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Protein Kinase Cδ Inhibition of S-Phase Transition in Capillary Endothelial Cells Involves the Cyclin-dependent Kinase Inhibitor $p27^{Kip1}$*

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Distinct protein kinase C (PKC) isoforms differentially regulate cellular proliferation in rat microvascular endothelial cells (EC). Overexpression of PKCα has little effect on proliferation, whereas PKCδ slows endothelial cell proliferation and induces S-phase arrest. Analyses were performed on EC overexpressing PKCα (PKCαEC) or PKCδ (PKCδEC) to determine the role of specific cell cycle regulatory proteins in the PKCδ-induced cell cycle arrest. Serum-induced stimulation of cyclins D1, E, and A-associated kinase activity was delayed by 12 h in the PKCδEC line in association with S-phase arrest. However, the protein levels for cyclins D1, E, and A were similar. Nuclear accumulation of cyclin D1 protein in response to serum was also delayed in PKCδEC. In the PKCδEC line, serum induced $p27^{Kip1}$ but not $p16^{Ink4a}$ or $p21^{Cip1}$. Serum did not affect $p27^{Kip1}$ levels in the control vascular endothelial cell line. Immunoprecipitation-Western blotting analysis of $p27^{Kip1}$ showed serum stimulation of the vascular endothelial cell line resulted in increased amounts of cyclin D1 bound to $p27^{Kip1}$. In the PKCδEC line, serum did not increase the amount of cyclin D1 bound to $p27^{Kip1}$. Transfection of full-length $p27^{Kip1}$ antisense into the PCKδEC line reversed the S-phase arrest and resulted in normal cell cycle progression, suggesting a critical role for $p27^{Kip1}$ in the PKCδ-mediated S-phase arrest.

The vascular endothelium is a dynamic organ controlling hemostasis, vasodilation, and wound healing. The endothelium is influenced by shear stress, hypoxia, and chemotactic/mitogenic gradients that promote migration and division of its cells. Endothelial cellular division is an important component of the angiogenic response to many stimuli (2–4). As several different external agents promote or inhibit endothelial cell proliferation, the secondary messengers mediating these responses are being actively investigated. The protein kinase C (PKC)[1] family of Ser-Thr kinases is a common intracellular signaling pathway that coordinates a diverse array of signals that arise in the extracellular environment. Activation of the PKC pathway by phorbol esters, for example, induces endothelial cell proliferation and angiogenesis *in vivo* (5–7). In contrast, inhibition of the PKC pathway by prolonged treatment with phorbol esters inhibits mitogenesis of endothelial cells (8, 9). The molecular mechanisms regulating endothelial cell proliferation in response to mitogens and PKC activation are poorly understood. However, it is likely that specific components of the cell cycle regulatory apparatus may govern these responses. Recent studies have suggested that individual isozymes modulate specific cell cycle transitions in specific cell types. The G_1-S transition is regulated by the PKCη isozyme in NIH3T3 cells (10), whereas in vascular smooth muscle cells PKCα/ε regulate this transition (11). Overexpression of PKCα and PKCδ affect cellular proliferation and cell cycle progression in several different cell types. PKCα promotes cellular proliferation in human breast cancer and other cells (12, 13). In contrast, overexpression of the PKCδ isoform in Chinese hamster ovary fibroblasts in the presence of phorbol ester induces G_2/M-phase arrest (14).

The components of the cell cycle regulatory apparatus governing progression through the G_1 phase are increasingly well understood (15–17). The cyclin-dependent kinases (CDKs) are serine-threonine holoenzymes, consisting of a regulatory and catalytic subunit that phosphorylate target substrates to promote progression through the G_1 phase of the cell cycle. The phosphorylation of the pRB protein is mediated in part by cyclin D1/Cdk4 and cyclin E/Cdk2 (18, 19). The phosphorylation of pRB inactivates its ability to block G_1 phase progression. Phosphorylation of pRB is associated with release of E2F/DP proteins from their binding site on the pRB protein and progression through G_1 into a phase of DNA synthesis. The activity of the CDKs is inhibited by members of the $p21^{Waf1/Cip1}$ family ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) and the INK family ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$). These proteins inhibit CDK enzymatic activity in part through binding to the CDK regulatory subunit, thereby inhibiting holoenzyme association. The p21 family proteins are referred to as "universal inhibitors" because of the ability to block the activity of the cyclin D, cyclin E, and cyclin protein kinase A. As the CDK holoenzymatic activity is directed at nuclear substrates, the activity of the

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[1] The abbreviations used are: PKC, protein kinase C; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; EC, endothelial cell(s); V-EC, vector EC (cell line); PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GST, glutathione *S*-transferase; MACS, magnetic activated cell separation system; pRB, retinoblastoma protein; JAB, JAK-binding protein.

CDK inhibitor (CKI) is in part determined by its subcellular distribution. Thus, the CKI is inhibitory in the nuclear but not the cytoplasmic location.

In recent studies we showed that overexpression of PKCδ, but not PKCα, in EC inhibited cellular proliferation through an arrest in S-phase (1). These findings were consistent with several other studies in which the loss of PKCδ expression was associated with increased cellular proliferation or transformation (20–22). Together these studies suggested a role for PKCδ as an inhibitor of cellular proliferation, which may play an important role in slowing cell cycle progression in normal cells. The current studies were performed to understand the molecular mechanisms governing PKCδ-mediated cell cycle arrest. We report here that PKCδ overexpression (PKCδEC) delays serum-induced expression of kinase activity associated with cyclins D1, E, and A. However, the cyclin protein levels induced by serum in the PKCδEC were unchanged compared with the induction seen in control cells. Expression of the INK family members (p16^{Ink4a} and p18^{Ink4c}) and the CKI p21^{Cip1} was unchanged. PKCδEC contained higher nuclear levels of the cyclin-dependent kinase inhibitor p27^{Kip1} than vector controls. p27^{Kip1}-antisense reduced p27^{Kip1} levels and relieved the cell cycle defect induced by PKCδ, strongly suggesting that increased expression of p27^{Kip1} was responsible for the prolongation of S-phase in PKCδEC.

MATERIALS AND METHODS

Cell Culture—Cells used in this study were those reported by Harrington *et al.* (1). The parent endothelial cells were derived from the rat epididymal fat pad and were stably transfected with full-length cDNA encoding the PKC isoforms alpha (PKCαEC) and delta (PKCδEC) or vector (pc-DNA1) without insert (V-EC). Cells were cultured in medium 199 supplemented with 15% fetal calf serum and 2 mM L-glutamine with 50 mM neomycin used as the selection marker. Cells were removed from culture dishes using 0.05% trypsin, 0.02% EDTA, pelleted by centrifugation, and inoculated onto 150-cm^2 dishes at 1×10^4 cells/cm^2. Cells were allowed to attach overnight before being washed three times with PBS and incubated for 72 h in medium 199 alone. At the end of this incubation cells were stimulated by the addition of serum-containing media as described previously (1). Cultures of endothelial cells were analyzed at 12, 24, or 36 h after stimulation.

Western Blots and Immunoprecipitation-Western Analysis—Western blotting was performed as described previously (25) using antibodies to p21^{Cip1} (F-5), p27^{Kip1} (M-197), p16^{Ink4a} (F-12), p18^{Ink4c} (11256, a generous gift from Dr. Y. Xiong), cyclin A (H-432), cyclin E (M-20), cyclin D1 (HD-11, Santa Cruz Biotechnology, Santa Cruz, CA or HD-1, a gift from Drs. J. Koh and E. Harlow). In addition, α-tubulin monoclonal antibody (5H1) (23–25) was used in Western blot analysis. Cell homogenates (50 μg) were subjected to electrophoresis in an SDS-12% polyacrylamide gel and transferred electrophoretically to a polyvinylidene difluoride membrane (Micron Separations Inc., Westborough, MA). The concentration of the protein was assessed using the Bradford assay (Bio-Rad Laboratories, Hercules, CA) prior to loading. After transfer, the gel was stained with Coomassie Blue as a control for blotting efficiency. The blotting membrane was incubated for 2 h at 25 °C in Tween 20-PBS buffer supplemented with 5% (w/v) dry milk to block nonspecific binding sites. Following a 6-h incubation with primary antibody at a 1:1000 or 1:2500 (α-tubulin) dilution in T-PBS buffer containing 0.05% (v/v) Tween 20, the membrane was washed with the same buffer. For detection of proteins, the membrane was incubated with horseradish peroxidase-conjugated second antibodies (Santa Cruz Biotechnology) and washed again. The protein levels were visualized by the enhanced chemiluminescence system (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and quantitation was performed by densitometry using Bio-Rad molecular analysis software.

For immunoprecipitation-Western blot analysis, cells were washed twice with PBS containing 1 mM phenylmethylsulfonyl fluoride, scraped into immunoprecipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 7.5) with 1 mg/ml of leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) (Sigma) at 4 °C and centrifuged at 10,000 × *g* for 10 min. p27^{Kip1} was immunoprecipitated using agarose beads pre-coated with saturating amounts of M-197. Precipitated complexes were separated by electrophoresis, and Western blotting was performed using antibodies for cyclins A, E, and D1 as described above.

Cyclin-dependent Immune Complex Kinase Assays—Immunoprecipitation kinase assays were performed essentially as described previously using either Histone H1 (Sigma) or pRB substrate prepared from the vector pGEX-Rb (a gift from Dr. E. Harlow) (25–28). Cells were Dounce-homogenized in lysis buffer (150 mM NaCl, 50 mM HEPES, pH 7.2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, and 0.1 mM sodium orthovanadate (Sigma)), at 4 °C. Lysates were centrifuged at 10,000 × *g* for 5 min, and protein concentrations were determined using the Bradford assay. The supernatants (100 μg) were precipitated for 12 h at 4 °C with protein A-agarose beads pre-coated with saturating amounts of the cyclins A, E, and D1 antibodies. Immunoprecipitated proteins on beads were washed twice with 1 ml of lysis buffer and twice with kinase buffer (50 mM HEPES (pH 7.0), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM dithiothreitol). The beads were resuspended in 40 μl of kinase buffer containing the protein substrate (2 μg), 10 μM ATP, and 5 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq, Amersham Pharmacia Biotech). The samples were incubated for 30 min at 30 °C with occasional mixing. The samples were boiled in SDS-polyacrylamide gel sample buffer containing and separated by electrophoresis. Phosphorylated proteins were quantified after exposure to autoradiographic film (Labscientific, Inc., Livingston, NJ) by densitometry using ImageQuant version 1.11 (Molecular Dynamics computing densitometer, Sunnyvale, CA). The specificity of the phosphorylated pRB band was confirmed by several criteria. The phosphorylated GST-pRB band was inhibited in a concentration-dependent manner by the addition of p16^{Ink4a}, as described previously (29), and no signal was detected in control experiments in which substrate was omitted from the reaction mixture.

Transient Expression of p27^{Kip1} Antisense and Cell Cycle Analysis—To select transfected cells, co-transfection experiments were conducted using magnetic separation of transfected cells using CD4 as the marker and the magnetic activated cell separation system (MACS) (30, 31). The cDNA for p27^{Kip1} (32) (a gift from Dr. T. Hunter) was cloned into the tetracycline-regulated expression vector pBPSTR-1 (33) (a gift from Dr. S. Reeves) to form the antisense (pBPSTR-1(p27AS)) and sense (pBPSTR-1(p27S)) expression plasmids. A comparison was made between cells transfected with p27^{Kip1} antisense and the 27^{Kip1} sense expression plasmid and cells transfected with the empty expression vector cassette pBPSTR-1. Cells were stained with the cell-permeable DNA binding dye Hoechst 33342 (10 μg/ml) for 2 h before harvesting, and all subsequent solutions contained Hoechst 33342 (26, 34). Cells were harvested 48 h after transfection using EDTA (5 mg/ml) in PBS. Cells were pelleted by centrifugation and resuspended in PBS containing anti-CD4 coated magnetic beads. Cells were incubated with beads for 20 min, and CD4-positive cells obtained using a separating column. Cell cycle analysis was performed in a flow cytometer with a 360–365 nM argon-iron laser. Western blotting was performed on the cells as described above.

RESULTS

Serum Induction of Cyclin-dependent Kinase Activity—In our previous studies a delay in S-phase exit, in response to serum, was observed in a cell line overexpressing PKCδ (PKCδEC) (1). Serum induced extracellular signal-regulated kinase-2 activity equally in both the PKCδEC and parental ECs (1). Serum treatment of fibroblasts and primary myocytes induces the G_1 cyclins, cyclin D1 and cyclin E, and their associated kinase activity (18, 19). The induction of these kinases is required for cell cycle progression. To determine whether the proliferative defect observed in the PKCδEC stable line was associated with alterations in cyclin-associated kinase activity, the cell lines PKCδEC, PKCαEC, and V-EC were analyzed. Cells were stimulated with serum, cell extracts were harvested at sequential time points, and immunoprecipitation kinase assays were performed. These experiments were conducted on three separate occasions with similar findings. Cyclin D1 kinase activity, assessed using GST-pRB as substrate, was increased 15-fold at 24 h after serum addition in the V-EC (Fig. 1*A*). In the PKCδEC, cyclin D1 kinase activity was induced only 4-fold at 24 h (Fig. 1*A*), with maximal induction at 36 h in the PKCδEC line (Fig. 1*A*). The cyclin E and cyclin A kinase activ-

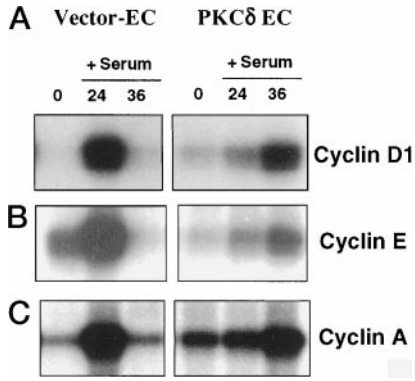


FIG. 1. **Effect of PKCδ overexpression on cyclin dependent kinase activity.** Immunoprecipitation kinase assays were performed on cell extracts derived from either a control stable cell line containing the empty expression vector cassette (*Vector-EC*) or the PKCδEC stable cell line. Analyses were performed after the addition of serum for the time points indicated (0–36 h). Immune complex kinase activity was assessed for cyclin D1 using GST-pRB as the substrate (*A*). Cyclin A and cyclin E kinase activities were assessed using histone H1 as substrate (*B* and *C*). The induction of each of the cyclin-dependent kinases occurred in a delayed manner in the PKCδEC stable cell line.

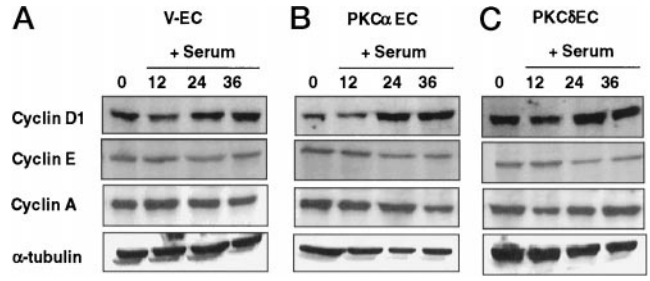


FIG. 2. **Cyclin protein abundance in microcapillary endothelial cell lines encoding PKCα and PKCδ.** Western blot analysis was performed on whole cell extracts derived from the cell lines V-EC (*A*), PKCαEC (*B*), or PKCδEC (*C*). Cells were treated with serum for the time points indicated (0–36 h). Western blot analysis was performed for cyclin D1, cyclin E, and cyclin A after serum treatment for the time points 0–36 h. Western blotting of the membranes for α-tubulin is shown. No significant differences in the total amount of cyclin proteins was observed between the V-EC, PKCαEC, and PKCδEC lines.

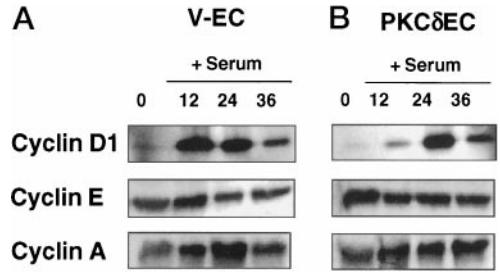


FIG. 3. **Nuclear protein abundance determined by Western blotting.** Nuclear fractions of the V-EC (*A*) and PKCδEC (*B*) lines were compared by Western blotting after serum treatment for the time points indicated. Cyclin D1 levels in the nuclear fraction were induced in a delayed manner in the PKCδEC line compared with the V-EC line. Cyclin D1 levels peaked at 12 h in the V-EC line and at 24 h in the PKCδEC line. No differences were observed in the relative abundance of cyclin A or cyclin E.

ities were induced 3- and 10-fold, respectively, by serum at 24 h in the V-EC line (Fig. 1, *B* and *C*). Kinase activity then decreased, approaching basal activity by 36 h. The addition of serum to the PKCδEC induced cyclin E kinase activity with a peak 5–6-fold induction at 36 h (Fig. 1*B*). Cyclin A kinase activity in the PKCδEC was only slightly increased after 24 h of serum treatment and continued to increase, peaking at 36 h (Fig. 1*C*). These studies demonstrate that serum induces activity of kinases associated with cyclins A, D1, and E in endothelial cells and that stable overexpression of the PKCδ isoform is associated with a delayed induction of these cyclin-dependent kinases.

Serum Regulation of the Cyclins—The activity of the cyclin-dependent kinases (CDK) is dependent in part upon the relative abundance of both the cyclin subunit and the presence of CDK inhibitors (CKI) (15–17). To determine whether the altered activity of the cyclin-dependent kinases in the PKCδEC was caused by differences in the abundance of cyclin proteins, Western blotting was performed. We hypothesized that the delayed induction of cyclin-dependent kinase activity in the PKCδEC was the result of delayed induction of the cyclin proteins. In the V-EC line (Fig. 2*A*), the addition of serum resulted in a modest induction of cyclin D1 at 24 h. The induction of cyclin D1 by serum was also observed in the PKCαEC and PKCδEC lines (Fig. 2, *B* and *C*). When the levels of cyclins A and E were compared following serum treatment, there were no significant differences between the V-EC and PKCαEC or PKCδEC lines (Fig. 2, compare *A*, *B*, and *C*). These results suggest that differences in the levels of cyclin D1, cyclin E, and cyclin A were not responsible for the inhibition of serum-induced cell cycle progression consequent upon PKCδ overexpression.

To examine further possible mechanisms for the delayed induction of cyclin kinase activity in serum-treated PKCδEC, we analyzed the nuclear abundance of the cyclins upon serum treatment. The cyclin D1 protein undergoes nuclear-cytoplasmic shuttling (35), which is critical for DNA synthesis in fibroblasts (36). We hypothesized that the S-phase arrest in the PKCδEC line may have been the result of delayed entry of cyclins to the nuclear compartment. To examine this possibility, nuclear fractions of serum-treated cells were examined (Fig. 3). The addition of serum to the V-EC line was associated with an increase in nuclear cyclin D1 levels at 12 h, which subsequently began to decrease at 24 and 36 h (Fig. 3*A*). The increase in nuclear cyclin D1 levels with serum treatment and subsequent decrease is consistent with previous observations in fibroblasts (35). Cyclin E nuclear abundance was relatively unchanged, and cyclin A levels increased at 12 h, peaking at 24 h. In the PKCδEC line (Fig. 3*B*), the induction of nuclear cyclin D1 was minor at 12 h and peaked at 24 h. The increase in nuclear cyclin D1 levels in the PKCδEC line was therefore delayed when compared with the V-EC line. The changes in cyclin E and cyclin A with the addition of serum in the PKCδEC were not significantly different from the V-EC line.

p27^{Kip1} Levels in the Presence of PKCδ Overexpression—Cyclin-dependent kinase activity is modulated *in vitro* by the cyclin-dependent kinase inhibitors, which include the INK4s (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, and p19^{Ink4d}), specific inhibitors of Cdk4 or Cdk6, and the CIP/KIP family (p21^{Cip1}, p27^{Kip1} and p57^{Kip2}) of "universal inhibitors" (15, 37, 38). We first hypothesized that increased INK4 protein abundance may contribute to the delayed serum-induced cyclin-dependent kinase activity in the PKCδEC line. Western blotting for p16^{Ink4a} abundance, however, showed no difference between the V-EC and the PKCδEC lines (Fig. 4). Western blotting was also performed for p18^{Ink4c} and p21^{Cip1}. No difference was observed in the abundance of either p18^{Ink4c} (data not shown) or p21^{Cip1} (Fig. 4) in PKCδEC as compared with the control cells or PKCαEC. In starved cells p27^{Kip1} levels were increased 2-fold in the PKCδEC (Fig. 4*C*) when compared with the PKCαEC or V-EC lines (Fig. 3, *A* and *B*). Furthermore, p27^{Kip1} levels increased

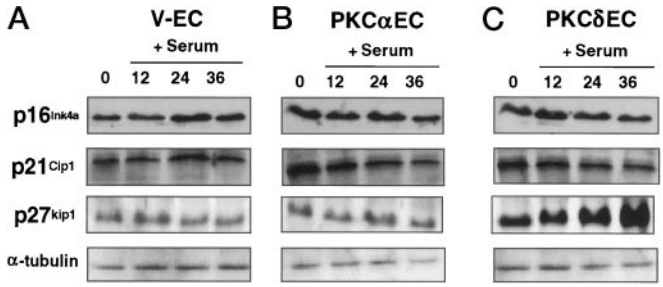


FIG. 4. **Cyclin-dependent kinase inhibitors assessed by Western blotting.** Western blotting of serum-treated cell lines V-EC (*A*), PKCαEC (*B*), and PKCδEC (*C*). Cells were treated with serum for 0–36 h. Analyses were performed on p16^{Ink4a}, p21^{Cip1}, p27^{Kip1}, and α-tubulin abundance. The levels of p27^{Kip1} were increased in response to the addition of serum in the PKCδEC lines (*C*) but were not increased in the V-EC (*A*) or PKCαEC (*B*) lines.

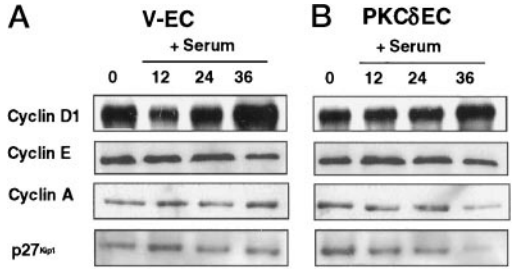
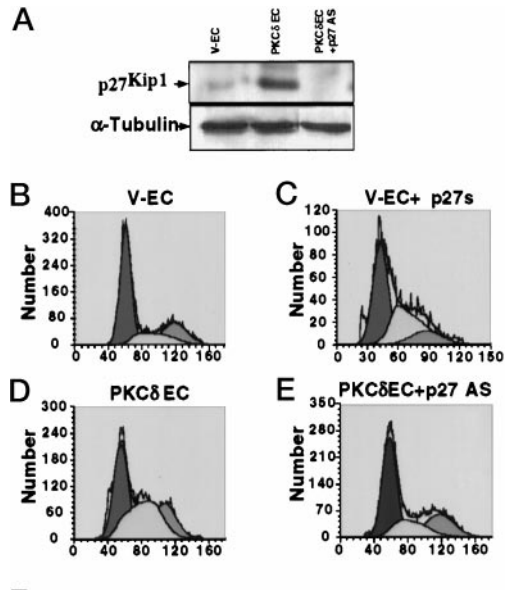


FIG. 5. **p27^{Kip1} Immunoprecipitation-Western blotting of the PKCδEC line.** The V-EC (*A*) and PKCδEC (*B*) cell lines were treated with serum for the time points indicated in hours. Immunoprecipitation was performed with the p27^{Kip1} antibody, and the precipitates were electrophoresed on an SDS-gel. Western blotting was then performed on the immunoprecipitate with antibodies to cyclin D1, cyclin E, cyclin A, and p27^{Kip1}. The relative amount of cyclin D1 bound to p27^{Kip1} increased in the serum-treated V-EC line at 36 h (*A*) compared with the relative binding of cyclin E. In the PKCδEC (*B*) line at 36 h, the relative amount of cyclin D1 compared with cyclin E bound to p27^{Kip1} was not significantly changed.



F

	G_0-G_1	S	G_2-M
V-EC	62 ± 4	24 ± 4	14± 6
V-ECp27s	42± 4	40 ± 4	18± 0.3
PKCδEC	33.5 ± 6	47 ± 11	19.5± 12
PKCδEC +p27AS	52± 6	30.6± 8	17.4±7.9
PKCδEC +p27s	39 ± 8	42± 2	19± 10

FIG. 6. **p27^{Kip1} antisense and the S-phase arrest of the PKCδ EC line.** *A*, Western blotting was performed for p27^{Kip1} and α-tubulin of the V-EC line, the PKCδEC line, or the PKCδEC line transfected with the expression plasmid pBSTR1 encoding p27^{Kip1} antisense (*p27AS*) and enriched by MACS sorting as described under "Materials and Methods" (41, 42). B–E, fluorescence-activated cell sorting analysis was performed on the cell lines indicated, with the proportion of cells in each phase of the cell cycle shown in *F*. Cell cycle analysis was performed on the V-EC line (*B*), the V-EC line transfected with the p27-sense expression plasmid (*pBPSTR1-p27S*) with subsequent MACS sorting (*C*), the PKCδEC line (*D*), and the PKCδEC line transfected with the p27^{Kip1} antisense (*AS*) plasmid with MACS sorting (*E*).

4-fold after 24 h of serum treatment in the PKCδEC line (Fig. 4*C*) but were unchanged in the PKCαEC or V-EC (Fig. 4, *A* and *B*).

p27^{Kip1} Immunoprecipitation Assays—These observations suggested that the p27^{Kip1} level increased in response to serum in the PKCδEC line but that this increase was not observed in the PKCαEC or the V-EC line. p27^{Kip1} is capable of binding cyclin D1/Cdk4 and cyclin E/Cdk2. It is thought that p27^{Kip1} inhibits cyclin E/Cdk2 activity but may not inhibit phosphorylation of pRB by cyclin D1/Cdk4 under certain circumstances (39). Thus, the relative binding of the CIP (p21/p27^{Kip1}) family proteins to cyclin D1 complexes compared with cyclin E complexes may determine the cytostatic activity of the complex (39). Immunoprecipitations were therefore performed on the V-EC and the PKCδEC lines using a p27^{Kip1}-specific antibody (Fig. 5). The relative amount of p27^{Kip1} in each immunoprecipitate was examined by Western blotting (Fig. 5, *bottom panel*). The relative amount of cyclin protein bound to the p27^{Kip1} was also assessed by Western blotting and was compared with the amount of p27^{Kip1} in the immunoprecipitate. The relative amount of cyclin D1 bound to p27^{Kip1} increased at 36 h in the V-EC line (Fig. 5*A*). The binding of cyclins E and A to p27^{Kip1} was not significantly changed after serum addition (Fig. 5*A*). In the PKCδEC line (Fig. 5*B*), the relative increase in cyclin D1 compared with cyclin E binding, found in the V-EC line at 36 h, did not occur. The relative binding of cyclins E and A to p27^{Kip1} was also unchanged. The lack of increase in binding of p27^{Kip1} to cyclin D1 may have caused by the relative delay in nuclear entry of cyclin D1 in the PKCδEC line compared with the V-EC line (Fig. 3). These results suggest that in the V-EC line there is a modest increase in the amount of cyclin D1 bound to p27^{Kip1} compared with cyclin E after 36 h of serum treatment. This increase in cyclin D1/p27^{Kip1} is not observed in the PKCδEC line.

p27^{Kip1} Antisense and the Cell Cycle Delay Induced by PKCδEC—The analysis of the CKI in the PKCδEC line indicated preferential induction of p27^{Kip1} but not the other CKI in response to serum. Because p27^{Kip1} overexpression is capable of inducing cell cycle arrest in fibroblasts (32), these findings raised the possibility that p27^{Kip1} may be involved in the PKCδ-induced S-phase arrest in endothelial cells. To examine the role of p27^{Kip1} in PKCδEC cell cycle delay, we used a p27^{Kip1} antisense construct. Transfection of rat microvascular endothelial cells with the p27^{Kip1} antisense expression plasmid reduced basal p27^{Kip1} levels 2-fold (Fig. 6*A*). The p27^{Kip1} antisense vector was also used to transfect the PKCδEC line. The increased p27^{Kip1} levels were reduced 10-fold by the p27^{Kip1} antisense expression plasmid. The overexpression of the empty expression vector cassette did not affect p27^{Kip1} levels. These studies demonstrated that the p27^{Kip1} antisense expression plasmid can reduce p27^{Kip1} levels in the PKCδEC line.

To determine the effect of p27^{Kip1} antisense on the cell cycle

arrest induced by PKCδEC, cells were transfected with the p27^{Kip1} antisense expression plasmid in conjunction with CD4 expression plasmid, and magnetic cell sorting was conducted with cell cycle analysis of the transfected cells. The cell cycle histograms are shown in Fig. 6, *B–E*. The control V-EC were transfected with the p27^{Kip1} expression vector. The relative proportion of cells in S-phase was increased from 24 to 40% (Fig. 6*F*). An increased proportion of the PKCδEC were in S-phase (47%) compared with the V-EC line, as described previously (1). Overexpression of the p27^{Kip1} antisense in the PKCδEC line reduced the proportion of cells in S-phase by 17% (Fig. 6*F*), with the resultant histograms similar to the V-EC line. These results suggest that the increase in p27^{Kip1} found in the PKCδEC line may play an important role in the increased proportion of cells found in S phase. The reversal of the S-phase arrest by p27^{Kip1} antisense was observed in three additional PKCδEC stable lines (data not shown).

DISCUSSION

The molecular mechanisms by which specific isozymes of the PKC family regulate cellular proliferation are poorly understood. The current studies extend our previous findings that PKCδ delays S-phase progression in rat microvascular endothelial cells (1). The PKCδ-mediated inhibition of S-phase progression was associated with a delay in the induction of the kinase activities associated with cyclins D1, E, and A. An investigation of the abundance of the cyclin-dependent kinase inhibitors associated with the inhibition of the cyclin kinase activity revealed an increase in the abundance of the "universal inhibitor," p27^{Kip1}. The relative abundance of p21^{Cip1} and the INK4 protein family was unchanged, suggesting that the induction of p27^{Kip1} was a relatively specific change. In addition, overexpression of an antisense expression plasmid for p27^{Kip1}, which was shown to reduce p27^{Kip1} protein levels, was also shown to reverse the S-phase arrest observed in the PKCδEC lines. These studies are consistent with a model in which the induction of p27^{Kip1} may play an important and specific role in PKCδ-mediated S-phase arrest in microvascular endothelial cells.

The pRB protein is a critical regulator of cell cycle progression, and the phosphorylation of pRB during G_1 phase coincides with passage of the cell through the restriction point in G_1 (15, 16). pRB undergoes continued phosphorylation throughout the S-phase (40), and the cyclin D1-dependent phosphorylation, which is required for cyclin D1 to promote cell cycle progression, occurs on specific phosphorylation sites that can be assessed in immunoprecipitation assays using a pRB fragment containing this site (25–28). Upon phosphorylation by cyclin D1, cyclin E kinase further phosphorylates pRB at distinct sites (41). Cyclin E kinase activity phosphorylates and inactivates additional substrates that contribute to cell cycle progression in a pRB-independent manner (42). In the current studies the induction of cyclin D1-dependent pRB phosphorylation was maximally induced at 24 h in the V-EC and PKCαEC but occurred in a delayed manner in the PKCδEC. The induction of cyclin E kinase activity by serum was also delayed in the PKCδEC. These data are consistent with the role of p27^{Kip1} as a "universal inhibitor" of both cyclin E and cyclin D1 kinase activity. The phosphorylation of pRB coincides with the loss of the ability of pRB to bind and inhibit E2F/DP complexes. The corresponding induction of "free E2F activity" activates genes involved in DNA synthesis. In the current studies, the delayed induction of cyclin A kinase activity, a marker of S-phase entry, in the PKCδEC line, is consistent with the delayed entry into S-phase. pRB is a poor substrate for cyclin E kinase, and cyclin E overexpression can promote S-phase entry independently of pRB, suggesting that cyclins D1 and E function in parallel pathways to promote S-phase entry (21, 42). The current studies suggest that PKCδ inhibits these parallel pathways in EC lines.

In the current studies, PKCδ induced p27^{Kip1} in rat microvascular endothelial cells. The induction of p27^{Kip1} by serum was enhanced in the PKCδEC in association with S-phase arrest. Antisense p27^{Kip1} expression blocked the PKCδEC-induced S-phase arrest. Overexpression of p27^{Kip1}, initially characterized as a protein homologous to p21^{Cip1} (32), can delay cell cycle progression in fibroblasts (43, 44). In recent studies p27^{Kip1} reduced the proliferation of smooth muscle induced by angioplasty and mediated the inhibition of smooth muscle cell proliferation by fibrillar collagen (45, 46). Together these studies suggest that p27^{Kip1} may be an important inhibitor of vascular remodeling (45). Our finding that p27^{Kip1} is involved in the cell cycle delay by PKCδ extends the known cytostatic signaling pathways in which p27^{Kip1} is involved. p27^{Kip1} also mediates the cytostatic effects of rapamycin and cAMP (32, 47–49).

In the current studies, p27^{Kip1} immunoprecipitation assays were performed to assess the effect of the PKC isoforms on the multimeric complexes bound to p27^{Kip1}. The cyclin/CDK complex to which p27^{Kip1} is bound determines its functional activity. p27^{Kip1} associates with cyclin E in a variety of cell types during quiescence (47, 50). When bound to cyclin D1/Cdk4, p27^{Kip1} may not be inhibitory (47, 51–53), whereas cyclin E/Cdk2 activity is inhibited by p27^{Kip1}. In the current studies, we compared the relative proportion of p27^{Kip1} bound to either cyclin D1 or cyclin E after 36 h of serum stimulation. In the PKCδEC line, p27^{Kip1} was bound to both cyclins D1 and E after 36 h stimulation; however, there was relatively more cyclin D1 bound to p27^{Kip1} in the V-EC line (Fig. 4). Thus, in the PKCδEC line the serum-induced increase in cyclin D1 binding to p27^{Kip1} is reduced. It is thought that the removal of p27^{Kip1} from the cyclin E/CDK complex is an essential step for S-phase entry. Through binding cyclin D1/Cdk4, p27^{Kip1} is sequestered from cyclin E/Cdk2, reducing its inhibition by p27^{Kip1} (47, 51–53). Thus, in the PKCδEC line it may be expected that p27^{Kip1} is incorporated proportionately more in an inhibitory complex with cyclin E than is the case in the V-EC line. The failure of p27^{Kip1} to bind increasing amounts of cyclin D1 may be the result of the delayed nuclear entry of cyclin D1 in response to serum (Fig. 3). Thus, these studies suggest that PKCδ overexpression both increases the amount of p27^{Kip1} induced in the cell in response to serum stimulation and also alters the multiprotein complex with which p27^{Kip1} is associated in the cell.

The mechanisms responsible for the increased p27^{Kip1} levels in the PKCδEC line remain to be fully evaluated. The abundance of p27^{Kip1} is regulated primarily at a post-translational level, and p27^{Kip1} protein levels decrease after mitogenic stimulation in quiescent NIH3T3 cells (50, 54, 55). The degradation of p27^{Kip1} upon mitogen stimulation is dependent upon prior phosphorylation. Cyclin E/Cdk2-induced phosphorylation of p27^{Kip1} on T187 in murine fibroblasts (56) and phosphorylation of p27^{Kip1} by cyclin E/Cdk2 enhanced degradation of p27^{Kip1}. The delayed induction of cyclin E/Cdk2 activity in the PKCδEC line (Fig. 1C) may have delayed phosphorylation of p27^{Kip1}, in turn delaying its phosphorylation-dependent degradation. The binding of the JAB-1 gene product to p27^{Kip1} causes p27^{Kip1} degradation (57). An alternate mechanism may be that JAB-1 is a downstream target of PKCδ, with phosphorylation resulting in functional inactivation of JAB-1.

The mechanism by which p27^{Kip1} inhibits cell cycle progression may vary with the cell type, although our studies are consistent with the model in which p27^{Kip1} inhibits cell cycle

progression in part by binding to Cdk2 and thereby reducing cyclin E/Cdk2 kinase activity (32). Increased p27^{Kip1} levels induced by overexpression in VSMCs was associated with reduced Cdk2 activity (45). Consistent with our findings overexpression of p27^{Kip1} in the nuclear, rather than the cytoplasmic, compartment was required for the cell cycle arrest (58), indicating that the subcellular distribution of p27^{Kip1} is important in the inhibition of cellular proliferation. In recent studies an alternate mechanism of p27^{Kip1} action was proposed. In LAP-3 cells, derived from NIH3T3 cells, p27^{Kip1} overexpression induced a cell cycle arrest associated with a specific E2F pocket protein complex. One consequence of the p27^{Kip1}-Cdk2 association was disruption of the interaction between Cdk2 and both the E2F-p130 and the E2F-p107 repressor complexes (59). The p130/p107 complexes that were induced by p27^{Kip1} were similar to the complexes induced by serum starvation (59). Further studies will be directed at analyzing the effect of p27^{Kip1} in the presence of the PKCδEC on components of the E2F-130 and E2F-p107 complexes. The present studies indicate, however, that alterations in cyclin E protein abundance do not appear to be important in the cell cycle effects mediated by p27^{Kip1}, arguing against an indirect effect of p27^{Kip1} on E2F-p130/p107 complex activity.

The results presented here are consistent with recent studies in which PKC isozymes were implicated in the inhibition of cellular proliferation and cell cycle progression. PKC has been shown to inhibit cell cycle progression in intestinal epithelial cells (60), IMR-90 fibroblasts (61), melanoma cells (62), and vascular endothelial cells (63, 64). Both the PKCα and PKCδ isozymes have been implicated in the inhibition of cellular proliferation in different cell types. Overexpression of PKCα inhibited cell cycle progression in Chinese hamster ovary cells (65), B16 melanoma cells (66), and F9 teratocarcinoma cells (67). In rat microvascular capillary endothelial cells (EC), PKCα does not inhibit cell cycle progression but rather promotes migration of the endothelial cells in response to growth factors (1). The studies suggest the cell cycle regulatory effect of the PKC isozymes may be cell type specific. PKCδ is the only isoform to undergo tyrosine phosphorylation (68), and PKCδ was inactivated by tyrosine phosphorylation in v-Src (21) and v-Ras (22) transformed cells, raising the possibility that inactivation of PKCδ may promote unregulated cellular proliferation and transformation. In view of the current findings that p27^{Kip1} is required for the cell cycle inhibitory function of PKCδ and the prior observations that PKCδ inactivation may play an important role in oncogene induced transformation, future studies are warranted to examine the role of p27^{Kip1} in oncogene/PKC-induced transformation.

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REFERENCES

1. Harrington, E. O., Loffler, J., Nelson, P. R., Kent, K. C., Simons, M., and Ware, J. A. (1997) *J. Biol. Chem.* **272,** 7390–7397
2. Yang, E. Y., and Moses, H. L. (1990) *J. Cell Biol.* **111,** 731–741
3. Ausprunk, D. H., and Folkman, J. (1977) *Microvasc. Res.* **14,** 53–65
4. Yancopoulos, G. D., Klagsbrun, M., and Folkman, J. (1998) *Cell* **93,** 661–664
5. Montesano, R., and Orci, L. (1985) *Cell* **42,** 469–477
6. Hu, D. E., and Fan, T. P. (1995) *Inflammation* **19,** 39–54
7. Wright, P. S., Cross-Doersen, D., Miller, J. A., Jones, W. D., and Bitonti, A. J. (1992) *J. Cell. Physiol.* **152,** 448–457
8. Presta, M., Tiberio, L., Rusnati, M., Dell'Era, P., and Ragnotti, G. (1991) *Cell Regul.* **2,** 719–726
9. Daviet, I., Herbert, J. M., and Maffrand, J. P. (1990) *FEBS Lett.* **259,** 315–317
10. Livneh, E., Shimon, T., Bechor, E., Doki, Y., Schieren, I., and Weinstein, I. B. (1996) *Oncogene* **12,** 311–320
11. Sasaguri, T., Kosaka, C., Hirata, M., Masuda, J., Shimokado, K., Fujishima, M., and Ogata, J. (1993) *Exp. Cell Res.* **208,** 311–320
12. Hirai, S., Izumi, Y., Higa, K., Kaibucji, K., Mizuno, K., Osada, S., Suzuki, K., and Ohno, S. (1994) *EMBO J.* **13,** 2331–2340
13. Ways, D. K., Kukoly, C. A., deVente, J., Hooker, J. L., Bryant, W. O., Posekany, K. J., Fletcher, D. J., Cook, P. P., and Parker, P. J. (1995) *J. Clin. Invest.* **95,** 1906–1915
14. Watanabe, T., Ono, Y., Taniyama, Y., Hazama, K., Igarashi, K., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10159–10163
15. Pestell, R. G., Albanese, C., Reutens, A. T., Lee, R. J., and Arnold, A. (1999) *Endocr. Rev.*, in press
16. Weinberg, R. A. (1995) *Cell.* **81,** 323–330
17. Sherr, C. J. (1994) *Cell.* **79,** 551–555
18. Resnitzky, D., and Reed, S. I. (1995) *Mol. Cell. Biol.* **15,** 3463–3469
19. Xiong, W., Pestell, R. G., Watanabe, G., Rosner, M. R., and Hershenson, M. B. (1997) *Am. J. Physiol.* **272,** L1205–L1210
20. Lu, Z., Hornia, A., Jiang, Y.-W., Zang, Q., and Foster, D. A. (1997) *Mol. Cell. Biol.* **17,** 3418–3428
21. Zang, Q., Lu, Z., Curto, M., Barile, N., Shalloway, D., and Foster, D. A. (1997) *J. Biol. Chem.* **272,** 13275–13280
22. Denning, M. F., Dlugosz, A. A., Howett, M. K., and Yuspa, S. H. (1993) *J. Biol. Chem.* **268,** 26079–26081
23. Caceres, A., Binder, L. I., Payne, M. R., Bender, P., Rebhun, L., and Steward, O. (1983) *J. Neurosci.* **4,** 394–410
24. Watanabe, G., Pena, P. P., Albanese, C., Wilsbacher, L. D., Young, J. B., and Pestell, R. G. (1997) *J. Biol. Chem.* **272,** 20063–20069
25. Watanabe, G., Pena, P., Shambaugh, G. E., III, Haines, G. K., III, and Pestell, R. G. (1998) *Dev. Brain Res.* **108,** 77–87
26. Watanabe, G., Albanese, C., Lee, R. J., Reutens, A., Vairo, G., Henglein, B., and Pestell, R. G. (1998) *Mol. Cell. Biol.* **18,** 3212–3222
27. Watanabe, G., Lee, R. J., Albanese, C., Rainey, W. E., Batlle, D., and Pestell, R. G. (1996) *J. Biol. Chem.* **271,** 22570–22577
28. Lee, R. J., Watanabe, G., and Pestell, R. G. (1999) *Methods in Molecular Medicine*: *Fibroblast Growth Factor Family of Polypeptides.* Humana Press Inc., in press
29. Byeon, I.-J. L., Li, J., Ericson, K., Selby, T. L., Tevelev, A., Kim, H.-J., O'Maille, P., and Tsai, M.-D. (1998) *Molec. Cell.* **1,** 421–431
30. Miltenyi, S., Muller, W., Weichel, W., and Radbruch, A. (1990) *Cytometry* **11,** 231–238
31. Siebenkotten, G., Behrens-Jung, U., Miltenyi, S., Petry, K., and Radbruch, A. (1998) In *Cell Separation Methods and Applications* (Recktenwald, D., and Radbruch, A., eds) pp. 271–281
32. Toyoshima, H., and Hunter, T. (1994) *Cell* **78,** 67–74
33. Paulus, W., Baur, I., Boyce, F. M., Breakefield, X. O., and Reeves, S. A. (1996) *J. Virol.* **70,** 62–67
34. Lybarger, L., Dempsey, D., Franek, K. J., and Chervenak, R. (1996) *Cytometry* **25,** 211–220
35. Pagano, M., Theodorus, A. M., Tam, S. W., and Draetta, G. F. (1994) *Genes & Dev.* **8,** 1627–1639
36. Diehl, J. A., and Sherr, C. J. (1997) *Mol. Cell. Biol.* **17,** 7362–7374
37. Hunter, T., and Pines, J. (1994) *Cell.* **79,** 573–582
38. Sherr, C. J., and Roberts, J. M. (1995) *Genes & Dev.* **9,** 1149–1163
39. LaBaer, J., Garret, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. (1997) *Genes & Dev.* **11,** 847–862
40. Mittnacht, S., Lees, J. A., Desai, D., Morgan, D. O., and Weinberg, R. A. (1994) *EMBO J.* **13,** 118–127
41. Lundberg, A. S., and Weinberg, R. A. (1998) *Mol. Cell. Biol.* **18,** 753–761
42. Zhao, J., Dynlacht, B., Imai, T., Hori, T., and Harlow, E. (1998) *Genes & Dev.* **12,** 456–461
43. Coats, S., Flanagan, W. M., Nourse, J., and Roberts, J. M. (1996) *Science* **272,** 877–880
44. Rivard, N., L'Allemain, G., Bartek, J., and Pouyssegur, J. (1996) *J. Biol. Chem.* **271,** 18337–18341
45. Chen, D., Krasinski, K., Chen, D., Sylvester, A., Chen, J., Nisen, P. D., and Andres, V. (1997) *J. Clin. Invest.* **99,** 2334–2341
46. Koyama, H., Raines, E. W., Bornfeldt, K. E., Roberts, J. M., and Ross, R. (1996) *Cell.* **87,** 1069–1078
47. Polyak, K., Kato, J. Y., Solomon, M. J., Sherr C. J., Massague, J., Roberts, J. M., and Koff, A. (1994) *Genes & Dev.* **8,** 9–22
48. Kato, J.-y., Matsuoka, M., Stromm, D. K., and Sherr, C. J. (1994) *Mol. Cell. Biol.* **14,** 2713–2721
49. Luo, Y., Marx, S. O., Kiyokawa, H., Koff, A., Massague, J., and Marks, A. R. (1996) *Mol. Cell. Biol.* **16,** 6744–6751
50. Agrawal, D., Dong, K. F., Wang, Y. Z., Kayda, D., and Pledger, W. J. (1995) *Cell Growth & Differ.* **6,** 1199–1205
51. Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994) *Cell* **78,** 59–66
52. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995) *Genes & Dev.* **9,** 1831–1845
53. Soos, T., Kiyokawa, J. H., Yan, J. S., Rubin, M. S., Giordano, A., DeBlasio, A., Bottega, S., Wong, B., Mendelsohn, J., and Koff, A. (1996) *Cell Growth & Differ.* **7,** 135–146
54. Winston, J., Dong, F., and Pledger, W. J. (1996) *J. Biol. Chem.* **271,** 11253–11260
55. Firpo, E. J., Koff, A., Solomon, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M. H., Massague, J., Crabtree, G. R., and Roberts, J. M. (1994) *Mol. Cell. Biol.* **14,** 4889–4901
56. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Clurman, B. E. (1997) *Genes & Dev.* **11,** 1464–1478
57. Tomoda, K., Kubota, Y., and Kato, J-Y. (1999) *Nature* **398,** 160–164
58. Reynisdottir, I., and Massague, J. (1997) *Genes & Dev.* **11,** 492–503
59. Shiyanov, P., Hayes, S., Chen, N., Pestov, D. G., Lau, L. F., and Raychaudhuri, P. (1997) *Mol. Biol. Cell* **8,** 1815–1827
60. Frey, M. R., Saxon, M. L., Zhao, X., Rollins, A., Evans, S. S., and Black, J. D. (1997) *J. Biol. Chem.* **272,** 9424–9435
61. Hamada, K., Takuwa, N., Zhou, W., Kumada, M., and Takuwa, Y. (1996) *Biochim. Biophys. Acta* **1310,** 149–156

62. Coppock, D. L., Tansey, J. B., and Nathanson, L. (1993) *Cell. Growth & Differ.* **3,** 485–494
63. Zhou, W., Takuwa, N., Kumada, M., and Takuwa, Y. (1993) *J. Biol. Chem.* **268,** 23041–23048
64. Kosaka, C., Sasaguri, T., Ishida, A., and Ogata, J. (1996) *Am. J. Physiol.* **39,** C170–C178
65. Yamaguchi, K., Ogita, K., Nakamura, S., and Nishizuka, Y. (1995) *Biochem. Biophys. Res. Commun.* **210,** 639–647
66. Gruber, J. T., Ohno, S., and Niles, R. M. (1992) *J. Biol. Chem.* **267,** 13356–13360
67. Kindregan, H. C., Rosenbaum, S. E., Ohno, S., and Niles, R. M. (1994) *J. Biol. Chem.* **269,** 27756–27761
68. Gschwendt, M., Keilbassa, K., Kittstein, W., and Marks, F. (1994) *FEBS Lett.* **347,** 85–89

Protein Kinase Cδ Inhibition of S-Phase Transition in Capillary Endothelial Cells Involves the Cyclin-dependent Kinase Inhibitor p27 ^{Kip1}

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