The Cyclin-dependent Kinase Inhibitor p21cip1 Mediates the Growth Inhibitory Effect of Phorbol Esters in Human Venous Endothelial Cells*

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Long-term application of the phorbol ester phorbol 12,13-dibutyrate (PDBo) inhibits the proliferation of human venous endothelial cells. The cyclin-dependent kinase inhibitor p21cip1 is a potential candidate mediating the PDBo-induced delayed entry of the cells into S-phase (by ~10 h when compared with cells stimulated with basic fibroblast growth factor (bFGF)). Levels of p21cip1 (protein and mRNA) rapidly rise (within ~2 h) in endothelial cells treated with the active isomer β-PDBo, but not with α-PDBo; this effect is blocked by the mitogen-activated protein kinase kinase-1 (Mek1) inhibitor PD088509 and by the protein kinase C (PKC) antagonists GF109203X and rottlerin (selective for PKC-δ), but not Gö 6976 (selective for Ca2+-dependent PKC isoforms). Rapamycin blocks the PDBo-induced accumulation of p21cip1 (but not of the cognate mRNA), indicating an action of PKC on p21cip1 mRNA translation. If endothelial cells are recruited into the cell cycle by bFGF, p21cip1 mRNA and protein levels rise initially (within 2 h) and decline subsequently such that p21cip1 drops to a minimum prior to the initiation of DNA synthesis (i.e. after ~12 h). In bFGF-stimulated cells, changes in p21cip1 mRNA and protein are strictly linked. In contrast, the levels of p21cip1 mRNA decline substantially (>10 h) before the protein decreases in PDBo-stimulated cells. Thus, PKC (presumably PKC-δ) regulates the amounts of p21cip1 in endothelial cells at the level of mRNA accumulation and translation, leading to a rapid and robust induction; following persistent PKC activation, p21cip1 remains elevated despite reduced mRNA levels, indicating an enhanced stability of the protein. The bFGF-mediated increase in p21cip1 is blocked by the Mek1 inhibitor, but not by GF109203X; hence, in endothelial cells, induction of p21cip1 by PKC- and growth factor-dependent signaling is achieved by distinct pathways that converge and require activation of the mitogen-activated protein kinase cascade. The β-PDBo-induced delayed S-phase entry and drop in p21cip1 are reversed if GF109203X is added 4 h after β-PDBo to prevent persistent PKC activation. These observations indicate a cause and effect relation between sustained p21cip1 elevations and the delay in S-phase entry induced by β-PDBo.

The addition of phorbol esters to the culture medium inhibits the growth of endothelial cells (1). This observation is difficult to reconcile with the fact that stimulation of the cellular targets of phorbol esters, i.e. the isoforms of protein kinase C, leads to sustained activation of several signaling pathways that are required for the recruitment of quiescent cells into the cell cycle. These include activation of the MAP1 kinase cascade, which results in stimulation of gene transcription (2, 3), as well as the p70 S6 kinase, which enhances the efficiency of mRNA translation (4, 5). Previous studies have addressed this paradox and have shown that the PKC activator PDBo exerts a bidirectional effect on growth of human vascular endothelial cells; short-term application of phorbol esters efficiently recruits quiescent endothelial cells into the G1-phase of the cell cycle, whereas persistent activation of protein kinase C subsequently slows the progression though G1 and thereby delays entry of the cells into S-phase (6, 7); thus, the net effect of continuous treatment with phorbol esters is a suppression of endothelial cell proliferation (1, 6).

Signaling pathways that are linked to inhibition of endothelial cell growth are of potential relevance to cancer therapy. Angiogenesis, i.e. the de novo formation of blood vessels from preexisting capillaries, is a prerequisite for the growth of solid tumors and their metastases (8, 9) as well as for the chronic inflammatory response that causes cartilage destruction in rheumatoid arthritis (10). Tumor cells, hypoxic tissue, and leukocytes secrete several endothelial mitogens in a paracrine manner. These growth factors (e.g. fibroblast growth factor, vascular endothelial growth factor, adenosine, interleukin-8, and adhesion molecules) stimulate the growth of endothelial cells by recruiting them from G0-phase to G1-phase via the stimulation of tyrosine kinase receptors and G-protein-coupled receptors (8, 9, 11–13). The ideal antiangiogenic regimen ought to block the action of all endothelial mitogens in a reversible fashion. In theory, this may be achieved by inhibiting a signaling pathway onto which all receptor-generated intracellular stimuli converge to drive the cells through the cell cycle, i.e. the components of the cell cycle machinery that are required for progression through G1. The delay in S-phase entry induced by phorbol esters in endothelial cells is thus of interest, as persistent application of phorbol esters results in delayed activation of cyclin-dependent kinases in G1 (5, 6). We have therefore compared the effects of phorbol esters and of a physiological stimulus, i.e. bFGF, on regulatory components that impinge on the cyclin-dependent kinases responsible for G1-phase progression. Here, we report that in primary cultures of human endothelial cells, continuous protein kinase C activation leads to a

* The abbreviations used are: MAP, mitogen-activated protein; PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; bFGF, basic fibroblast growth factor; HUVECs, human umbilical venous endothelial cells; FCS, fetal calf serum.

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sustained elevation of the cyclin-dependent kinase inhibitor p21\(^{cip1}\). This effect is likely to account for the delayed S-phase entry and the resulting growth inhibition.

**EXPERIMENTAL PROCEDURES**

**Materials—**\(y\)-\(^{32}\)P\]ATP and \(\beta\)\]H\]thymidine were obtained from NEN Life Science Products. Recombinant bovine bFGF and histone H1 were obtained from Boehringer Mannheim (Mannheim, Federal Republic of Germany). Endothelial cell growth supplement was from Technoclone (Vienna, Austria); fetal calf serum was from Life Technologies, Inc.; heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA). The PKC inhibitor GF109203X (bisindolylmaleimide I) was from Calbiochem, and the heparin was from Novo-Nordisk (Bagsvaerd, Denmark); and cell culture dishes were from Costar Corp. (Cambridge, MA).

**Cell Culture—**Human umbilical venous endothelial cells (HUVECs) were obtained according to Jaffe et al. (14). Cells were obtained by enzymatic digestion with 0.2% trypsin and seeded in 96-well plates (1–2 \(\times\) 10\(^3\) cells per well) in medium 199, 2.5% FCS, 100 mg/ml streptomycin, and 100 units/ml penicillin, 0.25 mg/ml histone H1, the samples were boiled in Laemmli sample buffer and resolved on a 10% SDS-polyacrylamide gel. The gel was dried and exposed to Kodak X-ray film. Aliquots of the immunoprecipitates were also withdrawn for immunoblots with the cyclin E antisemur. The affinity-purified antisemur against cyclin E was used at a dilution of 1:2000 for immunostaining. The antigen-antibody complexes were visualized as described above.

**Cyclin E-dependent Kinase Activity and Cyclin E Immunoblots—**HUVECs were synchronized in G\(_0\)-phase and stimulated as described above. At each time point, 5 \(\times\) 10\(^4\) cells were harvested by scraping them off in 0.8 ml of lysis buffer D (\(\phi\) adjusted to 7.5 with NaOH) containing 0.5 M NaCl, 2.5 mM Tris-\(\mathrm{HCl}\), 1 mM NaF, 0.5 mM sodium fluoride, and p21\(^{cip1}\) and p27\(^{kip1}\) (see above) or antisera against cyclin D1 (1:300), CDK4 (1:300), and pRb (1:1000) were used for immunostaining. The antigen-antibody complexes were visualized as described above.

**Northern Blots—**Total cellular RNA was prepared by homogenizing the cells (4–6 \(\times\) 10\(^5\) cells) in guanidinium isothiocyanate, followed by extraction of the homogenate with phenol/chloroform/3-methylbutan-1-ol. Aliquots (20–25 \(\mu\)g) of each RNA sample were applied to a 1% agarose gel containing formaldehyde and transferred to Hybond N\(^+\) nylon membranes by vacuum blotting. The coding sequence of p21\(^{cip1}\) was excised from the plasmid pmCMV\_\(\beta\)-galid by digestion with BamHI (18); the p21\(^{cip1}\) and p53 probes were labeled using the Amersham Megaprime labeling kit. Hybridization was performed overnight at 65 °C in a solution containing 10 \(\times\) SSPE (SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5 \(\times\) Denhardt’s solution, and 100 mg/ml salmon sperm DNA and the labeled cDNA probe. Blots were washed with 2 \(\times\) SSC, twice with 2 \(\times\) SSC and 0.1% SDS, and twice with 0.1 \(\times\) SSC for 15 min each at 65 °C. The membranes were exposed to Kodak X-Omat AR5 films at −80 °C. Each experiment was carried out at least three times on different primary cultures of human endothelial cells.
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Fig. 1. Comparison between the onset of DNA synthesis (A), the protein expression of p21<sup>cip1</sup> and p27<sup>kip1</sup> (B), and the p21<sup>cip1</sup> mRNA accumulation (C) in human endothelial cells. A, quiescent endothelial cells (1.5 × 10<sup>5</sup> cells/well) were stimulated with 20 ng/ml bFGF + 10% FCS (□), 10% FCS alone (○), or 1 µM β-PDBu (●); at the time points indicated, 0.5 µCi of [3H]thymidine was added for 4 h. The amount of [3H]thymidine incorporated was determined as outlined under "Experimental Procedures." Assays were done in sextuplicate. B, in a parallel experiment, cells from the same primary culture (5 × 10<sup>5</sup> cells/dish) were stimulated with 20 ng/ml bFGF + 10% FCS or 1 µM β-PDBu and were harvested in 100 µl of lysis buffer A at the indicated time points. After removal of insoluble proteins, lysates were resolved by SDS gel electrophoresis, and the blots were analyzed using monoclonal antibodies against p27<sup>kip1</sup> (upper row) and p21<sup>cip1</sup> (lower row) as described under "Experimental Procedures." Lane Std, a cellular lysate (20 µg) prepared from UV-irradiated human fibroblasts that was used as a standard for p21<sup>cip1</sup> and p27<sup>kip1</sup>. C, growth-arrested synchronized endothelial cells (5 × 10<sup>5</sup> cells/dish) were stimulated with 20 ng/ml bFGF + 10% FCS or 1 µM β-PDBu. At the indicated time points, cells were harvested, and total RNA was prepared as outlined under "Experimental Procedures"; an aliquot thereof (20 µg) was separated on a 1% agarose gel and blotted onto a nylon membrane, which was probed with p21<sup>cip1</sup>. The RNA blots were also reprobed with a p53- and a 28 S RNA-specific probe to verify that the differences observed cannot be accounted for by unequal loading (not shown).

RESULTS

Expression Pattern of p21<sup>cip1</sup> Protein and mRNA during G<sub>1</sub>-phase Progression—When applied to quiescent (G<sub>0</sub>) endothelial cells in a pulsatile manner (1.5 h), the sole addition of β-PDBu suffices to promote the entry of 40–60% of the total cell population into the cell cycle as assessed by propidium iodide staining and fluorescence-activated cell sorter analysis (data not shown). However, if β-PDBu is applied continuously, the subsequent onset of [3H]thymidine incorporation into DNA, i.e. S-phase entry, is delayed by ~10 h compared with cells that have been treated with physiological stimuli such as bFGF or FCS (Fig. 1A). This phenomenon has been attributed to a phorbol ester-dependent delay in the activation of cyclin-dependent kinases in G<sub>1</sub>-phase. To identify the relevant upstream signaling events that may regulate cell cycle progression, we evaluated the expression of the cyclin-dependent kinase inhibitors p21<sup>cip1</sup> and p27<sup>kip1</sup>. Quiescent endothelial cells were stimulated with either bFGF or PDBu; the expression pattern of the cyclin-dependent kinase inhibitors was assessed at the level of the protein in the subsequent cell cycle (Fig. 1B) and compared with the onset of DNA synthesis (Fig. 1A). In G<sub>0</sub>, endothelial cells contain abundant amounts of p27<sup>kip1</sup>, but only low levels of p21<sup>cip1</sup> (cf. Fig. 2A). At the earliest time point that was assessed after mitogenic stimulation (4 h), the cellular levels of p21<sup>cip1</sup> were elevated in cells treated with bFGF or β-PDBu, whereas the levels of p27<sup>kip1</sup> remained essentially unaltered (Fig. 1B; see also Fig. 2A); the increase in p21<sup>cip1</sup> was maintained throughout G<sub>1</sub>-phase and dropped as cells incorporated [3H]thymidine. In bFGF-stimulated cells, the drop in p21<sup>cip1</sup> was observed after 12 h; as the incorporation of [3H]thymidine started to decline, p21<sup>cip1</sup> reaccumulated (Fig. 1B, lanes labeled bFGF 20+ and 24+). In β-PDBu-treated cells, p21<sup>cip1</sup> remained elevated up to
24 h, and the subsequent decline coincided again with the entry into S-phase (Fig. 1B, lane labeled PDBu 28\(^+\)). In contrast, stimulation of endothelial cells with \(\beta\)-PDBu did not defer but rather accelerated the decline in p27\(^{kip1}\) (Fig. 1B). Although we have attempted to optimize the synchronization of the cells in G\(_0\), there was some variability in individual cell batches with respect to the onset (i.e. 11–14 and 25–30 h for bFGF and \(\beta\)-PDBu, respectively) and the duration of \([\text{H}]\)thymidine incorporation (cf. also Figs. 1 and 6, A and B). This variation is not unexpected in experiments with primary cell cultures. However, we stress that irrespective of this variation, the decline in p21\(^{cip1}\) always occurred prior to the onset of DNA synthesis. The changes in the p21\(^{cip1}\) protein levels of bFGF-stimulated cells were paralleled by appropriate changes in the p21\(^{cip1}\) mRNA (Fig. 1C); this finding is consistent with a previous report that showed that in a human fibroblast cell line, serum stimulation resulted in an oscillation of the p21\(^{cip1}\) mRNA along the cell cycle (19). In bFGF-stimulated cells, the decline in the protein levels of p21\(^{cip1}\) and of its cognate mRNA coincided, indicating that the protein turned over rapidly (Fig. 1, compare B and C). In contrast, if the cells had been stimulated with \(\beta\)-PDBu, p21\(^{cip1}\) mRNA declined substantially (>12 h) before the drop in the protein (Fig. 1, compare B and C). This discrepancy between the p21\(^{cip1}\) mRNA and protein levels can only be accounted for by a \(\beta\)-PDBu-induced stabilization of the protein.

**Signaling Pathways Involved in the \(\beta\)-PDBu-mediated Induction and Regulation of p21\(^{cip1}\)**—To characterize the initial induction of p21\(^{cip1}\), endothelial cells were subjected to short-term incubations in the presence of 1 \(\mu\)M PDBu. The addition of the phorbol ester to quiescent cells led to a rapid accumulation of p21\(^{cip1}\) (Fig. 2A). In contrast, the levels of p27\(^{kip1}\), which were readily detectable in quiescent cells, remained unaffected by the short-term incubation with \(\beta\)-PDBu. However, an appropriate stimulus did regulate the levels of p27\(^{kip1}\) in endothelial cells. Incubation of endothelial cells with transforming growth factor-\(\beta\), which is known to redistribute cellular levels of p27\(^{kip1}\) (20), led to the accumulation of p27\(^{kip1}\) (data not shown). We stress that a virtually identical increase in p21\(^{cip1}\) was also observed if asynchronously growing cells were stimulated with \(\beta\)-PDBu and irrespective of whether total cellular protein or a detergent extract was used for analysis (data not shown). The accumulation of p21\(^{cip1}\) was paralleled by a rise in the cognate mRNA, which reached a maximum within 2–4 h (Fig. 2A). The levels of p21\(^{cip1}\) protein and mRNA also increased if quiescent endothelial cells were stimulated with bFGF (Fig. 2A), albeit to a lesser extent than following the addition of \(\beta\)-PDBu (cf. Fig. 1B). In contrast, we did not detect any \(\beta\)-PDBu-induced change in the levels of p53 irrespective of whether the determination was done by immuno blotting cellular lysates or by RNA blots (Fig. 2C).

The experiments depicted in Figs. 1 and 2 suggested that direct activation of protein kinase C by \(\beta\)-PDBu resulted in an initial rapid increase in p21\(^{cip1}\). Growth factor receptors with tyrosine kinase activity are capable of recruiting phospholipase C\(_\gamma\) via the interaction of the SH2 domain with autophosphorylated tyrosine residues; the resulting activation of phospholipase C is expected to trigger PKC activation (21). We have therefore assessed whether the bFGF-induced increase in p21\(^{cip1}\) resulted from the activation of a PKC isofrom. This was clearly not the case as GF109203X did not block the response to bFGF while blunting the response to \(\beta\)-PDBu (Figs. 3A and 4, A and C). In contrast, incubation of endothelial cells with PD098059, an inhibitor of MAP kinase kinase-1 (Mek1; see Ref. 14), blocked the induction of p21\(^{cip1}\) by both \(\beta\)-PDBu and bFGF (Fig. 3A). Hence, the bFGF receptor- and PKC-dependent stimulation converges upstream of MAP kinase and depend on the activation of Mek1 to raise p21\(^{cip1}\) levels in endothelial cells. This response is not restricted to endothelial cells and can also be observed in other cell types, e.g. A431 cells. Previous experiments have verified that PD098059 effectively inhibits MAP kinase activation in endothelial cells at the concentration employed (17). A discrepancy is evident if the amounts of p21\(^{cip1}\) mRNA and protein are compared in quiescent cells; while the mRNA is readily detectable, the level of the protein is very low. This discrepancy has been noted earlier and has been interpreted as evidence that the cellular concentration of p21\(^{cip1}\) is presumably regulated not only at the level of mRNA transcription, but also at the level of mRNA translation (22). We have tested this hypothesis by employing the immunosuppressant rapamycin. The p70 S6 kinase regulates the translation of mRNA transcripts, and rapamycin blocks the activation of the p70 S6 kinase irrespective of the upstream signaling pathway employed by the stimulus (5). At concentrations \(\geq 1\) nM, rapamycin attenuated the \(\beta\)-PDBu-induced increase in p21\(^{cip1}\) protein levels (Fig. 3B, lanes labeled R), whereas the accumulation of p21\(^{cip1}\) mRNA was virtually unaffected (Fig. 3C, lanes labeled R); in contrast, the Mek1 inhibitor PD098059 and the PKC inhibitor GF109203X blocked the \(\beta\)-PDBu-induced increase in p21\(^{cip1}\) protein and mRNA levels (Fig. 3, B and C, lanes labeled PD and GF). Hence, \(\beta\)-PDBu raises p21\(^{cip1}\) levels in endothelial cells not only by inducing the accumulation of the cognate mRNA, but also by regulation at the translational level.

**Protein Kinase C-dependent Induction of p21\(^{cip1}\)**—We have

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verified that the PDBu-induced increase in p21cip1 was mediated via activation of a protein kinase C isoform (presumably the δ isoform) based on the following criteria. (i) The half-maximum response to β-PDBu was observed in the nanomolar concentration range (Fig. 4A). (ii) The effect of phorbol esters was stereospecific, as the inactive isomer α-PDBu failed to affect the levels of p21cip1 (Fig. 4B). (iii) The addition of GF109203X (bisindolylmaleimide I), a inhibitor of typical (i.e. Ca2+-dependent) and novel (i.e. Ca2+-independent) protein kinase C isoforms (23), blunted the effect of β-PDBu (Figs. 3A and 4A). We noted that the sole addition of GF109203X caused a modest increase in p21cip1. This effect was even more pronounced for other PKC inhibitors such as staurosporine and H-7, which actually enhanced the effect of β-PDBu (Fig. 4B). These compounds, however, are less specific as protein kinase C inhibitors, as they also act as potent inhibitors of several other serine/threonine kinases (23). In addition, prolonged incubation of endothelial cells with staurosporine (but not with GF109203X) caused the cells to detach from the matrix and promote cell death (data not shown). (iv) The induction of p21cip1 was blocked by 10 μM rottlerin; this inhibitor is selective for PKC-δ at this concentration (24). In contrast, Gö 6976, a compound structurally related to GF109203X and staurosporine, which inhibits the α- and β-isoforms of PKC with IC50 values in the nanomolar range (25), was ineffective up to 1 μM (Fig. 4C).

In several human cell lines, p21cip1 is induced by oxidative stress caused by intracellular glutathione depletion with di-ethyl maleate, an effect that is prevented by the glutathione precursor N-acetylcysteine (26). Similarly, N-acetylcysteine blocks several effects of phorbol esters (27–29). We have therefore determined the level of p21cip1 in the presence of these compounds or, alternatively, stimulated the endothelial cells to generate an endogenous free radical, namely NO. Preincubation with N-acetylcysteine or diethyl maleate neither inhibited nor potentiated, respectively, the increase in p21cip1 induced by β-PDBu (Fig. 4D). Finally, treatment of the cells with the Ca2+-ionophore A23187 (calcimycin), under conditions that lead to a very pronounced activation of NO synthase (30) and subsequently to cell death (data not shown), had no effect on p21cip1 levels (Fig. 4D).

Functional Relevance of the Phorbol Ester-induced Increase in p21cip1—To be functionally relevant for the delay in G1-phase progression, p21cip1 must form a complex with a cyclin-dependent kinase that is active in early G1-phase. We therefore immunoprecipitated cyclin D1 from lysates of cells that were stimulated with either bFGF or β-PDBu. Equivalent amounts of cyclin D1 and of the associated cyclin-dependent kinase CDK4 were recovered (Fig. 5A). However, even at the earliest time point investigated (i.e. 3 h after stimulation; Fig. 5A), these complexes contained higher amounts of p21cip1 when isolated from lysates of β-PDBu-treated cells (Fig. 5A, lanes labeled IP). While a significant portion of p21cip1 was immunoprecipitated with the cyclin D1 antisera, only low levels of p27kip1 were recovered in the complex, and the majority of p27kip1 was detected in the supernatant from the immunoprecipitation (Fig. 5A, compare lanes labeled IP and SN). At all time points investigated (up to 12 h after stimulation), complexes of cyclin D1 and CDK4 that were immunoprecipitated from β-PDBu-treated cells contained higher levels of p21cip1 than those isolated from bFGF-stimulated cells (data not shown).

These findings predict a reduced activity of the cyclin D1-CDK4 complex. The retinoblastoma protein pRB represents one of the key endogenous substrates of cyclin D1-CDK4, and hyperphosphorylation of pRB is an essential step in G1-phase progression prior to S-phase entry (31). This modification is associated with a reduced mobility of pRB on SDS-polyacrylamide gels. We have therefore enriched pRB by immunoprecipitation from cellular lysates and analyzed the electrophoretic mobility of the protein after stimulation of the cells with bFGF and β-PDBu. As can be seen from Fig. 5B, the time point at which pRB is phosphorylated to a significant extent is delayed by several hours in β-PDBu-stimulated cells. The induction of cyclin E synthesis and the activation of cyclin E-CDK2 are downstream of cyclin D1-CDK4 activation. Accordingly, cyclin E synthesis (data not shown) and cyclin E-dependent kinase activity (as assessed by histone H1 phosphorylation after immunoprecipitation of the complexes) were delayed in PDBu-stimulated cells when compared with cells that had been recruited into the cell cycle with bFGF (Fig. 5C).

Reversal of the PDBu-induced Delay in S-Phase Entry—
Taken together, the findings presented so far indicate that
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activity of PKC by phorbol esters raises the levels of p21cip1 to inhibit activation of G1-phase cyclins and that the sustained increase in p21cip1 results in delayed S-phase entry. This interpretation predicts that the effect of β-PDBu should override the physiological stimulation by bFGF when both stimuli are presented in combination. In addition, if the sustained PKC activation is prevented, both the delay in S-phase entry and in the drop in p21cip1 should be prevented. Both predictions have been verified. (i) β-PDBUs, even when added 4 h after stimulation with bFGF, suppressed [3H]thymidine incorporation in endothelial cells (Fig. 6A, △), and this effect of β-PDBu was reversed by the PKC inhibitor GF109203X (Fig. 6A, □). (ii) If GF109203X was added to the endothelial cells 4 h after stimulation by β-PDBU, essentially no delay in the onset of DNA-synthesis was observed (Fig. 6B, △). If GF109203X was added 12 h after β-PDBU, the delay was only partially prevented (Fig. 6B, ▽); conversely, if the order of addition was reversed, i.e. GF109203X was added before β-PDBU, the cells were obviously not recruited into the cell cycle, and no [3H]thymidine incorporation was observed (data not shown). In parallel experiments,
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we have assessed the pattern of \( p21^{cip1} \) expression in cells that were first stimulated with \( \beta\)-PDBu and subsequently received GF109203X (Fig. 6C). This regimen reversed the delay in the drop in \( p21^{cip1} \) such that the pattern of expression was comparable to that observed in bFGF-treated cells. The decline in \( p21^{cip1} \) levels again preceded the onset of DNA synthesis.

**DISCUSSION**

Previous studies have demonstrated that induction of \( p21^{cip1} \) is an important element in the coordinated response that leads to cell cycle arrest following exposure to DNA damage (32–34). In addition, up-regulation of \( p21^{cip1} \) has been proposed to participate in cell senescence (18) and in cell cycle withdrawal upon terminal differentiation (20, 35–37). In this study, we show that in primary cultures of human endothelial cells, the levels of \( p21^{cip1} \) vary along the cell cycle; \( p21^{cip1} \) is low in quiescent cells, increases as cells are recruited into G1-phase by a mitogenic stimulus, drops prior to the onset of S-phase, and subsequently reaccumulates. This pattern is observed regardless of whether the cells are stimulated with bFGF, a physiological growth factor, or subjected to a long-term incubation with \( \beta\)-PDBu. However, bFGF leads to a transient induction of \( p21^{cip1} \), the protein turns over rapidly, and the decline in mRNA and protein levels is tightly linked. While the accumulation of \( p21^{cip1} \) in response to cell damage or growth inhibitors can be rationalized from a teleological point of view, the induction of an inhibitor of G1-phase progression by mitogens seems counterintuitive. However, expression of a cyclin-dependent kinase inhibitor may be required for efficient complex formation between CDK4 and cyclin D1 such that low concentrations of \( p21^{cip1} \) act as an assembly factor, whereas high concentrations inhibit the kinase activity (38). This dual action of \( p21^{cip1} \) is evident in endothelial cells; in contrast to bFGF, activation of protein kinase C causes a prolonged elevation of \( p21^{cip1} \) in G1-phase. \( \beta\)-PDBu elicits this effect by regulating at least three distinct processes, namely (i) the accumulation and (ii) translation of \( p21^{cip1} \) mRNA; and (iii) upon long-term incubation of the endothelial cells in the presence of the phorbol ester, \( p21^{cip1} \) is stabilized such that the protein levels remain elevated despite a reduction in the cognate mRNA. The resulting robust and sustained elevation of \( p21^{cip1} \) is functionally relevant as it accumulates in complexes with cyclin D1-CDK4. Accordingly, the phosphorylation of pRb and the induction of cyclin E-dependent kinase activity are delayed in \( \beta\)-PDBu-treated endothelial cells as shown here and described previously (6, 7). In contrast, the fluctuations in the levels of \( p27^{kip1} \), a cyclin-dependent kinase inhibitor related to \( p21^{cip1} \) (22, 31), do not coincide with the delayed onset of DNA synthesis. The \( \beta\)-PDBu-induced delayed drop in \( p21^{cip1} \) and S-phase entry can be appropriately reversed by the PKC inhibitor GF109203X. This substantiates our conclusion that the two effects are causally related. Although we obviously cannot rule out the participation of additional signaling pathways, the elevation of \( p21^{cip1} \) and the resulting inhibition of cyclin D1-dependent kinase activity are per se sufficient to account for the delayed progression through G1 induced by \( \beta\)-PDBu.

Our experiments identify PKC-\( \delta \) as the candidate isoform that mediates induction of \( p21^{cip1} \) in response to \( \beta\)-PDBu. This interpretation is supported by the observation that GF109203X and rottlerin antagonized the action of \( \beta\)-PDBu. GF109203X inhibits classical (\( Ca^{2+}\)-dependent) and novel (\( Ca^{2+}\)-independent) PKC isoforms with nanomolar affinities (23, 25); at the concentration at which rottlerin inhibited the effect of \( \beta\)-PDBu on the expression of \( p21^{cip1} \), the compound is selective for PKC-\( \delta \) (IC \(_{50} \) = 3–6 \( \mu \)M), whereas 10–50-fold higher concentrations are required to inhibit the other \( Ca^{2+}\)-independent as well as classical PKC isoforms (24). In contrast, Gö 6976, which selectively inhibits \( Ca^{2+}\)-dependent PKC isoforms with IC \(_{50} \) values in the nanomolar range (25), did not affect the \( \beta\)-PDBu-dependent induction of \( p21^{cip1} \). H-7 and staurosporine raised the basal levels of \( p21^{cip1} \). This was also seen, albeit to a lesser extent, in response to GF109203X. H-7 and staurosporine, which inhibit several protein kinase C isoforms as well as other serine/threonine kinase isoforms (23), have previously been reported to increase the expression of \( p21^{cip1} \) (39). Contrary to GF109203X, H-7 and staurosporine potentiated the effect of \( \beta\)-PDBu. It is at present not clear whether this phenomenon reflects a balance between protein kinase C isoforms, which exert opposite effects on the expression level of \( p21^{cip1} \) and which have different sensitivities for inhibitors, or the involvement of other kinases.

DNA damage and inhibition of DNA and RNA synthesis cause the accumulation of the tumor suppressor protein p53, which induces the expression of \( p21^{cip1} \); this signaling pathway is understood in considerable detail (see Ref. 40 for review). Several findings, however, argue against a role of p53 in the \( \beta\)-PDBu-dependent induction of \( p21^{cip1} \); while \( p21^{cip1} \) and its mRNA rapidly increased in endothelial cells stimulated with the phorbol ester, the level of p53 remained unaffected. Phorbol esters also raise \( p21^{cip1} \) levels in human cells that are deficient in p53 (41) or that contain a mutated form of p53 (e.g., A431 cells). Additional p53-independent mechanisms that result in the induction of \( p21^{cip1} \) are less well characterized; these include oxidative stress (26). However, our experiments rule out that induction of \( p21^{cip1} \) by phorbol esters can simply be accounted for by a stress-induced response of endothelial cells. Previous experiments indicate that stimulation of quiescent cells with serum-derived growth factors (20, 36) or epidermal growth factor (42) leads to a transient accumulation of \( p21^{cip1} \). Transient induction of \( p21^{cip1} \) was also seen in endothelial cells stimulated with bFGF. However, our work clearly shows that bFGF and \( \beta\)-PDBu use distinct but convergent signaling pathways to raise \( p21^{cip1} \) levels; contrary to \( \beta\)-PDBu, the effect of bFGF does not depend on the activation of GF109203X-sensitive protein kinase C isoforms. Nevertheless, induction of \( p21^{cip1} \) by both \( \beta\)-PDBu and bFGF is abolished by the Mek1 inhibitor PD98059. This compound has also recently been shown to suppress the nerve growth factor-stimulated accumulation of \( p21^{cip1} \) in NIH3T3 cells expressing the nerve growth factor receptor TRKA (43). Induction of \( p21^{cip1} \) is also seen after transfection with inducible forms of Raf-1, the kinase that is upstream of Mek1 (43, 44). Hence, we conclude that in endothelial cells, the signals elicited by physiological mitogens and protein kinase C activation converge at the level of the MAP kinase cascade and require its activation to induce the expression of \( p21^{cip1} \). However, this pathway is clearly not universally utilized in all cell types; for instance, in the monocytic cell line U937, stimulation of MAP kinase fails to raise \( p21^{cip1} \) levels, and the induction by phorbol esters depends on the transcription factor Sp1 (45). Furthermore, contrary to human endothelial cells, where PKC-\( \delta \) mediates the phorbol ester-dependent accumulation of \( p21^{cip1} \), it is the \( \alpha \)-isoform of PKC that raises \( p21^{cip1} \) levels and that induces a growth arrest in a human epithelial cell line (46). Taken together, these data show that the mechanisms by which induction of \( p21^{cip1} \) is achieved are distinct in different cells, and this diversity may thus also be exploited to selectively arrest a given cell type in an intact organism. Cellular immortalization is associated with abrogation of one or several regulatory pathways that are required for the normal control of the cell cycle. We stress that our experiments were carried out on primary cultures of human endothelial cells. Thus, we believe that the observations in this study are relevant to the normal endothelial cell cycle in vivo.
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