Differential Regulation of p27Kip1 Expression by Mitogenic and Hypertrophic Factors: Involvement of Transcriptional and Posttranscriptional Mechanisms

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Abstract. Platelet-derived growth factor-BB (PDGF-BB) acts as a full mitogen for cultured aortic smooth muscle cells (SMC), promoting DNA synthesis and cell proliferation. In contrast, angiotensin II (Ang II) induces cellular hypertrophy as a result of increased protein synthesis, but is unable to drive cells into S phase. In an effort to understand the molecular basis for this differential growth response, we have examined the downstream effects of PDGF-BB and Ang II on regulators of the cell cycle machinery in rat aortic SMC. Both PDGF-BB and Ang II were found to stimulate the accumulation of G1 cyclins with similar kinetics. In addition, little difference was observed in the expression level of their catalytic partners, Cdk4 and Cdk2. However, while both factors increased the enzymatic activity of Cdk4, only PDGF-BB stimulated Cdk2 activity in late G1 phase. The lack of activation of Cdk2 in Ang II-treated cells was causally related to the failure of Ang II to stimulate phosphorylation of the enzyme on threonine and to downregulate p27Kip1 expression. By contrast, exposure to PDGF-BB resulted in a progressive and dramatic reduction in the level of p27Kip1 protein. The time course of p27Kip1 decline was correlated with a reduced rate of synthesis and an increased rate of degradation of the protein. Importantly, the repression of p27Kip1 synthesis by PDGF-BB was associated with a marked attenuation of Kip1 gene transcription and a corresponding decrease in Kip1 mRNA accumulation. We also show that the failure of Ang II to promote S phase entry is not related to the autocrine production of transforming growth factor-β1 by aortic SMC. These results identify p27Kip1 as an important regulator of the phenotypic response of vascular SMC to mitogenic and hypertrophic stimuli.

Key words: growth factors • cell cycle • CDK inhibitors • gene expression • smooth muscle cells

Introduction

The proliferation of normal mammalian cells is controlled by an intricate network of biochemical pathways that ensure that each cell cycle event is performed correctly and in proper sequence (Murray and Hunt, 1993). Growth factor-induced signals are required for progression through the G1 phase and must converge, in late G1, on the cell cycle engine to ensure the commitment of cells to enter S phase (Pardee, 1989). The regulation of G1 progression and G1/S transition is governed, at least in part, by the concerted action of cyclin-dependent kinases (Cdk)s1 and their regulatory cyclin subunits (Daetla, 1994; Sherr, 1994; Grana and Reddy, 1995). When quiescent cells resume cycling in response to growth factors, D-type cyclins (D1, D2, and D3) progressively accumulate during G1 phase and assemble with their catalytic partners, Cdk4 and Cdk6. The activity of Cdk4/Cdk6 is first detected in mid-G1 and increases as cells approach the G1/S boundary. One major target of Cdk4/Cdk6 is the retinoblastoma pro-

1 Abbreviations used in this paper: Ang II, angiotensin II; CAK, Cdk-activating kinase; Cdk, cyclin-dependent kinase; D,R,B, 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole; GA PDH, glyceroldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; MAP, mitogen-activated protein; PDGF-BB, platelet-derived growth factor-BB; pRb, retinoblastoma protein; SMC, smooth muscle cells; TGF-β1, transforming growth factor-β1; TNA, TGF-β1 neutralizing antibody.
tein (pRb), which upon phosphorylation dissociates from bound transcription factors, such as E2F, enabling them to activate genes required for DNA replication (Weinberg, 1995). Cyclin E is expressed at maximum level in late G1 and associates with Cdk2. Biochemical and genetic data indicate that cyclin E-Cdk2 activity is essential for entry into S phase (van den Hauwe and Harlow, 1993; K Noblich et al., 1994; Ohtsubo et al., 1995; K Rude et al., 1997).

The activity of Cdks is regulated by a combination of mechanisms. These include the synthesis of the cyclin and Cdk, the assembly of these proteins into complexes, the phosphorylation of a conserved threonine residue by Cdk-activating kinase (CAK), and the interaction with Cdk inhibitory proteins (Morgan, 1995). Cdk inhibitors fall into two gene families (Sherr and Roberts, 1995). The Ink4 family of proteins, which includes p16^\text{INK4A}, p15^\text{INK4B}, p18^\text{INK4C}, and p19^\text{INK4D}, specifically interacts with Cdk4 and Cdk6 to prevent cyclin D-Cdk assembly or enters into stable ternary complexes with cyclin D-Cdk, resulting in complexes that are catalytically inactive (Serrano et al., 1993; Guan et al., 1994; Hannon and Beach, 1994; Chan et al., 1995; Hirai et al., 1995). The second family of inhibitors includes p21^\text{CIP1/WAF1} (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993; Noda et al., 1994), p27^\text{kip1} (Polyak et al., 1994a; Toyoshima and Hunter, 1994), and p57^\text{kip2} (Lee et al., 1995; Matsuoka et al., 1995), which are all structurally unrelated to the Ink4 proteins. The Cip/kip family binds to and inhibits a broader range of Cdks than the Ink4 family and displays a preference for fully assembled cyclin-Cdk complexes. They inhibit the kinase activity of G1 Cdk by stoichiometric binding to the cyclin-Cdk complex or by physically blocking the phosphorylation of the Cdk subunit by CAF (Sherr and Roberts, 1995). A mong them, p27^\text{kip1} was first identified in transforming growth factor \(\beta\) (TGF-\(\beta\))-treated cells (Polyak et al., 1994b; Slingerland et al., 1994). The expression of p27^\text{kip1} is increased in serum-starved or density-arrested cells (Fi rolo et al., 1994; Kato et al., 1994; Nourse et al., 1994) and in cells exposed to antiproliferative signals like TGF-\(\beta\), rapamycin (Nourse et al., 1994), and \(\alpha\) M P (Kato et al., 1994; L'Alemman et al., 1997). In contrast, the level of p27^\text{kip1} declines in response to mitogenic factor stimulation (Kato et al., 1994; Nourse et al., 1994; Coats et al., 1996; Winston et al., 1996; this study). Thus, in addition to D-type cyclins, p27^\text{kip1} may play an essential role in connecting mitogenic signaling pathways to cell cycle activation. Ectopic expression of p27^\text{kip1} causes cell cycle arrest in G1 phase (Polyak et al., 1994a; Toyoshima and Hunter, 1994) and, conversely, antisense inhibition of p27^\text{kip1} expression suppresses quiescence in fibroblasts (Coats et al., 1996; R Ivard et al., 1996).

In cultured arterial smooth muscle cells (SMC), the peptide growth factor platelet-derived growth factor (PDGF-BB) acts as a full mitogen, promoting DNA synthesis and cell division (Raines et al., 1990; Grainger et al., 1994). The mitogenic action of PDGF-BB is initiated by its interaction with two structurally related tyrosine kinase receptors that dimerize upon ligand binding, leading to activation of the intrinsic kinase domain and intermolecular autophosphorylation (Claesson-Welsh, 1994). The phosphorylated tyrosine residues serve as docking sites for multiple SH2-containing signaling molecules that include Src, phosphoinositide 3-kinase (PI3-kinase), phospholipase C-\(\gamma\) (PLC-\(\gamma\), SHP-2, Grb2, Shc, and Nck. Recruitment and activation of these effector proteins catalyze the formation of second messengers and propagate the signal to downstream serine/threonine kinases, such as protein kinase C, mitogen-activated protein (MAP) kinases, and p70 S6 kinase, ultimately resulting in increased gene expression and DNA synthesis (Claesson-Welsh, 1994; Hendrin, 1997).

In contrast to PDGF-BB, many investigators, including ourselves, have shown that the peptide angiotensin II (Ang II) induces cellular hypertrophy in cultured aortic SMC as a result of increased protein synthesis, but is unable to drive cells into S phase (Geisterfer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Grainger et al., 1994; Giasson and M eloche, 1995). On the other hand, Ang II was reported to exert weak mitogenic effects on SMC of resistance arteries (Dube et al., 1992) and on aortic SMC isolated from spontaneously hypertensive rats (Buskens et al., 1992; Itazaki et al., 1995). In vivo, a number of studies have shown that infusion of Ang II stimulates SMC DNA synthesis and proliferation in normal and injured rat arteries (Damen et al., 1991; van Kleef et al., 1992; deBlois et al., 1996; Su et al., 1998). However, results of in vivo studies are difficult to interpret since the effect of Ang II may be indirect or Ang II may simply act as a comitogen. It has been postulated that Ang II may be a bifunctional growth factor that activates both proliferative and anti proliferative (TGF-\(\beta\)) signals in vascular SMC (Gibbons et al., 1992; Kibuchi et al., 1993). According to this model, the autocrine production of TGF-\(\beta\) would determine whether vascular SMC grow by hypertrophy or hyperplasia in response to Ang II.

In cultured aortic SMC, the hypertrophic action of Ang II is initiated by its interaction with the G protein-coupled AT\(\_1\) receptor, which stimulates the activity of PLC-\(\beta\) to generate the second messengers inositol 1,4,5-trisphosphate (InsP\(3\)) and diacylglycerol, and inhibits the activity of adenylyl cyclase (Catt et al., 1993; Timmermans et al., 1993). These early signaling events subsequently lead to the activation of multiple serine/threonine kinases, which include the MAP kinases ERK1/ERK2 (Duff et al., 1992; Tsuda et al., 1992; Servant et al., 1996) and p70 S6 kinase (Giasson and M eloche, 1995). Ang II also induces tyrosine phosphorylation of multiple proteins in aortic SMC (Molloy et al., 1993; Leduc et al., 1995) and stimulates the activity of cytosolic tyrosine kinases, such as p125\text{FAK} (Polte et al., 1994; Giasson et al., 1997), Pyk2 (Giasson et al., 1997), Src (Ishida et al., 1995), and the Janus kinases Jak2 and Tyk2 (Marrero et al., 1995; Giasson et al., 1997). Despite the fact that Ang II and PDGF-BB activate similar signal transduction pathways, only the latter is able to induce proliferation of aortic SMC.

In an effort to understand the molecular basis for this differential response, we have examined the downstream effects of PDGF-BB and Ang II on regulators of the cell cycle machinery. We show that while both factors are able to stimulate the activity of Cdk4, only PDGF-BB increases the enzymatic activity of Cdk2 in late G1 phase. The lack of activation of Cdk2 in Ang II-treated cells is associated with the failure of Ang II to downregulate p27^\text{kip1} expression. We also show that p27^\text{kip1} abundance is regulated by
multiple transcriptional and posttranscriptional mechanisms in vascular SM C.

**Materials and Methods**

**Cell Culture**

Rat aortic SM C were cultured to 80% confluence and synchronized in the quiescent state as described previously (Glaissonn and M. eloeho, 1995). The cells were stimulated with 100 mM A ng II (Hukabel Scientific) or 50 ng/ml PDGF-BB (Oncogene Science) for the indicated times at 37°C. M. eloeho mink lung epithelial cells (obtained from Dr. Maureen O’Connor, Biochemistry Research Institute of Montreal, Canada) were grown in MEM containing 10% FBS.

**Protein Synthesis, DNA Synthesis, and Cell Number Measurements**

For protein synthesis measurements, quiescent aortic SM C in 6-well plates were stimulated with A ng II or PDGF-BB for 72 h in serum-free medium containing 0.5 µCi/ml [3H]leucine. For DNA synthesis measurements, quiescent aortic SM C in 35-mm petri dishes were stimulated for the indicated times with A ng II or PDGF-BB and pulse-labeled with 2 µCi/ml [3H]thyminidine for the last 2-4 h. A fter the stimulation, the medium was aspirated and the cells were incubated for a minimum of 30 min in cold 5% TCA. The wells were then washed once with TCA and three times with tap water. The radioactivity incorporated into TCA-precipitable material was measured by liquid scintillation counting after solubilization in 0.1 M NaOH. For determination of cell number, quiescent aortic SM C in 6-well plates were stimulated with A ng II or PDGF-BB for 72 h and then trypanized and counted using a hemacytometer.

**Immunoblot Analysis**

Cells were washed twice with ice-cold PBS and lysed in Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 10 µM pepstatin A, 1% Triton X-100) for 30 min at 4°C. L ysat es were clarified by centrifugation at 13,000 g for 10 min and equal amounts of lysate proteins (30-85 µg) were subjected to electrophoresis on 12 or 15% acrylamide gels. Proteins were electrophoretically transferred to Hybond-C nitrocellulose membranes (Coomassie blue-stained and un-stained) and were subjected to electrophoresis on 12 or 15% acrylamide gels. Proteins were electroblotted to nitrocellulose membranes (Coomassie blue-stained and unstained) and were subjected to electrophoresis on 12 or 15% acrylamide gels. The membranes were blocked in TBS containing 5% nonfat dry milk and 0.1% Tween 20 for 1 h at 37°C before incubation for 1 h at 25°C with 5 µg/ml of primary antibody to cyclin D1 (DCS-6), cyclin D2 (DCS-3.1), or cyclin D3 (DCS-22; NeoMark), Cdk4 (SC-545), or p27Kip1 (85-163), Cdk2 (SC-260), or p27Kip1 (85-163, Santa Cruz Biotechnology) in blocking solution. After washing four times in TBS, 0.1 Tween 20, the membranes were incubated with 1 h for HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:10,000) in blocking solution. Immunoblot reactive bands were visualized by enhanced chemiluminescence (Nycomed Amersham, Inc.)

For coprecipitation studies, total lysate proteins (200-500 µg) were incubated with 3 h at 4°C with anticyclin E antibody and the immune complexes were collected with protein A-Sepharose beads (Pharmacia Biotech). The beads were washed five times with Triton X-100 lysis buffer, resuspended in denaturing sample buffer, and the eluted proteins were analyzed by immunoblotting.

**Protein Kinase Assays**

The phosphatase activity of Cdk2 was measured by immune complex kinase assay using histone H1 as substrate as described previously (Meloeho, 1995). In brief, lysate proteins (200 µg) were subjected to immunoprecipitation with 1 µg of anti-Cdk2 antibody preadsorbed to protein A-Sepharose beads for 2 h at 4°C. The immune complexes were washed three times with Triton X-100 lysis buffer and once with kinase asay buffer (20 mM Hepes, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol). Histone H1 kinase activity was assayed by resuspending the beads in a total volume of 40 µl of kinase assay buffer containing 0.25 mg/ml histone H1 (Boehringer Mannheim Corp.), 100 µM ATP, and 10 µCi [γ-32P]ATP. The reactions were initiated by the addition of A TP, incubated at 30°C for 5 min, and stopped by addition of 2X denaturing sample buffer. The samples were analyzed by SDS-gel electrophoresis and the bands corresponding to histone H1 were excised and counted.

For inhibition experiments, extracts of PDGF-F-BB-stimulated cells containing active Cdk2 were mixed with boiled (5 min at 100°C) extracts of A ng II-stimulated cells (1:1 ratio; 200 µg protein of each lysate) for 1.5 h at 4°C before immunoprecipitation of Cdk2 and kinase assay. Immunodepletion of p27Kip1 was performed by incubating 200 µg of A ng II-treated cellular extract with 5 µg of anti-p27Kip1 antibody and 0.5 µg of normal rabbit serum and Cdk2 was immunoprecipitated as described above. The immunoprecipitated complexes were washed five times with Triton X-100 lysis buffer. Proteins were eluted by heating at 95°C for 5 min in denaturing sample buffer and analyzed by SDS-gel electrophoresis on 10% acrylamide gels. The proteins were then electrophoretically transferred to PVDF membranes (Millipore) in 25 mM Tris, 192 mM glycine, and 20% methanol, and visualized by autoradiography.

**Phosphorys 32 Labeling and Immunoprecipitation**

Quiescent aortic SM C in 100-mm petri dishes were stimulated for 10 or 20 h with A ng II or PDGF-BB and labeled for the last 5 h in bicarbonate- and phosphate-free Hepes-buffered MEM containing 0.5 mM/ltr [32P]phosphoric acid. The cells were then washed twice with ice-cold PBS and lysed in Triton X-100 lysis buffer. After clarification, the lysates were precleared for 1 h with 5 µl of normal rabbit serum and Cdk2 was immunoprecipitated as described above. Immune complexes were washed five times with Triton X-100 lysis buffer. Proteins were eluted by heating at 95°C for 5 min in denaturing sample buffer and analyzed by SDS-gel electrophoresis on 10% acrylamide gels. The proteins were then electrophoretically transferred to PVDF membranes (Millipore) in 25 mM Tris, 192 mM glycine, and 20% methanol, and visualized by autoradiography.
Phosphoamino Acid Analysis
The labeled band corresponding to Cdk2 was excised from the PVDF membrane and subjected to partial acid hydrolysis in 5.7 M HCl for 1 h at 110°C (Kamps, 1991). The resulting phosphoamino acids, along with unlabelled phosphoamino acid standards (0.2 mg/ml), were separated by one-dimensional thin layer electrophoresis using an optimized pH 2.5 buffer (Elkine and Weber, 1993). The standards were visualized by ninhydrin staining and the labeled amino acids by autoradiography.

Biosynthetic Labeling Experiments
To examine the turnover of p27Kip1 protein, quiescent aortic SM C in 100-mm petri dishes were pulse-labeled for 1 h with 166 μCi/ml of [35S]methionine and [35S]cysteine and then chased for the indicated times in serum-free medium containing excess methionine and cysteine and either Ang II or PDGF-BB. The cells were then washed twice with ice-cold PBS and lysed in Triton X-100 lysis buffer. Lysates (500 μg proteins) were pre-cleared for 1 h with 5 μl of normal rabbit serum and the resulting supernatants were incubated with protein A-Sepharose beads predesorbed with 2 μg of anti-p27Kip1 for 4 h at 4°C. Immune complexes were washed five times with Triton X-100 lysis buffer. Proteins were eluted by heating at 95°C for 5 min in denaturing sample buffer and analyzed by SDS-gel electrophoresis on 12% acrylamide gels. The p27Kip1 protein was detected by fluorography and quantified using a PhosphorImager apparatus.

For labeling newly synthesized proteins, cells were stimulated for the indicated times, rinsed with methionine- and cysteine-free medium, and incubated with 250 μCi/ml of [35S]methionine and [35S]cysteine. Labeling was allowed to proceed for the last 20 min. Cell lysis and immunoprecipitation of p27Kip1 were conducted as described above.

Northern Blot Analysis
Total RNA was extracted by a modified version of the guanidinium thiocyanate procedure as described (Chomczynski and Sacchi, 1987; Chomczynski, 1993). Equal amounts of total RNA (15–25 μg) were denatured and resolved by electrophoresis in a 1% agarose gel containing 1.8% formaldehyde. The RNA was transferred to Hybond-N membranes (Amersham) and fixed with 10% dimethyl sulfoxide, 10% formamide, and 5 μl/ml of 20% deionized sodium sulfate DNA, and 0.25 μg/ml yeast tRNA at 55°C for 2 h. The membranes were washed extensively at 60°C in 0.5× SSC, 0.1% SDS. The extent of hybridization was analyzed with a PhosphorImager apparatus.

TGF-β1 Bioassay
TGF-β1 bioassay was conducted essentially as described previously (Gibbons et al., 1992). Recombinant TGF-β1 and TGF-β1 neutralizing antibody (TNA) was a generous gift from Dr. M. au rene O'Connor. In brief, Mv1Lu cells were plated at a density of 5 × 10⁴ cells per well in 24-well plates. A filter 6 h of serum exposure, the cells were washed with serum-free medium, then incubated with conditioned medium or TGF-β1 in serum-free medium for 24 h. The rate of DNA synthesis was measured by pulse-labeling cells with 2 μCi/ml [3H] thymidine during the last 6 h of incubation. For each experiment, a standard curve was constructed with increasing concentrations of recombinant TGF-β1. A ng II conditioned medium was obtained from aortic SM C stimulated for 24 h with Ang II and was added to Mv1Lu cells at two dilutions (1:5 and 1:10). TNA was purified by protein A–agarose chromatography. The antibody was used at a concentration of 10–15 μg/ml, which completely blocks the growth inhibitory effect of TGF-β1 in Mv1Lu cells.

Results
PDGF-BB, but Not Ang II, Induces DNA Synthesis in Aortic SM C
We compared the ability of the vascular growth factors PDGF-BB and Ang II to stimulate the rate of DNA synthesis in quiescent rat aortic SM C. As previously reported (Eisterfer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Giasson and Meloche, 1995), treatment of aortic SM C with Ang II had no significant effect on DNA synthesis, as measured by [3H] thymidine incorporation (Fig. 1 A). In contrast, addition of PDGF-BB strongly increased the rate of DNA synthesis, which reached a peak (180-fold over basal level) at 24 h after stimulation. PDGF-BB also induced cellular division as reflected by an increase in SM C number and by the small ratio between [3H] thymidine incorporation and cell number (Fig. 1 B).

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Ang II Fails to Stimulate the Activity of Cdk2 in Aortic SMC

In an effort to understand the molecular basis for this differential response of aortic SMC to vascular growth factors, we examined the downstream effects of PDGF-BB and Ang II on regulators of the cell cycle machinery. We first analyzed the regulated expression of G1 cyclins. Fig. 2A shows that both PDGF-BB and Ang II stimulated the accumulation of D-type cyclins with similar kinetics. The expression of cyclin D1, D2, and D3 started to increase at 4 h poststimulation and reached a maximal level by 12-16 h. Cyclin E expression was already detectable in quiescent cells. Treatment with PDGF-BB caused a small but significant increase in cyclin E expression, which was delayed compared with D-type cyclins (Fig. 2A). Ang II had little effect on cyclin E expression. It should also be noted that PDGF-BB promoted higher levels of cyclin D1 accumulation than Ang II in aortic SMC. This is in agreement with previous observations showing that the extent of cyclin D1 accumulation is correlated with the mitogenic potential of growth factors and their ability to induce sustained ERK1/ERK2 activation (Lavoie et al., 1996; and data not shown). Little difference was observed in the expression level of the catalytic subunits Cdk4 and Cdk2, which were present in all extracts, including those prepared from quiescent cells (Fig. 2B). However, we noted that treatment with PDGF-BB results in the late appearance of a faster migrating species of Cdk2, which is indicative of phosphorylation of the enzyme on threonine 160 (Gu et al., 1992). Only the slower migrating form of Cdk2 was observed in Ang II-stimulated cells.
We next measured the enzymatic activity of CAK, Cdk4, and Cdk2 after treatment of aortic SMC with the two vascular growth factors. CAK, Cdk4, and Cdk2 were selectively immunoprecipitated from cell lysates and their activity assayed in vitro using GST-Cdk2, GST-pRb, and histone H1 as substrates, respectively. As observed in other cellular models (Sclafani, 1996), CAK enzymatic activity was the same in quiescent and growth factor-treated aortic SMC (Fig. 3 A). Both Ang II and PDGF-BB increased the Rb kinase activity of Cdk4, which became detectable at eight hours and remained elevated up to the end of G1 phase (Fig. 3 B). Notably, Ang II treatment of aortic SMC induced a delayed Rb kinase activity as compared with PDGF-BB treatment. These results most likely reflect the quantitative differences in the ability of Ang II and PDGF-BB to regulate the expression of cyclin D1 (Fig. 2 A). Both factors equally stimulated Cdk4 activity after 16 h of exposure. As expected, stimulation of aortic SMC with the mitogenic factor PDGF-BB strongly increased Cdk2-associated histone H1 kinase activity, which was first detected at 12 h and reached a maximum in S phase (Fig. 3 C). In contrast, treatment with Ang II failed to induce any detectable Cdk2 activity over the same period of time. Thus, we carried out a series of experiments to explain the inability of Ang II to activate Cdk2. Since CAK-mediated phosphorylation of threonine 160 on Cdk2 is required for kinase activation (Morgan, 1995), we first analyzed the phosphorylation state of Cdk2 after immunoprecipitation from 32P-labeled cells stimulated with Ang II or PDGF-BB. Fig. 4 A shows that the lack of activation of Cdk2 in Ang II-treated cells was associated with the failure of Ang II to stimulate phosphorylation of the enzyme on threonine. On the other hand, addition of PDGF-BB resulted in the phosphorylation of Cdk2 on threonine, tyrosine, and serine residues after 20 h (Fig. 4 B). Indeed, it has been reported in HeLa cells that most of the phosphorylation of Cdk2 on tyrosine (tyrosine 15) occurs on Cdk2 molecules that are also phosphorylated on threonine 160 (Gü et al., 1992). The absence of CAK-mediated threonine phosphorylation of Cdk2 in Ang II-treated cells was not attributable to the inability of Cdk2 to form complexes with cyclin E. Immunoblot analysis showed that cyclin E immunoprecipitates from quiescent aortic SMC already contained a significant amount of Cdk2 and that treatment with PDGF-BB caused a further increase in complex formation that became apparent only after 16 h of stimulation (Fig. 4 C). In addition, detailed kinetic analysis of Cdk2 phosphorylation and activity revealed that 10–12 h of PDGF-BB stimulation (thus before induction of cyclin E expression and increased cyclin E–Cdk2 complex formation) is sufficient to promote Cdk2 phosphorylation on threonine (Fig. 4 A; and data not shown) and to activate the enzyme (Fig. 3 C). These results indicate that mechanisms other than increased cyclin E expression or cyclin E–Cdk2 complex assembly account for the inability of Ang II to induce threonine 160 phosphorylation of Cdk2 and to stimulate the activity of the enzyme in aortic SMC.

Differential Modulation of p27Kip1 Expression by PDGF-BB and Ang II

In addition to its ability to disrupt the catalytic activity of phosphorylated cyclin-bound Cdks (Russo et al., 1996), the inhibitor p27Kip1 can also sterically interfere with the phosphorylation of Cdks by CAK (Kato et al., 1994; Polyak et al., 1994a; Aprelikova et al., 1995). To determine whether p27Kip1 was a determinant factor in the differential regulation of Cdk2 activation by mitogenic and hypertrophic factors, we compared the levels of p27Kip1 protein expression. The expression of p27Kip1 protein was elevated in quiescent aortic SMC and decreased progressively upon
treatment of cells with the mitogenic factor PDGF-BB (Fig. 5 A). The decrease in p27Kip1 level was already evident four hours after PDGF-BB exposure. After 20 h of stimulation with PDGF-BB, the expression of p27Kip1 was reduced by ~80%. In contrast, Ang II had a negligible effect on the expression of the Cdk inhibitor protein. Importantly, we found that mixing of boiled extract from Ang II-stimulated cells with an equal amount of extract from cells treated with PDGF-BB significantly reduced Cdk2-associated histone H1 kinase activity (Fig. 5 B). p27Kip1 previously has been shown to be heat-stable (Pollyak et al., 1994b), thus making it a good candidate for the inhibitory factor of Ang II-boiled extracts. Indeed, the Cdk2 inhibitory activity present in Ang II-treated cells was completely eliminated after immunodepletion of p27Kip1 with a specific antibody (Fig. 5 B). Preincubation of the anti-p27Kip1 antibody with a saturating amount of immunogenic peptide completely restored the Cdk2 inhibitory activity, confirming that p27Kip1 is the major factor responsi-
ble for this activity. Addition of boiled extracts from PDGF-BB-stimulated cells, which contain very low levels of p27Kip1 (Fig. 5 A), did not inhibit Cdk2 activity of extracts from cells exposed to PDGF-BB for 20 h (data not shown).

The inability of Ang II to downregulate expression of p27Kip1 in aortic SMC was also reflected by the strong and sustained association of the inhibitor with cyclin E–Cdk2 complexes. When cells exposed to Ang II or PDGF-BB were subjected to immunoprecipitation with anticyclin E antibody, the level of associated p27Kip1 was found to be significantly lowered after 16 h of PDGF-BB treatment compared with Ang II-stimulated cells (Fig. 5 C). From these results, we conclude that the failure of Ang II to downregulate p27Kip1 expression is responsible, at least in part, for the inability of Ang II to induce Cdk2 activation, DNA synthesis, and cellular division in aortic SMC.

The Abundance of p27Kip1 Is Regulated both at the Level of mRNA Expression and Protein Stability in Aortic SMC

We next addressed the question of how the levels of p27Kip1 are regulated by vascular growth factors. Studies in other cell systems have shown that the abundance of p27Kip1 is controlled by multiple posttranscriptional processes including degradation through the ubiquitin–proteasome pathway (Pagano et al., 1995) and changes in translation rates (Agrawal et al., 1996; Hengst and Reed, 1996; Millard et al., 1997). To determine the rate of p27Kip1 turnover, pulse-chase experiments were conducted on aortic SMC treated with Ang II or PDGF-BB. The rate of degradation of p27Kip1 was clearly increased in cells exposed to PDGF-BB (Fig. 6, A and B). Quantitation of the data revealed that the half-life of the protein was reduced to six hours, compared with that of arrested (8.9 h) or Ang II-treated cells (8.2 h).

The rate of synthesis of p27Kip1 was also affected by treatment with vascular growth factors. As shown in Fig. 6 C, the synthesis of p27Kip1 was dramatically repressed after two hours of PDGF-BB stimulation and this inhibition persisted for up to 20 h. Ang II treatment also resulted in the repression of p27Kip1 synthesis, but the effect was less marked and more transient, the rate of synthesis returning to basal level within 6–12 h of stimulation. To verify whether the decline in p27Kip1 synthesis was associated with a decrease in Kip1 mRNA accumulation, we measured the steady-state levels of Kip1 mRNA by Northern hybridization. Results of these experiments clearly demonstrated that expression of Kip1 mRNA is regulated in aortic SMC. PDGF-BB treatment resulted in a rapid and marked decrease of Kip1 mRNA, which was almost undetectable by two hours of stimulation, and then slowly returned to its quiescent level at ~12 h (Fig. 7). Ang II also reduced expression of Kip1 mRNA, but the effect was smaller in comparison to PDGF-BB. The time course of Kip1 mRNA downregulation and reappearance correlated well with the transient decrease in the rate of p27Kip1 synthesis seen after PDGF-BB and Ang II treatment (Fig. 6 C). This suggests that repression of p27Kip1 synthesis by vascular growth factors is likely attributable, at least in part, to a corresponding decrease of Kip1 mRNA abundance.

There was a tight temporal relationship between the decrease in the rate of p27Kip1 synthesis observed at two hours after stimulation, the increased rate of degradation of the protein, and the change in the total amount of p27Kip1. If we consider that the rate of p27Kip1 synthesis is almost null after two to three hours of PDGF-BB treatment and that the half-life of the protein is approximately six hours (Fig. 6), the level of p27Kip1 protein should be reduced by ~50% eight to nine hours after mitogenic stimulation. This estimation is consistent with the data presented in Fig. 5 A.
PDGF-BB Reduces the Rate of Kip1 Gene Transcription in Aortic SMC

To determine whether PDGF-BB–mediated downregulation of Kip1 mRNA involves a transcriptional mechanism, nuclear run-on transcription assays were performed on nuclei isolated from quiescent and growth factor-treated aortic SMC. Fig. 8 shows that PDGF-BB markedly decreased the rate of Kip1 transcription (~90% reduction of control value) after two hours of stimulation. Addition of Ang II also caused a significant attenuation of Kip1 transcription, but the effect was less pronounced than that of PDGF-BB. As a control, we also examined transcription of the gene encoding smooth muscle α-actin, which is known to be induced by Ang II, but not PDGF-BB, in vascular SMC (Corjay et al., 1990; Hautmann et al., 1997). In agreement with these studies, only Ang II enhanced smooth muscle α-actin transcription. No appreciable difference in the transcription of GAPDH gene was observed in response to Ang II or PDGF-BB treatment.

We next examined the effect of PDGF-BB and Ang II on the stability of Kip1 mRNA using two independent approaches: mRNA decay in the presence of the transcriptional inhibitor 5,6-Dichloro-1-b-D-ribofuranosylbenzimidazole (DRB) and pulse-chase analysis with [3H]uridine. With the use of DRB, the half-life of Kip1 mRNA was estimated to be 4.7 h in unstimulated cells (Fig. 9 A). Treatment with PDGF-BB or Ang II accelerated the degradation of Kip1 mRNA, decreasing the half-life to 2.2 h and 2.3 h, respectively. Comparable results were obtained with the pulse-chase method with a calculated half-life of 3.6 h in quiescent cells, and of 1.9 h and 2.0 h in cells stimulated with PDGF-BB and Ang II, respectively (Fig. 9 B). These findings indicate that both PDGF-BB and Ang II destabilize Kip1 mRNA to the same extent in aortic SMC. We found that the calculated half-life values of Kip1 mRNA are slightly longer than what would be expected from the results of Fig. 7. This is likely due to the inherent imprecision associated with the measure of low abundant messages with short half-lives (Harrold et al., 1991).

The Failure of Ang II to Promote S Phase Entry of Aortic SMC Is Not Explained by Autocrine Production of TGF-β1

TGF-β1 is the prototype of a family of growth factors that play important roles in cellular growth, differentiation, and morphogenesis (Massague, 1990). In particular, TGF-β1 is a potent growth inhibitor for many cell types, including vascular SMC (Owens et al., 1988). Several mechanisms have been proposed to explain how TGF-β1 inhibits proliferation and induces cell cycle arrest in G1 phase (Hannon and Beach, 1994; Reynisdottir et al., 1995). The observation that Ang II can induce hypertrophic or mitogenic effects in vascular SMC has led to the hypothesis that Ang II activates both proliferative and antiproliferative, specifically TGF-β1, signals (Gibbons et al., 1992). Therefore, we carried out a series of experiments to test the possibility that autocrine production of TGF-β1 may be responsible for the failure of Ang II to activate Cdk2 and induce DNA synthesis in aortic SMC. To determine whether Ang II stimulates the production of active TGF-β1, we used a highly sensitive bioassay that is based on the
ability of TGF-β to induce G<sub>1</sub> arrest in M<sub>v1L</sub>u cells. Fig. 10 A shows that addition of 10 μM TGF-β1 to M<sub>v1L</sub>u cells is sufficient to inhibit DNA synthesis by 90%. This inhibitory activity of TGF-β1 is reversed by coincubation with TNA, but not with normal rabbit IgG. However, conditioned medium from A ng II–treated aortic SMC did not inhibit M<sub>v1L</sub>u cells DNA synthesis, but rather had a significant stimulatory effect (Fig. 10 B). We also tested the effect of TNA on the ability of A ng II to stimulate DNA synthesis in aortic SMC. Coincubation of A ng II with normal rabbit IgG or TNA had essentially no effect on the rate of DNA synthesis (Fig. 10 C). Finally, we examined the effect of simultaneous exposure of aortic SMC to both PDGF-BB and A ng II. Simultaneous addition of A ng II or pretreatment with A ng II (data not shown) did not interfere with PDGF-BB–induced DNA synthesis (Fig. 10 C) or p27<sup>Kip1</sup> downregulation (Fig. 10 D), consistent with the idea that A ng II does not stimulate the synthesis of an antimitogenic factor. Together, these results demonstrate that the failure of A ng II to promote S phase entry of aortic SMC is not due to autocrine production of TGF-β1.

**Discussion**

Unlike cardiac and skeletal muscle cells, which undergo terminal and irreversible differentiation, vascular SMC display remarkable cellular plasticity that allows them to acquire a spectrum of different phenotypes in response to appropriate stimuli (Owens, 1995). In addition to their main function of contraction, vascular SMC can increase their mass through cellular proliferation, cellular hypertrophy, and production of extracellular matrix proteins. Changes in growth rates occur normally during development of the vascular system and after vascular injury, but also under pathological conditions such as hypertension and atherosclerosis (Schwartz et al., 1986; Owens, 1989; Ross, 1993). In animal models of hypertension, the increase in vascular mass is associated primarily with SMC hypertrophy in large arteries and with hyperplasia in small resistance vessels. SMC proliferation also plays a central role in the atherosclerotic process. The growth response of vascular SMC is clearly dependent on the nature of the growth stimulus. For example, in cultured rat aortic SMC, agonists like A ng II induce cellular hypertrophy as a result of increased protein synthesis (Gisterfer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Grainger et al., 1994; Giasson and McIre, 1995), whereas peptide growth factors like PDGF-BB cause a strong proliferative response (Raines et al., 1990; Grainger et al., 1994). However, much remains to be learned about the molecular determinants of vascular SMC hypertrophic versus hyperplastic growth response. Here, we present evidence that p27<sup>Kip1</sup> is an important regulator of the phenotypic response of vascular SMC. First, we show that treatment with the mitogenic factor PDGF-BB, but not with the hypertrophic factor A ng II, leads to a progressive and dramatic decline in the level of p27<sup>Kip1</sup> protein. The failure of A ng II to downregulate p27<sup>Kip1</sup> results in the increased association of the inhibitor with cyclin E–Cdk2 complexes and correlates with inhibition of threonine 160 phosphorylation of Cdk2. Since C A K is constitutively active in aortic SMC, the simplest interpretation of our data is that stoichiometric binding of p27<sup>Kip1</sup> to cyclin E–Cdk2 complexes prevents CAK from phosphorylating and activating Cdk2 in A ng II–treated cells. In support of this hypothesis, in vitro studies have shown that p27<sup>Kip1</sup> binding to preformed cyclin E–Cdk2 complexes blocks CAK–mediated threonine 160 phosphorylation of the enzyme (Polyak et al., 1994a; A Prelkova et al., 1995). Second, we further show that extracts from A ng II–stimulated cells contain enough CDK inhibitory activity to reduce by ~70% Cdk2–associated histone H1 kinase activity of PDGF-BB–treated cell extracts. The stability of this inhibitory activity to heat treatment and its reversal following immunodepletion of p27<sup>Kip1</sup> confirmed that p27<sup>Kip1</sup> is the major inhibitory factor present in these extracts.

Previous studies have shown that the abundance of
p27\(^{Kip1}\) is regulated by multiple posttranscriptional mechanisms. Our present results add another level of complexity by demonstrating that the levels of p27\(^{Kip1}\) are also controlled by transcriptional mechanisms in vascular SMC. Our data support a model where the reduction of p27\(^{Kip1}\) expression observed in response to mitogenic factors occurs by two mechanisms. The first mechanism is a rapid decrease in the rate of p27\(^{Kip1}\) synthesis that becomes minimal by two hours and slowly returns to quiescent value after \(\sim 20\) h. This lowered synthesis, combined with the significant turnover of the protein (see Fig. 6 B), is responsible for the initial decline in p27\(^{Kip1}\) protein levels, which can be easily detected by eight hours of PDGF-BB stimulation. Detailed kinetic analysis revealed that the reduction in the rate of p27\(^{Kip1}\) synthesis is tightly paralleled by a transient downregulation of \(Kip1\) mRNA accumulation. Importantly, these changes in \(Kip1\) mRNA levels coincide with a marked decrease in the rate of \(Kip1\) gene transcription, suggesting that transcriptional control is an important factor in regulating the synthesis of p27\(^{Kip1}\). While other studies have reported changes in the levels of \(Kip1\) mRNA in response to extracellular factors (Kwon et al., 1996; Liu et al., 1996), our findings provide the first demonstration that p27\(^{Kip1}\) expression is regulated at the level of gene transcription. We also show that both PDGF-BB and Ang II significantly decrease the stability of \(Kip1\) mRNA. The almost complete inhibition of \(Kip1\) gene transcription, coupled with the increased turnover of the mRNA, explains the marked downregulation of \(Kip1\) mRNA expression observed in PDGF-BB–treated cells. Further studies are clearly necessary to identify the cis-acting elements that target \(Kip1\) mRNA for degradation and the corresponding RNA-binding proteins. In addition to transcription, other levels of control may also be involved in the regulation of p27\(^{Kip1}\) synthesis. Figs. 6 C and 7 show that the rate of p27\(^{Kip1}\) synthesis is still repressed in PDGF-BB–treated cells after 12–20 h when \(Kip1\) mRNA has returned to control levels. One possibility is that \(Kip1\) mRNA is not being used efficiently by the translation machinery during G1 progression because of the binding of mRNA masking proteins (Spirin, 1996). In support of this idea, it was found that the accumulation of p27\(^{Kip1}\) protein observed during growth arrest of HL-60 cells is due to an increase in the amount of \(Kip1\) mRNA in polyribosomes (Millard et al., 1997). The second mechanism of p27\(^{Kip1}\) elimination is an increase in the degradation rate of the protein, which is mostly evident by eight hours of mitogenic stimulation. By contrast, treatment of vascular SMC was added alone or in combination with 10 \(\mu\)g/ml TNA to Mv1Lu cells. C, Quiescent aortic SMC were stimulated for 24 h with 100 nM Ang II in the absence or presence of 10 \(\mu\)g/ml TNA or normal rabbit IgG (NRS). In the same experiment, the cells were stimulated with 50 ng/ml PDGF-BB alone or in the presence of 100 nM Ang II. The rate of DNA synthesis was measured by \(^{3}H\)thymidine incorporation during the last 4–6 h of stimulation. Each value represents the mean \(\pm\) SEM of triplicate determinations. The data presented are representative of at least two different experiments with similar results. D, Expression of p27\(^{Kip1}\) protein. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB or with both agonists for a 20-h period. The expression of p27\(^{Kip1}\) protein was analyzed by immunoblotting.
with hypertrophic factors like Ang II less effectively represses p27Kip1 synthesis and does not affect the rate of degradation of the protein.

The signaling pathways that are involved in the regulation of Kip1 gene transcription remain to be identified. As mentioned earlier, PDGF-BB and Ang II activate several common signaling events in aortic SMC. However, significant differences are noted in the time course of these events. For example, PDGF-BB induces a sustained activation of the MAP kinases ERK1/ERK2, whereas Ang II has a very transient effect (Pleven et al., 1996; and data not shown). PDGF-BB and Ang II are also known to have different effects on the source and duration of the increase in cytosolic-free calcium in vascular SMC (Roe et al., 1999; Brinson et al., 1998). In addition, mitogenic and hypertrophic factors are likely to trigger unique signaling events. Studies in pulmonary arterial SMC have shown that PDGF-BB exclusively stimulates an increase in phosphatidylinositol 3,4,5-trisphosphate (Button et al., 1994), whereas only thrombin, which behaves as a hypertrophic factor, induces fosB mRNA levels (Rothman et al., 1994). However, these observations may not be generalized to other SMC types, since both PDGF-BB and Ang II activate PI3-kinase and induce fosB mRNA in rat aortic SMC (Saward and Zahradka, 1997; and data not shown). Characterization of the 5′ flanking region of the mouse Kip1 gene showed that a region between −326 to −615 is sufficient to confer maximal basal promoter activity (Kwon et al., 1996; Zhang and Lin, 1997). Constructs extending beyond −615 displayed lower basal promoter activity, suggesting that a negative regulatory element may be contained in the region between −615 and −1,609 (Kwon et al., 1996). However, these studies did not examine the serum or growth factor responsiveness of the various Kip1 gene promoter constructs. Work is in progress in our laboratory to identify specific regions within the promoter of the rat Kip1 gene which mediate PDGF-BB dependent transcriptional repression.

The turnover of p27Kip1 is also subject to regulation by mitogenic factors in vascular SMC. Given the recent demonstration that cyclin E-Cdk2 directly phosphorylates p27Kip1 on threonine 187 and promotes its elimination from the cell (Sheaff et al., 1997; Vlach et al., 1997), it is tempting to speculate that the different rates of p27Kip1 turnover observed in PDGF-BB or Ang II-treated cells are a reflection of their differential ability to activate Cdk2. In agreement of this idea, we found that in vivo phosphorylation of p27Kip1 increases after 8–12 h in cells exposed to PDGF-BB, but not in response to Ang II (data not shown). However, phosphorylation by Cdk2 is unlikely to be the sole mechanism that regulates the proteolysis of p27Kip1. Indeed, significant degradation of the inhibitor is observed during the first hours of growth factor stimulation, in the absence of detectable histone H1 kinase activity (Pagano et al., 1995; Arawal et al., 1996; this study). We also found that in vascular SMC and other cell types, p27Kip1 is significantly phosphorylated in G0 and early G1 phase (data not shown). These observations suggest that other protein kinases and/or mechanisms signal p27Kip1 for degradation. In this respect, it was reported that R as signaling is required for downregulation of p27Kip1 in rodent fibroblasts (Aktas et al., 1997; Takuwa and Takuwa, 1997; Kawada et al., 1997) and that RhoA is a necessary mediator of p27Kip1 degradation (Webber et al., 1997).

It has been postulated that the failure of Ang II to stimulate vascular SMC hyperplasia is due to autocrine production of the antimitogenic cytokine TGF-b1 by these cells (Gibbons et al., 1992; Kobuchi et al., 1993). However, our results do not support this model. First, active TGF-β1 was not detected in the supernatant of Ang II-treated aortic SMC. Second, the use of a neutralizing antibody against TGF-β1 in combination with Ang II did not potentiate DNA synthesis in these cells. Third, pretreatment of aortic SMC for four hours with Ang II before PDGF-BB stimulation (data not shown) or simultaneous addition of both factors did not affect the mitogenic response to PDGF-BB.

Previous in vivo studies have demonstrated that Cdk2 function is required for intimal SMC accumulation after angioplasty in the rat carotid artery (Abe et al., 1994; Morishita et al., 1994). In addition, Cdk2 expression is temporally correlated with vascular SMC proliferation after angioplasty (Wei et al., 1997). Moreover, it was reported that p27Kip1 is markedly upregulated after balloon angioplasty in the rat carotid artery and that high levels of p27Kip1 expression correlates with downregulation of Cdk2 kinase activity (Chen et al., 1997). Ectopic overexpression of p27Kip1 in injured arteries attenuated neointimal lesion formation. A recent study also presented evidence that polymerized collagen inhibits aortic SMC proliferation in vitro through α2 integrin-mediated upregulation of p27Kip1 (Koyama et al., 1996). Thus, the results presented here, together with these findings, clearly identify p27Kip1 as an important regulator of vascular SMC growth response.

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