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

Marc J. Servant, Philippe Coulombe, Benjamin Turgeon, and Sylvain Meloche

Research Centre, Centre hospitalier de l’Université de Montréal and Department of Pharmacology, University of Montreal, Montreal, Quebec, H2W 1T8 Canada

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)1 and their regulatory cyclin subunits (Draetta, 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-

1Abbreviations used in this paper: Ang II, angiotensin II; CAK, Cdk-activating kinase; Cdk, cyclin-dependent kinase; DRB, 5,6-Dichloro-1-b-D-ribofuranosylbenzimidazole; GA PDH, glyceraldehyde-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.
protein (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 Hove and Harlow, 1993; K noblich et al., 1994; Ohtsubo et al., 1995; K rude et al., 1997).

The activity of Cdk5 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, p15, p18, and p19, 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; Chen et al., 1995; Hira et al., 1995). The second family of inhibitors includes p21, p27, and p57, which upon phosphorylation dissociates from pRb, which upon phosphorylation dissociates from

In cultured arterial smooth muscle cells (SMC), 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-γ (PLC-γ), 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; Hellin, 1997).

In contrast to PDGF-BB, many investigators, including ourselves, have shown that the peptide angiotensin II (A ng 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, A ng II was reported to exert weak mitogenic effects on SM C of resistance arteries (D ubey et al., 1992) and on aortic SM C isolated from spontaneously hypertensive rats (Bunkenburg et al., 1992; Itazaki et al., 1995). In vivo, a number of studies have shown that infusion of A ng II stimulates SM C DNA synthesis and proliferation in normal and injured rat arteries (Daemen et al., 1991; van K leef et al., 1992; de Blois et al., 1996; Su et al., 1998). However, results of in vivo studies are difficult to interpret since the effect of A ng II may be indirect or A ng II may simply act as a comitogen. It has been postulated that A ng II may be a bifunctional growth factor that activates both proliferative and antiproliferative (TG F-β1) signals in vascular SM C (G ibbons et al., 1992; Kibuchi et al., 1993). A ccording to this model, the autocrine production of TG F-β1 would determine whether vascular SM C grow by hypertrophy or hyperplasia in response to A ng II.

In cultured aortic SMC, the hypertrophic action of A ng II is initiated by its interaction with the G protein-coupled A T1 receptor, which stimulates the activity of PLC-β to generate the second messengers inositol 1,4,5-trisphosphate (InsP3) 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 ERK 1/ERK 2 (Duff et al., 1992; Tsuda et al., 1992; Servant et al., 1996) and p70 S6 kinase (Giasson and M eloche, 1995). A ng II also induces tyrosine phosphorylation of multiple proteins in aortic SM C (M olloy et al., 1993; Leduc et al., 1995) and stimulates the activity of cytosolic tyrosine kinases, such as p125FAK (P olte et al., 1994; Giasson et al., 1997), Pyk2 (Giasson et al., 1997), Sr c (Ishida et al., 1995), and the Janus kinases Jak2 and Tyk2 (M arrero et al., 1995; Giasson et al., 1997). Despite the fact that A ng II and PDGF-BB activate similar signal transduction pathways, only the latter is able to induce proliferation of aortic SM C.

In an effort to understand the molecular basis for this differential response, we have examined the downstream effects of PDGF-BB and A ng II on regulators of the cell cycle machinery. W e 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 A ng II-treated cells is associated with the failure of A ng II to downregulate p27Kip1 expression. W e also show that p27Kip1 abundance is regulated by
Materials and Methods

Cell Culture
Rat aortic SM C were cultured to 80% confluence and synchronized in the quiescent state as described previously (Glaiss and Melloche, 1995). The cells were stimulated with 100 mM A ng II (H-ukabel Scientific) or 50 mg/ml PDGF-BB (Oncogene Science) for the indicated times at 37°C. M v3L u mink epithelial cells (obtained from Dr. M aureen O’Connor, Bio- technology Research Institute of Montreal, Canada) were grown in M EM 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]thymidine for the last 2-4 h. A fter the stimulation, the medium was aspirated and the cells were incubated for a maximum of 30 min in cold 5% TCA. The cells 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 were trypsinized 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 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin a, and 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 (Nycomer A mersham, Inc.) in 25 mM Tris, 192 mM glycine, and 20% methanol, and visualized by autoradiography. 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 were trypsinized 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 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin a, and 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 (Nycomer A mersham, Inc.) in 25 mM Tris, 192 mM glycine, and 20% methanol, and visualized by autoradiography. 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 were trypsinized 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 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin a, and 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 (Nycomer A mersham, Inc.) in 25 mM Tris, 192 mM glycine, and 20% methanol, and visualized by autoradiography. 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 were trypsinized 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 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin a, and 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 (Nycomer A mersham, Inc.) in 25 mM Tris, 192 mM glycine, and 20% methanol, and visualized by autoradiography. 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 were trypsinized 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 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin a, and 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 (Nycomer A mersham, Inc.) in 25 mM Tris, 192 mM glycine, and 20% methanol, and visualized by autoradiography. 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 were trypsinized and counted using a hemacytometer.
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 (K. amps, 1991). The resulting phosphoamino acids, along with unla-
beled phosphoamino acid standards (0.2 mg/ml), were separated by one-
dimensional thin layer electrophoresis using an optimized pH 2.5 buffer (Jelinek 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 p27\textsuperscript{kip1} protein, quiescent aortic SM C in 100-mm petri dishes were pulse-labeled for 1 h with 166 \mu Ci/ml of [\textsuperscript{35}S]me-thionine and [\textsuperscript{35}S]cysteine and then chased for the indicated times in ser-
um-free medium containing excess methionine and cysteine and either A ng II or PDGF-BB. The cells were then washed twice with ice-cold PBS and lysed in Triton X-100 lysis buffer. Lysates (50 \mu g proteins) were pre-
cleared for 1 h with 5 \mu l of normal rabbit serum and the superna-
tants were incubated with protein A-Sepharose beads preadsorbed with 2 \mu g of anti-p27\textsuperscript{kip1} 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 elec-
trophoresis on 12% acrylamide gels. The p27\textsuperscript{kip1} protein was detected by fluoro
graphy 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 \mu Ci/ml of [\textsuperscript{35}S]methionine and [\textsuperscript{35}S]cysteine. Labeling was allowed to proceed for the last 20 min. Cell lysis and immunoprecipi-
tation of p27\textsuperscript{kip1} were conducted as described above.

Nuclear Run-On Transcription Assays
Total RNA was extracted by a modified version of the guanidinium thiocyanate procedure as described (Chomczynski and Sacchi, 1987; Chom-
czynski, 1993). Equal amounts of total RNA (15–25 \mu g) were denatured and resolved by electrophoresis in a 1% agarose gel containing 1.8%
formaldehyde. The RNA was transferred to Hybond-N membranes and resolved by electrophoresis in a 1% agarose gel containing 1.8%
formaldehyde procedure as described (Chomczynski and Sacchi, 1987; Chom-
czynski, 1993). The standards were visualized by ninhydrin

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 A ng II to stimulate the rate of DNA syn-
thesis in quiescent rat aortic SM C. A s previously reported (Gisterfer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Giasson and Meloche, 1995), treatment of aortic SM C with A ng II had no significant effect on DNA synthesis, as measured by \textsuperscript{3}H 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 \textsuperscript{3}H leucine incor-
poration and cell number (Fig. 1 B). A ng II did not pro-
mote cell division, but caused cellular hypertrophy by in-
creasing the rate of protein synthesis per cell over a period of 72 h (Fig. 1 B). This finding suggests that cell cycle progression is not delayed in A ng II-stimulated cells, but rather, that cells are arrested in G\textsubscript{1} phase. Thus, PDGF-BB is a strong mitogenic factor for rat aortic SM C, promoting DNA synthesis and cellular division, whereas A ng II acts as a hypertrophic factor.
Servant et al. Regulation of p27Kip1 Expression in Vascular SMC

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.

Figure 1. Comparative effects of Ang II and PDGF-BB on the growth of aortic SMC. A, DNA synthesis. Quiescent rat aortic SMC were stimulated for different periods of time with 100 nM Ang II (○) or 50 ng/ml PDGF-BB (■) and the rate of DNA synthesis was measured by [3H]thymidine incorporation. Each value represents the mean ± SEM of triplicate determinations. B, Cell protein content. Quiescent aortic SMC were stimulated with Ang II or PDGF-BB for 72 h. The rate of protein synthesis was measured by [3H]leucine incorporation and the number of cells counted on parallel plates. The results are expressed as the relative ratio between [3H]leucine incorporation and cell number. Each value represents the mean ± SEM of triplicate determinations. Cell numbers for this experiment were: control (cont), 374,167 ± 53,428 cells; Ang II, 312,000 ± 28,369 cells; PDGF-BB, 917,500 ± 42,120 cells. The data are representative of at least three different experiments with similar results.

Figure 2. Effects of Ang II and PDGF-BB on the accumulation of G1 cyclins and the expression level of Cdk2 in aortic SMC. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for different times over a 24-h period. Equal amounts of lysate proteins were resolved by SDS-gel electrophoresis on 12% acrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with antibodies specific to the different cyclins and Cdk2. The proteins were visualized by chemiluminescence detection. Similar results were obtained in five different experiments.
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 stimulate 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 (Gu 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. 3 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. 5A). 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 inhibitory 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. 5B). p27Kip1 previously has been shown to be heat-stable (Polyak et al., 1994b), thus making it a good candidate for the inhibitory factor of Ang II-boiled extracts. The phosphorylated amino acids were separated by one-dimensional thin layer electrophoresis. The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) are indicated. C, Complex formation between Cdk2 and cyclin E. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for different times over a 24-h period. Cell lysates were prepared and subjected to immunoprecipitation with cyclin E-specific antibody. The immunoprecipitated proteins were then analyzed by immunoblotting with anti-Cdk2 antibody. Similar results were obtained in three different experiments.
The Synthesis of p27Kip1 was Dramatically Repressed after Treatment with Vascular Growth Factors. As shown in Fig. 6 C, downregulation and reappearance correlated well with the decline in 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. Preparation of cell extracts and immunoprecipitation were conducted as above. Similar results were obtained in four separate experiments.

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.
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-β-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

**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-β-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).
SMC were pulse-labeled with [3H]uridine for 12 h and chased in separate experiments. B, Pulse-chase method. Quiescent aortic PDGF-BB–treated cells. Data points represent the mean of three Northern hybridization using a 32P-labeled probe. The hybridization signals were quantified using a PhosphorImager apparatus.

The Journal of Cell Biology, Volume 148, 2000 552

Figure 9. Effect of Ang II and PDGF-BB on the stability of Kip1 mRNA. A, DRB method. Quiescent aortic SMC were either left untreated or stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for the indicated times in the presence of 25 μg/ml DRB. Total cellular RNA was extracted from the cells and analyzed by Northern hybridization using a [32P]-labeled Kip1 probe. The hybridization signals were quantified using a PhosphorImager apparatus. ○, Unstimulated cells; ●, Ang II–treated cells; ▲, PDGF-BB–treated cells. Data points represent the mean of three separate experiments. B, Pulse-chase method. Quiescent aortic SMC were pulse-labeled with [3H]uridine for 12 h and chased with unlabeled uridine and cytidine in the presence or absence of Ang II or PDGF-BB. The effective chase was started 2 h after addition of cold nucleosides (zero time point). At different times, total [3H]-labeled RNA was isolated and hybridized to Kip1 cDNA immobilized onto nitrocellulose filters. Data points are the mean of two separate experiments. For each method, the values are expressed as percentage of time zero. The solid lines represent the least-squares fit of the data obtained by linear regression analysis.

The ability of TGF-β to induce G1 arrest in Mv1Lu cells. Fig. 10A shows that addition of 10 pM TGF-β1 to Mv1Lu cells is sufficient to inhibit DNA synthesis by 90%. This inhibitory activity of TGF-β1 is reversed by coinubcation with TNA, but not with normal rabbit IgG. However, conditioned medium from Ang II–treated aortic SMC did not inhibit Mv1Lu cells DNA synthesis, but rather had a significant stimulatory effect (Fig. 10B). We also tested the effect of TNA on the ability of Ang II to stimulate DNA synthesis in aortic SMC. Coincubation of Ang II with normal rabbit IgG or TNA had essentially no effect on the rate of DNA synthesis (Fig. 10C). Finally, we examined the effect of simultaneous exposure of aortic SMC to both PDGF-BB and Ang II. Simultaneous addition of Ang II or pretreatment with Ang II (data not shown) did not interfere with PDGF-BB–induced DNA synthesis (Fig. 10C) or p27Kip1 downregulation (Fig. 10D), consistent with the idea that Ang II does not stimulate the synthesis of an antimitogenic factor. Together, these results demonstrate that the failure of Ang II to promote S phase entry of aortic SMC is not due to autocrine production of TGF-β.

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 Ang II induce cellular hypertrophy as a result of increased protein synthesis (Gleistfer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Grainger et al., 1994; Glasson and Meloche, 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 p27Kip1 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 Ang II, leads to a progressive and dramatic decline in the level of p27Kip1 protein. The failure of Ang II to downregulate p27Kip1 results in the increased association of the inhibitor with cyclin E–Cdk2 complexes and correlates with inhibition of threonine 160 phosphorylation of Cdk2. Since Cdk is constitutively active in aortic SMC, the simplest interpretation of our data is that stoichiometric binding of p27Kip1 to cyclin E–Cdk2 complexes prevents Cdk from phosphorylating and activating Cdk2 in Ang II–treated cells. In support of this hypothesis, in vitro studies have shown that p27Kip1 binding to preformed cyclin E–Cdk2 complexes blocks Cdk-mediated threonine 160 phosphorylation of the enzyme (Polyak et al., 1994a; Aprelikova et al., 1995). Second, we further show that extracts from Ang 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 p27Kip1 confirmed that p27Kip1 is the major inhibitory factor present in these extracts.

Previous studies have shown that the abundance of...
p27<sup>Kip1</sup> is regulated by multiple posttranscriptional mechanisms. Our present results add another level of complexity by demonstrating that the levels of p27<sup>Kip1</sup> are also controlled by transcriptional mechanisms in vascular SMC. Our data support a model where the reduction of p27<sup>Kip1</sup> expression observed in response to mitogenic factors occurs by two mechanisms. The first mechanism is a rapid decrease in the rate of p27<sup>Kip1</sup> synthesis that becomes minimal by two hours and slowly returns to quiescent value after ~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<sup>Kip1</sup> 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<sup>Kip1</sup> synthesis is tightly paralleled by a transient downregulation of <i>Kip1</i> 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<sup>Kip1</sup>. 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<sup>Kip1</sup> 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<sup>Kip1</sup> synthesis. Figs. 6 C and 7 show that the rate of p27<sup>Kip1</sup> 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 G<sub>1</sub> progression because of the binding of mRNA masking proteins (Spirin, 1996). In support of this idea, it was found that the accumulation of p27<sup>Kip1</sup> 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<sup>Kip1</sup> 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...
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 SM C. However, significant differences are noted in the time course of these events. For example, PDGF-BB induces a sustained activation of the MAP kinases ERK 1/ERK 2, 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 SM C (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 SM C have shown that PDGF-BB exclusively stimulates an increase in phosphatidylinositol 3,4,5-trisphosphate (Button et al., 1994), whereas only thornbin, which behaves as a hypertrophic factor, induces fosB mRNA levels (Rothman et al., 1994). However, these observations may not be generalized to other SM C types, since both PDGF-BB and Ang II activate PI3-kinase and induce fosB mRNA in rat aortic SM C (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 SM C. 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; A grawal et al., 1996; this study). We also found that in vascular SM C 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 (A ktas et al., 1997; Takuwa and Takuwa, 1997; K awada et al., 1997) and that RhoA is a necessary mediator of p27Kip1 degradation (Weber et al., 1997).

It has been postulated that the failure of Ang II to stimulate vascular SM C hyperplasia is due to autocrine production of the antimitogenic cytokine TGF-β1 by these cells (Gibbons et al., 1992; K obuchi 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 SM C. 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 SM C 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 SM C accumulation after angioplasty in the rat carotid artery (A be et al., 1994; M orishita et al., 1994). In addition, Cdk2 expression is temporally correlated with vascular SM C proliferation after angioplasty (Wei et al., 1997). More recently, 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 attenuates neointimal lesion formation. A recent study also presented evidence that polymerized collagen inhibits aortic SM C proliferation in vitro through α2 integrin-mediated upregulation of p27Kip1 (K oyama et al., 1996). Thus, the results presented here, together with these findings, clearly identify p27Kip1 as an important regulator of vascular SM C growth response.

We thank Drs. Jacques Pouysségur, Tomi Mäkelä, Maureen O’Connor, and Joan Massagué for valuable reagents, and Drs. Gilles L’Alem and Benoit Chabot for helpful discussions.

M. J. Servant and B. Turgeon are recipients of a studentship from the Heart and Stroke Foundation of Canada. P. Coulombe holds a studentship from the National Research Council of Canada. S. Meloche is a Scientist of the Medical Research Council of Canada. This work was supported by a grant from the Medical Research Council of Canada (M-T-34168).

Submitted: 19 Aug 1999
Revised: 23 Dec 1999
Accepted: 29 Dec 1999

References


The Journal of Cell Biology, Volume 148, 2000 554