins, thereby enhancing cell cycle progression and cell proliferation.

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