

Differential Regulation of p27^{Kip1} 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 threo-

nine and to downregulate p27^{Kip1} expression. By contrast, exposure to PDGF-BB resulted in a progressive and dramatic reduction in the level of p27^{Kip1} protein. The time course of p27^{Kip1} decline was correlated with a reduced rate of synthesis and an increased rate of degradation of the protein. Importantly, the repression of p27^{Kip1} 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- β 1 by aortic SMC. These results identify p27^{Kip1} 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 con-

certed action of cyclin-dependent kinases (Cdks)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-

Philippe Coulombe and Benjamin Turgeon contributed equally to this work.

Address correspondence to Dr. Sylvain Meloche, Research Centre, Centre hospitalier de l'Université de Montréal (CHUM), Hôtel-Dieu Campus, 3850 St. Urbain Street, Montreal, Quebec, H2W 1T8 Canada. Tel.: (514) 843-2733. Fax: (514) 843-2715. E-mail: meloches@ere.umontreal.ca

¹*Abbreviations used in this paper:* Ang II, angiotensin II; CAK, Cdk-activating kinase; Cdk, cyclin-dependent kinase; DRB, 5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole; GAPDH, 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- β 1, transforming growth factor- β 1; TNA, TGF- β 1 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 Heuvel and Harlow, 1993; Knoblich et al., 1994; Ohtsubo et al., 1995; Krude 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 genes families (Sherr and Roberts, 1995). The Ink4 family of proteins, which includes p16^{Ink4A}, p15^{Ink4B}, p18^{Ink4C}, and p19^{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^{Cip1/Waf1} (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993; Noda et al., 1994), p27^{Kip1} (Polyak et al., 1994a; Toyoshima and Hunter, 1994), and p57^{Kip2} (Lee et al., 1995; Matsuo 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 Cdks by stoichiometric binding to the cyclin-Cdk complex or by physically blocking the phosphorylation of the Cdk subunit by CAK (Sherr and Roberts, 1995). Among them, p27^{Kip1} was first identified in transforming growth factor β (TGF- β)-treated cells (Polyak et al., 1994b; Slingerland et al., 1994). The expression of p27^{Kip1} is increased in serum-starved or density-arrested cells (Firpo et al., 1994; Kato et al., 1994; Nourse et al., 1994) and in cells exposed to antiproliferative signals like TGF- β , rapamycin (Nourse et al., 1994), and cAMP (Kato et al., 1994; L'Allemain et al., 1997). In contrast, the level of p27^{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^{Kip1} may play an essential role in connecting mitogenic signaling pathways to cell cycle activation. Ectopic expression of p27^{Kip1} causes cell cycle arrest in G₁ phase (Polyak et al., 1994a; Toyoshima and Hunter, 1994) and, conversely, antisense inhibition of p27^{Kip1} expression suppresses quiescence in fibroblasts (Coats et al., 1996; Rivard 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- 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; Heldin, 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 Meloche, 1995). On the other hand, Ang II was reported to exert weak mitogenic effects on SMC of resistance arteries (Dubey et al., 1992) and on aortic SMC isolated from spontaneously hypertensive rats (Bunkenburg 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 (Daemen 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 antiproliferative (TGF- β 1) signals in vascular SMC (Gibbons et al., 1992; Koibuchi et al., 1993). According to this model, the autocrine production of TGF- β 1 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 AT₁ 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 ERK1/ERK2 (Duff et al., 1992; Tsuda et al., 1992; Servant et al., 1996) and p70 S6 kinase (Giasson and Meloche, 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^{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^{Kip1} expression. We also show that p27^{Kip1} abundance is regulated by

multiple transcriptional and posttranscriptional mechanisms in vascular SMC.

Materials and Methods

Cell Culture

Rat aortic SMC were cultured to 80% confluence and synchronized in the quiescent state as described previously (Giasson and Meloche, 1995). The cells were stimulated with 100 nM Ang II (Hukabel Scientific) or 50 ng/ml PDGF-BB (Oncogene Science) for the indicated times at 37°C. Mv1Lu mink lung epithelial cells (obtained from Dr. Maureen O'Connor, Biotechnology 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 SMC in 6-well plates were stimulated with Ang II or PDGF-BB for 72 h in serum-free medium containing 0.5 μ Ci/ml [3 H]leucine. For DNA synthesis measurements, quiescent aortic SMC in 35-mm petri dishes were stimulated for the indicated times with Ang II or PDGF-BB and pulse-labeled with 2 μ Ci/ml [3 H]thymidine for the last 2–4 h. After 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 SMC in 6-well plates were stimulated with Ang 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^{-4} M phenylmethylsulfonyl fluoride, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, 1% Triton X-100) for 30 min at 4°C. Lysates were clarified by centrifugation at 13,000 *g* for 10 min and equal amounts of lysate proteins (30–85 μ g) were subjected to electrophoresis on 12 or 15% acrylamide gels. Proteins were electrophoretically transferred to Hybond-C nitrocellulose membranes (Nycomed Amersham, Inc.) in 25 mM Tris, 192 mM glycine, and fixed for 10 min in methanol/acetic acid/glycerol (40:7:3). 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 2 μ g/ml of mAb to cyclin D1 (DCS-6), cyclin D2 (DCS-3.1), or cyclin D3 (DCS-22; NeoMarkers), or 1 μ g/ml of polyclonal antibody to cyclin E (SC-481), Cdk2 (SC-163), Cdk4 (SC-260), or p27^{Kip1} (SC-528; Santa Cruz Biotechnology) in blocking solution. After washing four times in TBS, 0.1% Tween 20, the membranes were incubated for 1 h with HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:10,000) in blocking solution. Immunoreactive bands were visualized by enhanced chemiluminescence (Nycomed Amersham, Inc.).

For coprecipitation studies, total lysate proteins (200–500 μ g) were incubated for 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 phosphotransferase activity of Cdk2 was measured by immune complex kinase assay using histone H1 as substrate as described previously (Meloche, 1995). In brief, lysate proteins (200 μ g) were subjected to immunoprecipitation with 1 μ g 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 assay 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 μ l of kinase assay buffer containing 0.25 mg/ml histone H1 (Boehringer Mannheim Corp.), 100 μ M ATP, and 10 μ Ci [γ - 32 P]ATP. The reactions were initiated by the addition of ATP, incubated at 30°C for

5 min, and stopped by addition of 2 \times 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-BB-stimulated cells containing active Cdk2 were mixed with boiled (5 min at 100°C) extracts of Ang II-stimulated cells (1:1 ratio; 200 μ g protein of each lysate) for 1.5 h at 4°C before immunoprecipitation of Cdk2 and kinase assay. Immunodepletion of p27^{Kip1} was performed by incubating 200 μ g of Ang II-treated cell extract with 5 μ g of anti-p27^{Kip1} antibody for 2 h at 4°C. The resulting supernatant was then used for the inhibition experiment. Specificity of p27^{Kip1} immunodepletion was assessed by preincubating the anti-p27^{Kip1} antibody with excess immunogenic peptide (50 μ g of SC-528P; Santa-Cruz Biotechnology) for 2 h at 4°C before incubation with Ang II-treated cell extract.

Cdk4 enzymatic assays were performed as described (Matsushime et al., 1994) with some modifications. After stimulation, the cells were washed twice with ice-cold PBS and lysed in Tween 20 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM EGTA, 20 mM β -glycerophosphate, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10^{-4} M phenylmethylsulfonyl fluoride, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, and 0.1% Tween 20). The cells were scraped from the plates and sonicated at 4°C (once for 10 s). Cellular lysates were clarified by centrifugation and 150 μ g of lysate proteins were precleared for 1 h with 5 μ l of normal rabbit serum and then incubated for 3 h at 4°C with 1 μ g of anti-Cdk4 antibody preadsorbed to protein A-Sepharose beads. The immune complexes were washed twice with Tween 20 lysis buffer and twice with kinase assay buffer (50 mM Hepes, pH 7.4, 10 mM MgCl₂, 2.5 mM EGTA, 1 mM dithiothreitol, 10 mM β -glycerophosphate, 1 mM sodium fluoride, and 0.1 mM sodium orthovanadate). pRb kinase activity was assayed by resuspending the beads in a total volume of 40 μ l of kinase assay buffer containing 1 μ g glutathione S-transferase (GST)-pRb protein (amino acids 792–928), 0.2 mg/ml BSA, 20 μ M ATP, and 10 μ Ci [γ - 32 P]ATP. The reactions were incubated at 30°C for 30 min and stopped by addition of 2 \times denaturing sample buffer. The samples were resolved by SDS-gel electrophoresis and the radioactivity incorporated into GST-pRb was counted.

The enzymatic activity of CAK was measured essentially as described (Musgrove et al., 1998). Cellular extracts (400 μ g protein) prepared in CAK lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 10% glycerol, 10 mM β -glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10^{-4} M phenylmethylsulfonyl fluoride, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, and 0.1% Tween 20) were precleared as described above and then incubated for 3.5 h at 4°C with 5 μ g of anti-Cdk7 antibody (06-377; Upstate Biotechnology) preadsorbed to protein A-Sepharose beads. The immune complexes were washed twice with lysis buffer and twice with kinase assay buffer (50 mM Hepes, pH 7.5, 30 mM MgCl₂, and 1 mM dithiothreitol). CAK activity was assayed by resuspending the beads in 40 μ l of kinase assay buffer containing 5 μ g of GST-Cdk2K33M, 90 μ M ATP, and 10 μ Ci [γ - 32 P]ATP. The reactions were incubated for 20 min at 30°C and stopped by addition of 2 \times denaturing sample buffer. The samples were analyzed by SDS-gel electrophoresis and the bands corresponding to GST-Cdk2K33M were excised and counted. No CAK kinase activity was detected in samples subjected to immunoprecipitation with beads alone.

The recombinant GST fusion proteins of pRb and Cdk2K33M were expressed in *Escherichia coli* by transformation with plasmids pGEX-Rb and pGEX-Cdk2K33M (obtained from Drs. Jacques Pouyssegur, Centre de Biochimie-CNRS, Nice, France, and Tomi P. Mäkelä, University of Helsinki, Helsinki, Finland, respectively) and purified as described (Matsushime et al., 1994).

Phosphorous 32 Labeling and Immunoprecipitation

Quiescent aortic SMC in 100-mm petri dishes were stimulated for 10 or 20 h with Ang II or PDGF-BB and labeled for the last 5 h in bicarbonate- and phosphate-free Hepes-buffered MEM containing 0.5 mCi/ml [32 P]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 μ l 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 unlabeled 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^{Kip1} protein, quiescent aortic SMC in 100-mm petri dishes were pulse-labeled for 1 h with 166 μ Ci/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 μ g proteins) were pre-cleared for 1 h with 5 μ l of normal rabbit serum and the resulting supernatants were incubated with protein A-Sepharose beads preadsorbed with 2 μ g of anti-p27^{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 electrophoresis on 12% acrylamide gels. The p27^{Kip1} 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 μ Ci/ml of [³⁵S]methionine and [³⁵S]cysteine. Labeling was allowed to proceed for the last 20 min. Cell lysis and immunoprecipitation of p27^{Kip1} 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 μ g) were denatured and resolved by electrophoresis in a 1% agarose gel containing 1.8% formaldehyde. The RNA was transferred to Hybond-N membranes (Nycomed Amersham, Inc.), fixed, and hybridized with ³²P-labeled probes. Hybridization was carried out in hybridization medium (5 \times SSC [1 \times SSC = 150 mM NaCl, 15 mM sodium citrate], 0.1% SDS, 5 \times Denhardt's solution [1 \times Denhardt's = 0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, and 0.02% BSA], 50% formamide, and 100 μ g/ml herring sperm DNA) containing the labeled probe (1–2 \times 10⁶ cpm/ml) for 16 h at 42°C. The membranes were washed twice at 25°C for 15 min in 2 \times SSC, 0.1% SDS, and twice at 60°C for 30 min in 0.5 \times SSC, 0.1% SDS. The extent of hybridization was analyzed with a PhosphorImager apparatus. The results were normalized to 18S ribosomal RNA.

The probes used were: 1.5-kb EcoRI fragment of human p27^{Kip1} cDNA (provided by Dr. Joan Massagué, Memorial Sloan-Kettering Cancer Center, NY) and a DNA oligonucleotide derived from the rat 18S ribosomal RNA sequence.

Nuclear Run-On Transcription Assays

Nuclei were prepared as described by Greenberg and Bender (1997). Vascular SMC were washed twice with ice-cold PBS and scraped from plates in PBS, 1 mM EDTA. Cell pellets were collected by centrifugation and resuspended in cold lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM CaCl₂, 2 mM MgCl₂, 1% NP-40). The cells were then disrupted in a Dounce homogenizer and the nuclei were sedimented at 500 *g* for 5 min. The nuclei were resuspended in 50 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA, and 40% glycerol and frozen in liquid nitrogen. For run-on transcription reactions, thawed nuclei (8 \times 10⁷) were resuspended in 400 μ l of reaction buffer containing 5 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 150 mM KCl, 5 mM dithiothreitol, 20 U/ml RNA guard (Pharmacia Biotech), 1 mM each of ATP, GTP, and CTP, and 600 μ Ci of α -³²P]UTP (Nycomed Amersham, Inc.; 800 Ci/mmol) and incubated at 37°C for 30 min. Transcription was stopped by the addition of 40 μ g DNase I in 1 ml of HSB buffer and incubated for 5 min at 30°C. Then, 10 μ l of 20 μ g/ml proteinase K in 0.5 M Tris-HCl, pH 7.4, 125 mM EDTA, and 5% SDS was added to the reaction mixture, followed by incubation for 30 min at 42°C. The ³²P-labeled RNA was extracted with phenol/chloroform and unincorporated nucleotides were removed by chromatography through a Sephadex G-50 (Pharmacia Biotech) column. Each plasmid DNA gene insert was denatured and immobilized to nitrocellulose membranes using a dot-blot

apparatus. The membranes were hybridized with ³²P-labeled RNA in 5 \times SSC, 5 \times Denhardt's, 50% formamide, 4 mM EDTA, 0.5 mg/ml salmon sperm DNA, and 0.25 mg/ml yeast tRNA at 55°C for 24 h. The membranes were washed extensively at 60°C in 0.5 \times SSC, 0.1% SDS. The extent of hybridization was analyzed with a PhosphorImager apparatus.

[³H]Uridine Pulse-Chase Experiments

Quiescent aortic SMC were pretreated for 2 h with 20 mM glucosamine (Sigma Chemical Co.) to deplete the UTP pool, washed, and pulse-labeled with 100 μ Ci/ml [³H]uridine (26 Ci/mmol; Nycomed Amersham, Inc.) for 12 h. The unincorporated [³H]uridine-containing medium was removed and the cells were incubated for an additional 2 h in serum-free medium containing 20 mM glucosamine, 5 mM uridine, and 5 mM cytidine. The chase was then continued for 4 h in the same medium containing Ang II or PDGF-BB. At various intervals, the cells were washed with PBS and total RNA was isolated as described above. Equivalent amounts of ³H-labeled RNA were hybridized to 5 μ g of linearized plasmid DNA immobilized onto a nitrocellulose membrane. Hybridization was performed at 45°C for 4 d, as described in the previous section. The membranes were washed extensively at 55°C in 0.5 \times SSC, 0.1% SDS. After drying, the radioactivity of each spot was determined by liquid scintillation counting.

TGF- β 1 Bioassay

TGF- β 1 bioassay was conducted essentially as described previously (Gibbons et al., 1992). Recombinant TGF- β 1 and TGF- β 1 neutralizing antibody (TNA) was a generous gift from Dr. Maureen O'Connor. In brief, Mv1Lu cells were plated at a density of 5 \times 10⁵ cells per well in 24-well plates. After 6 h of serum exposure, the cells were washed with serum-free medium, then incubated with conditioned medium or TGF- β 1 in serum-free medium for 24 h. The rate of DNA synthesis was measured by pulse-labeling cells with 2 μ Ci/ml [³H]thymidine during the last 6 h of incubation. For each experiment, a standard curve was constructed with increasing concentrations of recombinant TGF- β 1. Ang II conditioned medium was obtained from aortic SMC 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 μ g/ml, which completely blocks the growth inhibitory effect of TGF- β 1 in Mv1Lu cells.

Results

PDGF-BB, but Not Ang II, Induces DNA Synthesis in Aortic SMC

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 SMC. As previously reported (Geisterfer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Giasson and Meloche, 1995), treatment of aortic SMC 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 SMC number and by the small ratio between [³H]leucine incorporation and cell number (Fig. 1 B). Ang II did not promote cell division, but caused cellular hypertrophy by increasing 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 Ang II-stimulated cells, but rather, that cells are arrested in G₁ phase. Thus, PDGF-BB is a strong mitogenic factor for rat aortic SMC, promoting DNA synthesis and cellular division, whereas Ang II acts as a hypertrophic factor.

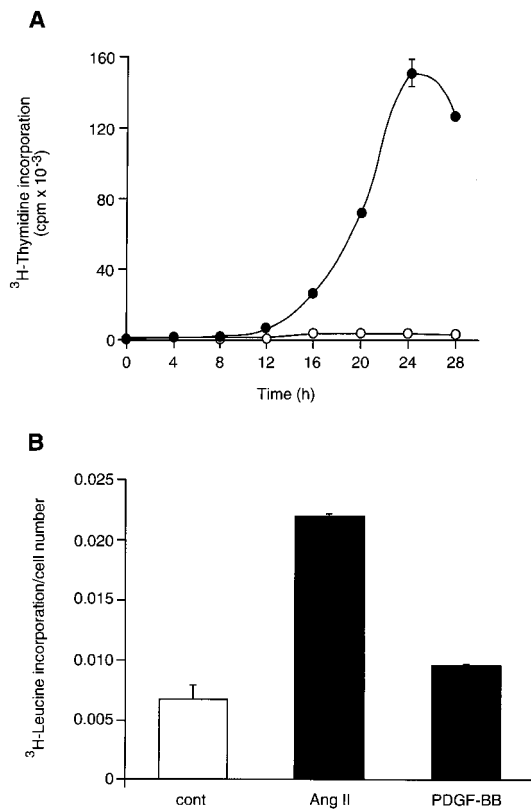


Figure 1. Comparative effects of Ang II and PDGF-BB on the growth of aortic SMC. **A**, DNA synthesis. Quiescent rat aortic SMC were stimulated for different periods of time with 100 nM Ang II (○) or 50 ng/ml PDGF-BB (●) and the rate of DNA synthesis was measured by [³H]thymidine incorporation. Each value represents the mean \pm 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 \pm SEM of triplicate determinations. Cell numbers for this experiment were: control (cont), $374,167 \pm 53,428$ cells; Ang II, $312,000 \pm 28,369$ cells; PDGF-BB, $917,500 \pm 42,120$ cells. The data are representative of at least three different experiments with similar results.

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. 2 A 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. 2 A). Ang II had little effect on cyclin E expression. It should also be noted that

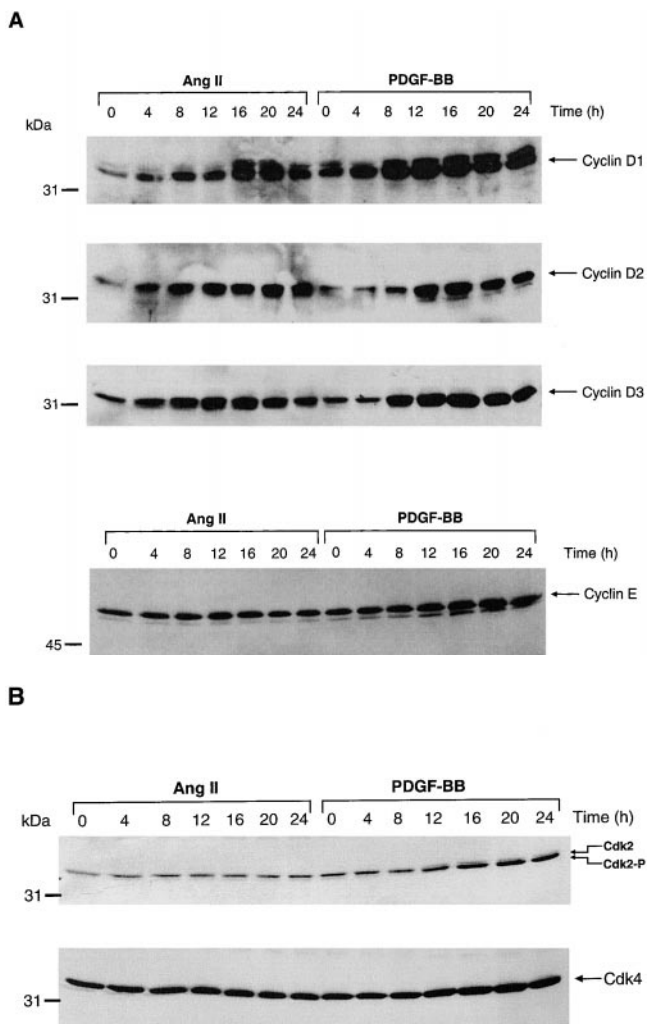


Figure 2. Effects of Ang II and PDGF-BB on the accumulation of G₁ cyclins and the expression level of Cdk2 in aortic SMC. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for different times over a 24-h period. Equal amounts of lysate proteins were resolved by SDS-gel electrophoresis 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.

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. 2 B). 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 in-

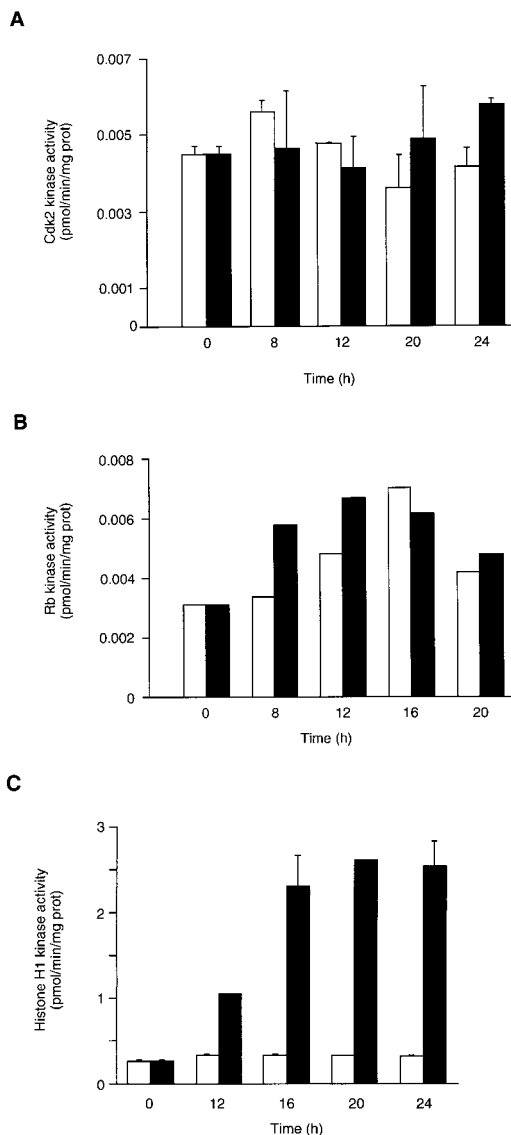


Figure 3. Regulation of CAK, Cdk4, and Cdk2 enzymatic activity by Ang II and PDGF-BB during G₁ progression. Quiescent aortic SMC were stimulated for different times with 100 nM Ang II (□) or 50 ng/ml PDGF-BB (■). Detergent lysates of the cells were prepared and equal amounts of proteins were subjected to immunoprecipitation with specific anti-Cdk7 (A), anti-Cdk4 (B), or anti-Cdk2 (C) antibodies. The phosphotransferase activity of the immunoprecipitates was assayed as described in Materials and Methods using GST-Cdk2K33M, GST-pRb, and histone H1 as substrates, respectively. The enzymatic activities are expressed as picomoles of phosphate incorporated into the substrate per min per mg of lysate protein. Similar results were obtained in three different experiments.

creased 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 (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. 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 p27^{Kip1} 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 p27^{Kip1} 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 p27^{Kip1} was a determinant factor in the differential regulation of Cdk2 activation by mitogenic and hypertrophic factors, we compared the levels of p27^{Kip1} protein expression. The expression of p27^{Kip1} protein was elevated in quiescent aortic SMC and decreased progressively upon

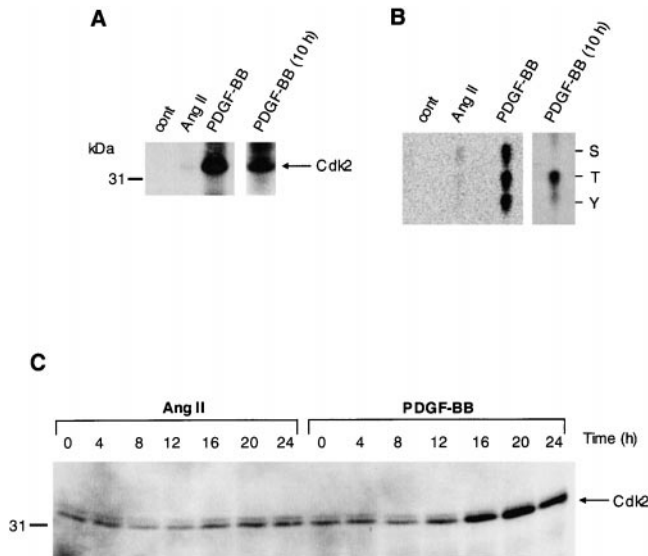


Figure 4. Ang II fails to stimulate the phosphorylation of Cdk2. **A**, Phosphorylation of Cdk2. Quiescent aortic SMC were stimulated for 20 h with 100 nM Ang II or for 10 and 20 h with 50 ng/ml PDGF-BB and labeled with [32 P]phosphoric acid during the last 5 h. Cell lysates were prepared and Cdk2 was immunoprecipitated using a specific antibody preadsorbed to protein A-Sepharose beads. The immunoprecipitated proteins were resolved by SDS-gel electrophoresis on 10% acrylamide gel, transferred to PVDF membrane, and analyzed by autoradiography. **B**, Phosphoamino acid analysis. The 32 P-labeled protein band corresponding to Cdk2 was excised from the PVDF membrane and subjected to partial acid hydrolysis. 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.

treatment of cells with the mitogenic factor PDGF-BB (Fig. 5 A). The decrease in p27^{Kip1} level was already evident four hours after PDGF-BB exposure. After 20 h of stimulation with PDGF-BB, the expression of p27^{Kip1} 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 for 20 h with PDGF-BB significantly reduced Cdk2-associated histone H1 kinase activity (Fig. 5 B). p27^{Kip1} 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. Indeed, the Cdk2 inhibitory activity present in Ang II-treated cells was completely eliminated after immunodepletion of p27^{Kip1} with a specific antibody (Fig. 5 B). Preincubation of the anti-p27^{Kip1} antibody with a saturating amount of immunogenic peptide completely restored the Cdk2 inhibitory activity, confirming that p27^{Kip1} is the major factor responsi-

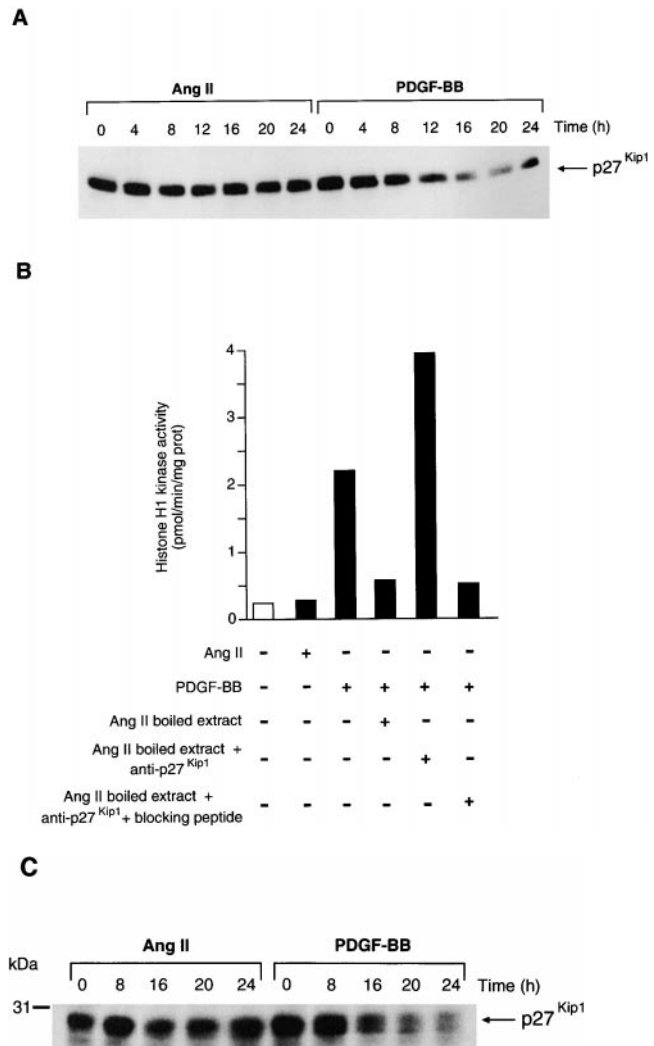


Figure 5. Differential modulation of p27^{Kip1} expression by Ang II and PDGF-BB. **A**, Expression of p27^{Kip1} protein. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for different times over a 24-h period. Equal amounts of lysate proteins were resolved by SDS-gel electrophoresis on 12% acrylamide gel and transferred to nitrocellulose membrane. The membrane was probed with anti-p27^{Kip1} antibody and the proteins visualized by chemiluminescence detection. **B**, Inhibitory activity of Ang II extracts. Quiescent aortic SMC were stimulated for 20 h with 100 nM Ang II or 50 ng/ml PDGF-BB. Extracts from PDGF-BB-treated cells, which contain active Cdk2, were prepared and mixed with boiled extracts from Ang II-stimulated cells as described in Materials and Methods. After incubation for 90 min at 4°C, Cdk2 was immunoprecipitated and histone H1 kinase activity was determined. p27^{Kip1} was immunodepleted from boiled extracts by incubation for 1 h at 4°C with anti-p27^{Kip1} antibody. Specificity of p27^{Kip1} immunodepletion was confirmed by preincubation of the antibody with excess immunogenic (blocking) peptide. The enzymatic activities are expressed as picomoles of phosphate incorporated into histone H1 per min per mg of lysate protein. **C**, Decreased association of p27^{Kip1} with cyclin E-Cdk2 complexes in PDGF-BB-treated cells. Detergent lysates of aortic SMC stimulated for the indicated times with 100 nM Ang II or 50 ng/ml PDGF-BB were subjected to immunoprecipitation with anticyclin E antibody and analyzed by immunoblotting with antibody to p27^{Kip1}. Similar results were obtained in three different experiments.

ble for this activity. Addition of boiled extracts from PDGF-BB-stimulated cells, which contain very low levels of p27^{Kip1} (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 p27^{Kip1} 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 p27^{Kip1} 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 p27^{Kip1} 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 p27^{Kip1} 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 p27^{Kip1} are regulated by vascular growth factors. Studies in other cell systems have shown that the abundance of p27^{Kip1} 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 p27^{Kip1} turnover, pulse-chase experiments were conducted on aortic SMC treated with Ang II or PDGF-BB. The rate of degradation of p27^{Kip1} 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 p27^{Kip1} was also affected by treatment with vascular growth factors. As shown in Fig. 6 C, the synthesis of p27^{Kip1} 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 p27^{Kip1} 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 p27^{Kip1} 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 p27^{Kip1} synthesis seen after PDGF-BB and Ang II treatment (Fig. 6 C). This suggests that repression of p27^{Kip1} synthesis by vascular growth factors is likely attributable, at least in part, to a corresponding decrease of *Kip1* mRNA abundance.

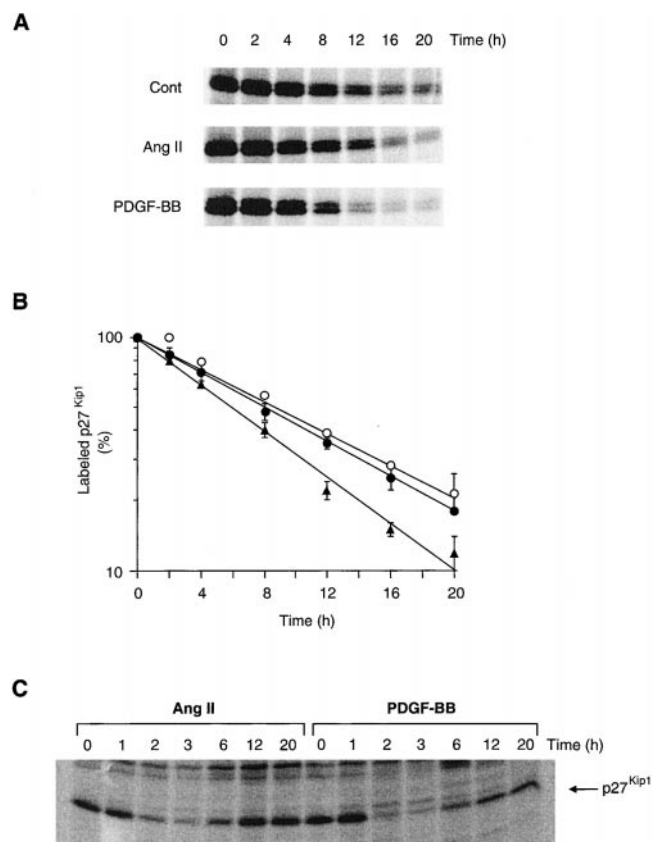


Figure 6. Downregulation of p27^{Kip1} expression is associated with increased turnover of the protein and repression of its synthesis. **A**, Turnover of p27^{Kip1} protein. Quiescent aortic SMC were pulse-labeled with [³⁵S]methionine/[³⁵S]cysteine for 60 min, after which the medium was changed with fresh medium alone or containing 100 nM Ang II or 50 ng/ml PDGF-BB. At different times, cell extracts were prepared and subjected to immunoprecipitation with anti-p27^{Kip1} antibody. After extensive washing, the immunoprecipitated proteins were separated by electrophoresis on 12% acrylamide gel and analyzed by fluorography using a PhosphorImager. **B**, Densitometric analysis of the data shown in **A**. ○, Unstimulated cells ($r = 0.958$); ●, Ang II-treated cells ($r = 0.969$); ▲, PDGF-BB-treated cells ($r = 0.950$). The data points represent mean \pm SEM of four separate experiments. **C**, Synthesis of p27^{Kip1}. Quiescent aortic SMC were stimulated with Ang II or PDGF-BB for the times indicated and metabolically labeled with [³⁵S]methionine/[³⁵S]cysteine during the last 20 min of incubation. Preparation of cell extracts and immunoprecipitation were conducted as above. Similar results were obtained in four separate experiments.

There was a tight temporal relationship between the decrease in the rate of p27^{Kip1} synthesis observed at two hours after stimulation, the increased rate of degradation of the protein, and the change in the total amount of p27^{Kip1}. If we consider that the rate of p27^{Kip1} 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 p27^{Kip1} 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.

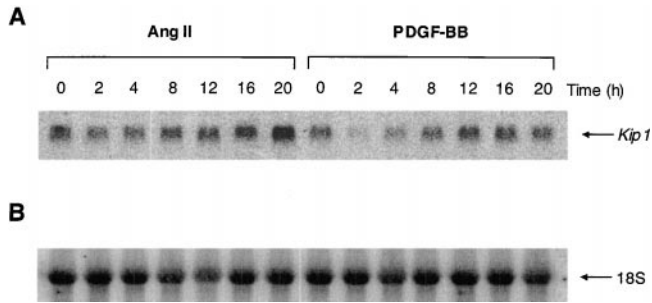


Figure 7. PDGF-BB produces a rapid and transient decrease of *Kip1* mRNA accumulation in aortic SMC. Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for the indicated times. Total cellular RNA was extracted from the cells and analyzed by Northern hybridization using a ^{32}P -labeled *Kip1* probe (A). The results were normalized by rehybridization of the blot with a 18S ribosomal RNA oligonucleotide probe (B). Similar results were obtained in five different experiments.

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 ($\sim 90\%$ 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).

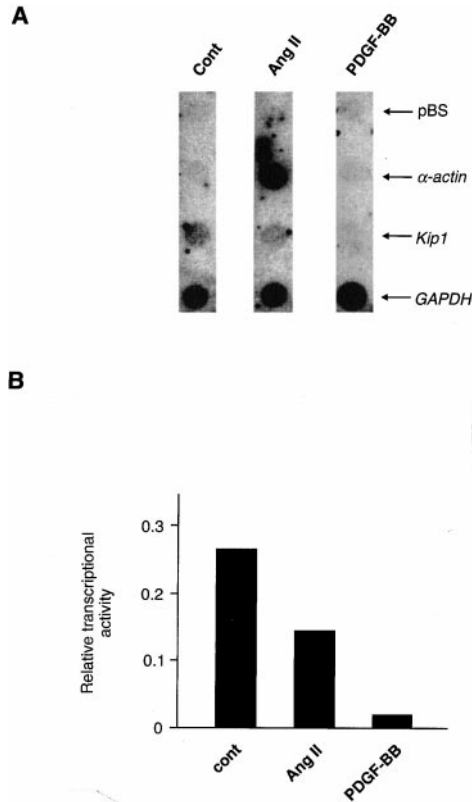


Figure 8. PDGF-BB reduces transcription of *Kip1* gene in aortic SMC. A, Quiescent aortic SMC were stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for 2 h. Nuclei were isolated and the ^{32}P -labeled nascent transcripts were hybridized to the indicated plasmid DNAs as described in Materials and Methods. The plasmid DNA inserts used in this experiment were pBluescript-SK vector (pBS), smooth muscle α -actin (α -actin), *Kip1*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). B, Densitometric analysis of the data shown in A expressed as the ratio *Kip1*/*GAPDH*. Similar results were obtained in two different experiments.

The Failure of Ang II to Promote S Phase Entry of Aortic SMC Is Not Explained by Autocrine Production of TGF- β 1

TGF- β 1 is the prototype of a family of growth factors that play important roles in cellular growth, differentiation, and morphogenesis (Massague, 1990). In particular, TGF- β 1 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- β 1 inhibits proliferation and induces cell cycle arrest in G_1 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- β 1, signals (Gibbons et al., 1992). Therefore, we carried out a series of experiments to test the possibility that autocrine production of TGF- β 1 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- β 1, we used a highly sensitive bioassay that is based on the

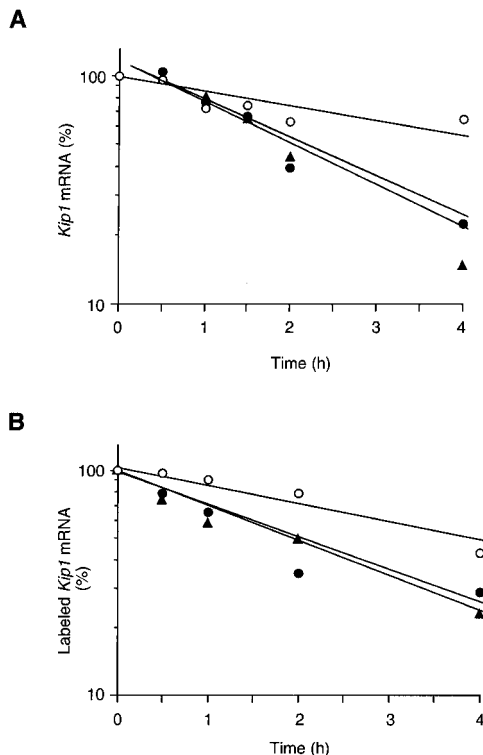


Figure 9. Effect of Ang II and PDGF-BB on the stability of *Kip1* mRNA. **A**, DRB method. Quiescent aortic SMC were either left untreated or stimulated with 100 nM Ang II or 50 ng/ml PDGF-BB for the indicated times in the presence of 25 μ g/ml DRB. Total cellular RNA was extracted from the cells and analyzed by Northern hybridization using a 32 P-labeled *Kip1* probe. The hybridization signals were quantified using a PhosphorImager apparatus. \circ , Unstimulated cells; \bullet , Ang II-treated cells; \blacktriangle , PDGF-BB-treated cells. Data points represent the mean of three separate experiments. **B**, Pulse-chase method. Quiescent aortic SMC were pulse-labeled with [3 H]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 3 H-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.

ability of TGF- β to induce G_1 arrest in Mv1Lu cells. Fig. 10 A shows that addition of 10 pM TGF- β 1 to Mv1Lu cells is sufficient to inhibit DNA synthesis by 90%. This inhibitory activity of TGF- β 1 is reversed by coincubation 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. 10 B). 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. 10 C). 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 inter-

fere with PDGF-BB-induced DNA synthesis (Fig. 10 C) or p27^{Kip1} downregulation (Fig. 10 D), 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 1.

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 (Geisterfer et al., 1988; Berk et al., 1989; Chiu et al., 1991; Grainger et al., 1994; Giasson 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 p27^{Kip1} 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 p27^{Kip1} protein. The failure of Ang II to downregulate p27^{Kip1} results in the increased association of the inhibitor with cyclin E-Cdk2 complexes and correlates with inhibition of threonine 160 phosphorylation of Cdk2. Since CAK is constitutively active in aortic SMC, the simplest interpretation of our data is that stoichiometric binding of p27^{Kip1} to cyclin E-Cdk2 complexes prevents CAK from phosphorylating and activating Cdk2 in Ang II-treated cells. In support of this hypothesis, in vitro studies have shown that p27^{Kip1} binding to preformed cyclin E-Cdk2 complexes blocks CAK-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 \sim 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^{Kip1} confirmed that p27^{Kip1} is the major inhibitory factor present in these extracts.

Previous studies have shown that the abundance of

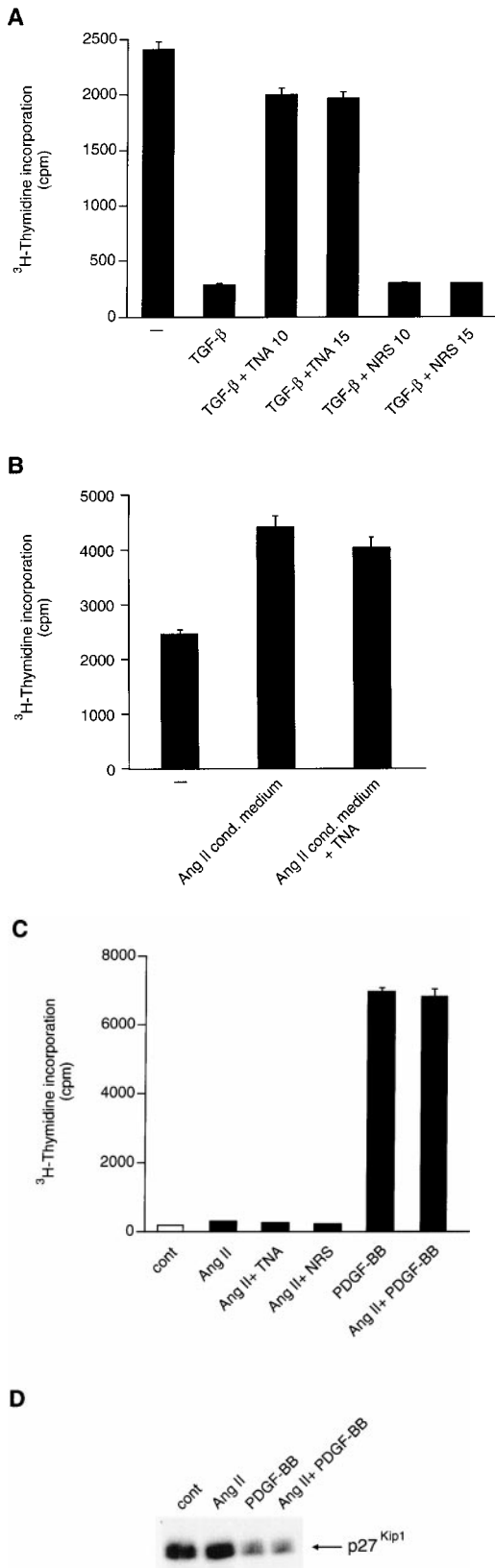


Figure 10. Ang II does not stimulate autocrine production of TGF-β1 in aortic SMC. **A**, TGF-β1 bioassay. Mv1Lu cells were treated for 24 h with 10 pM TGF-β1 in the absence or presence of 10–15 μg/ml of TNA or normal rabbit IgG (NRS). **B**, Conditioned medium from Ang II-stimulated aortic SMC (1:5 dilution)

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 ~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 μg/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 μg/ml TNA or 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 [³H]thymidine incorporation during the last 4–6 h of stimulation. Each value represents the mean ± 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 p27^{Kip1} 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 (Plevin 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., 1989; 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 p27^{Kip1} is also subject to regulation by mitogenic factors in vascular SMC. Given the recent demonstration that cyclin E-Cdk2 directly phosphorylates p27^{Kip1} 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 p27^{Kip1} 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 p27^{Kip1} 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 p27^{Kip1}. 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; Agrawal et al., 1996; this study). We also found that in vascular SMC and other cell types, p27^{Kip1} is significantly phosphorylated in G₀ and early G₁ phase (data not shown). These observations suggest that other protein kinases and/or mechanisms signal p27^{Kip1} for degradation. In this respect, it was reported that Ras signaling is required for downregulation of p27^{Kip1} in rodent fibroblasts (Aktas et al., 1997; Takuwa and Takuwa, 1997;

Kawada et al., 1997) and that RhoA is a necessary mediator of p27^{Kip1} degradation (Weber 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-β1 by these cells (Gibbons et al., 1992; Koibuchi 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). More recently, it was reported that p27^{Kip1} is markedly upregulated after balloon angioplasty in the rat carotid artery and that high levels of p27^{Kip1} expression correlates with downregulation of Cdk2 kinase activity (Chen et al. 1997). Ectopic overexpression of p27^{Kip1} 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 p27^{Kip1} (Koyama et al., 1996). Thus, the results presented here, together with these findings, clearly identify p27^{Kip1} as an important regulator of vascular SMC growth response.

We thank Drs. Jacques Pouyssegur, Tomi Mäkelä, Maureen O'Connor, and Joan Massagué for valuable reagents, and Drs. Gilles L'Allemain 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 (MT-14168).

Submitted: 19 August 1999

Revised: 23 December 1999

Accepted: 29 December 1999

References

- Abe, J., W. Zhou, J. Taguchi, N. Takuwa, K. Miki, H. Okasaki, K. Kurokawa, M. Kumada, and Y. Takuwa. 1994. Suppression of neointimal smooth muscle cell accumulation in vivo by antisense cdc2 and cdk2 oligonucleotides in rat carotid artery. *Biochem. Biophys. Res. Commun.* 198:16–24.
- Agrawal, D., P. Hauser, F. McPherson, F. Dong, A. Garcia, and W.J. Pledger. 1996. Repression of p27^{Kip1} synthesis by platelet-derived growth factor in BALB/c 3T3 cells. *Mol. Cell. Biol.* 16:4327–4336.
- Aktas, H., H. Cai, and G.M. Cooper. 1997. Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27^{Kip1}. *Mol. Cell. Biol.* 17:3850–3857.
- Aprelikova, O., Y. Xiong, and E.T. Liu. 1995. Both p16 and p21 families of cyclin-dependent kinase (CDK) inhibitors block the phosphorylation of cyclin-dependent kinases by the CDK-activating kinase. *J. Biol. Chem.* 270:18195–18197.
- Berk, B.C., V. Vekshtein, H.M. Gordon, and T. Tsuda. 1989. Angiotensin II-stimulated protein synthesis in cultured vascular smooth muscle cells. *Hypertension*. 13:305–314.
- Brinson, A.E., T. Harding, P.A. Diliberto, Y. He, X. Li, D. Hunter, B. Herman, H.S. Earp, and L.M. Graves. 1998. Regulation of a calcium-dependent tyrosine kinase in vascular smooth muscle cells by angiotensin II and platelet-derived growth factor. *J. Biol. Chem.* 273:1711–1718.

- Bunkenburg, B., T. van Amelsvoort, H. Rogg, and J.M. Wood. 1992. Receptor-mediated effects of angiotensin II on growth of vascular smooth muscle cells from spontaneously hypertensive rats. *Hypertension*. 20:746-754.
- Button, D., A. Rothman, C. Bongiorno, E. Kupperman, B. Wolner, and P. Taylor. 1994. Agonist-selective regulation of polyphosphoinositide metabolism in pulmonary artery smooth muscle cells. *J. Biol. Chem.* 269:6390-6398.
- Catt, K.J., K. Sandberg, and T. Balla. 1993. Angiotensin II receptor and signal transduction mechanisms. In *Cellular and Molecular Biology of the Renin-Angiotensin System*. M.K. Raizada, M.I. Phillips, and C. Summers, editors. CRC Press, Boca Raton, FL. 307-356.
- Chan, F.K., J. Zhang, L. Cheng, D.N. Shapiro, and A. Winoto. 1995. Identification of human and mouse p19, a novel Cdk4 and Cdk6 inhibitor with homology to p16ink4. *Mol. Cell. Biol.* 15:2682-2688.
- Chen, D., K. Krasinski, D. Chen, A. Sylvester, J. Chen, P.D. Nisen, and V. Andrés. 1997. Downregulation of cyclin-dependent kinase 2 activity in vascular smooth muscle cells by p27^{Kip1}, an inhibitor of neointima formation in the rat carotid artery. *J. Clin. Invest.* 99:2334-2341.
- Chiu, A.T., W.A. Roscoe, D.E. McCall, and P.B.M.W.M. Timmermans. 1991. Angiotensin II-1 receptors mediate both vasoconstrictor and hypertrophic responses in rat aortic smooth muscle cells. *Receptor*. 1:133-140.
- Chomczynski, P. 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*. 15: 532-537.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Claesson-Welsh, L. 1994. Platelet-derived growth factor receptor signals. *J. Biol. Chem.* 269:32023-32026.
- Coats, S., W.M. Flanagan, J. Nourse, and J.M. Roberts. 1996. Requirement for p27^{Kip1} for restriction point control of the fibroblast cell cycle. *Science*. 272: 877-880.
- Corjay, M.H., R.S. Blank, and G.K. Owens. 1990. Platelet-derived growth factor-induced destabilization of smooth muscle alpha-actin mRNA. *J. Cell. Physiol.* 145:391-397.
- Daemen, M.J., D.M. Lombardi, F.T. Bosman, and S.M. Schwartz. 1991. Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall. *Circ. Res.* 68:450-456.
- deBlois, D., M. Viswanathan, J.E. Su, A.W. Clowes, J.M. Saavedra, and S.M. Schwartz. 1996. Smooth muscle DNA replication in response to angiotensin II is regulated differently in the neointima and media at different times after balloon injury in the rat carotid artery. *Arterioscler. Thromb. Vasc. Biol.* 16: 1130-1137.
- Draetta, G.F. 1994. Mammalian G₁ cyclins. *Curr. Opin. Cell Biol.* 6:842-846.
- Dubey, R.K., A. Roy, and H.W. Overbeck. 1992. Cultured or renal arteriolar smooth muscle cells: mitogenic responses to angiotensin II. *Circ. Res.* 71: 1143-1152.
- Duff, J.L., B.C. Berk, and M.A. Corson. 1992. Angiotensin II stimulates the pp44 and pp42 mitogen-activated protein kinases in cultured rat aortic smooth cells. *Biochem. Biophys. Res. Commun.* 188:257-264.
- El-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell*. 75:817-825.
- Firpo, E.J., A. Koff, M.J. Solomon, and J.M. Roberts. 1994. Inactivation of a Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. *Mol. Cell. Biol.* 14:4889-4901.
- Geisterfer, A.A.T., M.J. Peach, and G.K. Owens. 1988. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ. Res.* 62:749-756.
- Giasson, E., and S. Meloche. 1995. Role of p70 S6 protein kinase in angiotensin II-induced protein synthesis in vascular smooth cells. *J. Biol. Chem.* 270: 5225-5231.
- Giasson, E., M.J. Servant, and S. Meloche. 1997. Cyclic AMP-mediated inhibition of angiotensin II-induced protein synthesis is associated with suppression of tyrosine phosphorylation signaling in vascular smooth muscle cells. *J. Biol. Chem.* 272:26879-26886.
- Gibbons, G.H., R.E. Pratt, and V.J. Dzau. 1992. Vascular smooth muscle cell hypertrophy vs. hyperplasia. *J. Clin. Invest.* 90:456-461.
- Grainger, D.J., C.M. Wittchell, P.L. Weissberg, and J.C. Metcalfe. 1994. Mitogens for adult rat aortic vascular smooth muscle cells in serum-free primary culture. *Cardiovasc. Res.* 28:1238-1242.
- Grana, X., and E.P. Reddy. 1995. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin dependent kinase inhibitors (CKIs). *Oncogene*. 11:211-219.
- Greenberg, M.E., and T.P. Bender. 1997. Identification of newly transcribed RNA. In *Current Protocols in Molecular Biology*. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, editors. John Wiley & Sons, Inc., New York. Suppl. 37:4.10.1-4.10.11.
- Gu, Y., J. Rosenblatt, and D.O. Morgan. 1992. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:3995-4005.
- Gu, Y., C.W. Turck, and D.O. Morgan. 1993. Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature*. 366:707-710.
- Guan, K.L., C.W. Jenkins, Y. Li, M.A. Nichols, X. Wu, C.L. O'Keefe, A.G. Matera, and Y. Xiong. 1994. Growth suppression by p18, a p16 ink4/MTS1- and p14 ink4B/MTS2-related Cdk6 inhibitor, correlates with wild-type pRb function. *Genes Dev.* 8:2939-2952.
- Hannon, G.J., and D. Beach. 1994. p15^{INK4B} is a potential effector of TGF- β induced cell cycle arrest. *Nature*. 371:257-261.
- Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G₁ cyclin-dependent kinases. *Cell*. 75:805-816.
- Harrold, S., G. Christine, B. Kobrin, S.L. Morisson, and C. Milcarek. 1991. A comparison of apparent mRNA half-life using kinetic labeling techniques vs decay following administration of transcriptional inhibitors. *Anal. Biochem.* 198:19-29.
- Hautmann, M.B., M.M. Thompson, E.A. Swartz, E.N. Olson, and G.K. Owens. 1997. Angiotensin II-induced stimulation of smooth muscle alpha-actin expression by serum response factor and the homeodomain transcription factor MHOX. *Circ. Res.* 81:600-610.
- Heldin, C.-H. 1997. Simultaneous induction of stimulatory and inhibitory signals by PDGF. *FEBS Lett.* 410:17-21.
- Hengst, L., and S.I. Reed. 1996. Translational control of p27^{Kip1} accumulation during the cell cycle. *Science*. 271:1861-1864.
- Hirai, H., M.F. Roussel, J.-Y. Kato, R.A. Ashmun, and C.J. Sherr. 1995. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol. Cell. Biol.* 15:2672-2681.
- Ishida, M., M.B. Marrero, B. Schieffer, T. Ishida, K.E. Bernstein, and B.C. Berk. 1995. Angiotensin II activates pp60c-src in vascular smooth muscle cells. *Circ. Res.* 77:1053-1059.
- Itazaki, K., M. Hara, N. Itoh, and M. Fujimoto. 1995. A long-term receptor stimulation is requisite for angiotensin II-dependent DNA synthesis in vascular smooth muscle cells from spontaneously hypertensive rats. *Eur. J. Pharmacol.* 291:417-425.
- Jelinek, T., and M.J. Weber. 1993. Optimization of the resolution of phosphoamino acids by one-dimensional thin-layer electrophoresis. *Biotechniques*. 15:629-630.
- Kamps, M.P. 1991. Determination of phosphoamino acid composition by acid hydrolysis of protein blotted to Immobilon. *Methods Enzymol.* 201:21-27.
- Kato, J., M. Matsuoka, K. Polyak, J. Massagué, and C.J. Sherr. 1994. Cyclic AMP-induced G₁ phase arrest mediated by an inhibitor (p27^{Kip1}) of cyclin-dependent kinase-4 activation. *Cell*. 79:487-496.
- Kawada, M., S. Yamagoe, Y. Murakami, K. Suzuki, S. Mizuno, and Y. Uehara. 1997. Induction of p27^{Kip1} degradation and anchorage independence by Ras through the MAP kinase signaling pathway. *Oncogene*. 15:629-637.
- Knoblich, J.A., K. Sauer, L. Jones, H. Richardson, R. Saint, and C.F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell*. 77:107-120.
- Koibuchi, Y., W.S. Lee, G.H. Gibbons, and R.E. Pratt. 1993. Role of transforming growth factor- β 1 in the cellular growth response to angiotensin II. *Hypertension*. 21:1046-1050.
- Koyama, H., E.W. Raines, K.E. Bornfeldt, J.M. Roberts, and R. Ross. 1996. Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of cdk2 inhibitors. *Cell*. 87:1069-1078.
- Krude, T., M. Jackman, J. Pines, and R.A. Laskey. 1997. Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. *Cell*. 88:109-119.
- Kwon, T.K., J.E. Nagel, M.A. Buchholz, and A.A. Nordin. 1996. Characterization of the murine cyclin-dependent kinase inhibitor gene p27^{Kip1}. *Gene*. 180: 113-120.
- L'Allemain, G., J.N. Lavoie, N. Rivard, V. Baldin, and J. Pouyssegur. 1997. Cyclin D1 expression is a major target of the cAMP-induced inhibition of cell cycle entry in fibroblasts. *Oncogene*. 14:1981-1990.
- Lavoie, J.N., G. L'Allemain, A. Brunet, R. Müller, and J. Pouyssegur. 1996. Cyclin D1 expression is regulated positively by the p42/p44mapk and negatively by the p38/HOGmapk pathway. *J. Biol. Chem.* 271:20608-20616.
- Leduc, I., P. Haddad, E. Giasson, and S. Meloche. 1995. Involvement of a tyrosine kinase pathway in the growth-promoting effects of angiotensin II on aortic smooth muscle cells. *Mol. Pharmacol.* 48:582-592.
- Lee, M.H., I. Reynisdottir, and J. Massagué. 1995. Cloning of p57^{KIP2}, a cyclin-dependent kinase inhibitor with unique domain structure and a tissue distribution. *Genes Dev.* 9:639-649.
- Liu, M., M.-H. Lee, M. Cohen, M. Bommakanti, and L.P. Freedman. 1996. Transcriptional activation of the Cdk inhibitor p21 by vitamin D₃ leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev.* 10:142-153.
- Marrero, M.B., B. Schieffer, W.G. Paxton, L. Heerdt, B.C. Berk, P. Lafontaine, and K.E. Bernstein. 1995. Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature*. 375:247-250.
- Massagué, J. 1990. The transforming growth factor-beta family. *Ann. Rev. Cell Biol.* 6:597-641.
- Matsuoka, S., M.C. Edwards, C. Bai, S. Parker, P. Zhang, A. Baldini, J.W. Harper, and S.J. Elledge. 1995. p57^{KIP2}, a structurally distinct member of the p21^{CIP1} Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev.* 9:650-662.
- Matsushime, H., D.E. Quelle, S.A. Shurtleff, M. Shibuya, C.J. Sherr, and J.-Y. Kato. 1994. D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* 14:2066-2076.
- Meloche, S. 1995. Cell cycle reentry of mammalian fibroblasts is accompanied by a sustained activation of p44mapk and p42mapk isoforms in the G₁ phase and their inactivation at the G₁/S transition. *J. Cell. Physiol.* 163:577-588.

- Millard, S.S., J.S. Yan, H. Nguyen, M. Pagano, H. Kiyokama, and A. Koff. 1997. Enhance ribosomal association of p27^{Kip1} mRNA is a mechanism contributing to accumulation during growth arrest. *J. Biol. Chem.* 272:7093–7098.
- Molloy, C.J., D.S. Taylor, and H. Weber. 1993. Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells. *J. Biol. Chem.* 268:7338–7345.
- Morgan, D.O. 1995. Principles of CDK regulation. *Nature*. 374:131–134.
- Morishita, R., G.H. Gibbons, K.E. Ellison, M. Nakajima, H. von der Leyen, L. Zhang, Y. Kaneda, T. Ogihara, and V.J. Dzau. 1994. Intimal hyperplasia after vascular injury is inhibited by antisense cdk2 kinase oligonucleotides. *J. Clin. Invest.* 93:1458–1464.
- Murray, A., and T. Hunt. 1993. The cell cycle, an introduction. W.H. Freeman and Company Press, New York.
- Musgrove, E.A., A. Swarbrick, C.S.L. Lee, A.L. Cornish, and R.L. Sutherland. 1998. Mechanisms of cyclin-dependent kinase inactivation by progestins. *Mol. Cell. Biol.* 18:1812–1825.
- Noda, A., Y. Ning, S.F. Venable, O.M. Pereira-Smith, and J.R. Smith. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* 211:90–98.
- Nourse, J., E. Firpo, W.M. Flanagan, S. Coats, K. Polyak, M.H. Lee, J. Massagué, G. Crabtree, and J.M. Roberts. 1994. Interleukin-2-mediated elimination of p27^{Kip1} cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature*. 372:570–573.
- Ohtsubo, M., A.M. Theodoras, J. Schumacher, J.M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G₁-to-S phase transition. *Mol. Cell. Biol.* 15:2612–2624.
- Owens, G.K. 1989. Control of hypertrophic versus hyperplastic growth vascular smooth muscle cells. *Am. J. Physiol.* 257:H1755–H1765.
- Owens, G.K. 1995. Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* 75:487–517.
- Owens, G.K., A.A.T. Geisterfer, Y.W.-H. Yang, and A. Komorika. 1988. Transforming growth factor- β -induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells. *J. Cell Biol.* 107:771–780.
- Pagano, M., S.W. Tam, A.M. Theodoras, P. Beer-Romero, G. Del Sal, V. Chau, P.R. Yew, G.F. Draetta, and M. Rolfe. 1995. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science*. 269:682–685.
- Pardee, A.B. 1989. G₁ events and regulation of cell proliferation. *Science*. 246: 603–608.
- Plevin, R., P.H. Scott, C.J.M. Robinson, and G.W. Gould. 1996. Efficacy of agonist-stimulated MEK activation determines the susceptibility of mitogen-activated protein (MAP) kinase to inhibition in rat aortic smooth muscle cells. *Biochem. J.* 318:657–663.
- Polte, T.R., A.J. Naftilan, and S.K. Hanks. 1994. Focal adhesion kinase is abundant in developing blood vessels and elevation of its phosphotyrosine content in vascular smooth muscle cells is a rapid response to angiotensin II. *J. Cell. Biochem.* 55:106–119.
- Polyak, K., M.H. Lee, H. Erdjument-Bromage, A. Koff, J.M. Roberts, P. Tempst, and J. Massagué. 1994a. Cloning of p27^{Kip1}, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell*. 78:59–66.
- Polyak, K., J. Kato, M.J. Solomon, C.J. Sherr, J. Massagué, J.M. Roberts, and A. Koff. 1994b. p27^{Kip1}, a cyclin-cdk inhibitor, links transforming growth factor- β and contact inhibition to cell cycle arrest. *Genes Dev.* 8:9–22.
- Raines, E.W., D.F. Bowen-Pope, and R. Ross. 1990. Peptide growth factors and their receptors. In *Handbook of Experimental Pharmacology*. M.B. Sporn, and A.B. Roberts, editors. Springer-Verlag Press, New York. 173–262.
- Reynisdottir, I., K. Polyak, A. Iavarone, and J. Massague. 1995. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF- β . *Genes Dev.* 9:1831–1845.
- Rivard, N., G. L'Allemain, J. Bartek, and J. Pouyssegur. 1996. Abrogation of p27^{Kip1} by cDNA antisense suppresses quiescence (G₀ state) in fibroblasts. *J. Biol. Chem.* 271:18337–18341.
- Roe, M.W., J.R. Hepler, T.K. Harden, and B. Herman. 1989. Platelet-derived growth factor and angiotensin II cause increases in cytosolic free calcium by different mechanisms in vascular smooth muscle cells. *J. Cell. Physiol.* 139: 100–108.
- Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*. 362:801–809.
- Rothman, A., B. Wolner, D. Button, and P. Taylor. 1994. Immediate-early gene expression in response to hypertrophic and proliferative stimuli in pulmonary arterial smooth muscle cells. *J. Biol. Chem.* 269:6399–6404.
- Russo, A.A., P.D. Jeffrey, A.K. Patten, J. Massagué, and N.P. Pavletich. 1996. Crystal structure of the p27^{Kip1} cyclin-dependent kinase inhibitor bound to the cyclin A-Cdk2 complex. *Nature*. 382:325–331.
- Saward, L., and P. Zahradka. 1997. Angiotensin II activates phosphatidylinositol 3-kinase in vascular smooth muscle cells. *Circ. Res.* 81:249–257.
- Schwartz, S.M., G.R. Campbell, and J.H. Campbell. 1986. Replication of smooth muscle cells in vascular disease. *Circ. Res.* 58:427–444.
- Sclafani, R.A. 1996. Cyclin dependent kinase activating kinases. *Curr. Opin. Cell Biol.* 8:788–794.
- Serrano, M., G.J. Hannon, and D. Beach. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*. 366:704–707.
- Servant, M.J., E. Giasson, and S. Meloche. 1996. Inhibition of growth factor-induced protein synthesis by a selective MEK inhibitor in aortic smooth muscle cells. *J. Biol. Chem.* 271:16047–16052.
- Sheaff, R.J., M. Groudine, M. Gordon, J.M. Roberts, and B.E. Clurman. 1997. Cyclin E-Cdk2 is a regulator of p27^{Kip1}. *Genes Dev.* 11:1464–1478.
- Sherr, C.J. 1994. G₁ phase progression: cycling on cue. *Cell*. 79:551–555.
- Sherr, C.J., and J.M. Roberts. 1995. Inhibitors of mammalian G₁ cyclin-dependent kinases. *Genes Dev.* 9:1149–1163.
- Slingerland, J.M., L. Hengst, C.H. Pan, D. Alexander, M.R. Stampfer, and S.I. Reed. 1994. A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor β -arrested epithelial cells. *Mol. Cell. Biol.* 14:3683–3694.
- Spirin, A.S. 1996. Masked and translatable messenger ribonucleoproteins in higher eukaryotes. In *Translational Control*. J.W.B. Hershey, M.B. Mathews, and N. Sonenberg, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 319–329.
- Su, E.J., D.M. Lombardi, J. Wiener, M.J.A.P. Daemen, M.A. Reidy, and S.M. Schwartz. 1998. Mitogenic effect of angiotensin II on rat carotid arteries and type II and III mesenteric microvessels but not type I mesenteric microvessels is mediated by endogenous basic fibroblast growth factor. *Circ. Res.* 82: 321–327.
- Takuwa, N., and Y. Takuwa. 1997. Ras activity late in G₁ phase required for p27^{Kip1} downregulation, passage through the restriction point, and entry into S phase in growth factor-stimulated NIH 3T3 fibroblasts. *Mol. Cell. Biol.* 17: 5348–5358.
- Timmermans, P.B.M.W.M., P.C. Wong, A.T. Chiu, W.F. Herblin, P. Benfield, D.J. Carini, R.J. Lee, R.R. Wexler, J.A.M. Saye, and R.D. Smith. 1993. Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol. Rev.* 45:205–251.
- Toyoshima, H., and T. Hunter. 1994. p27, a novel inhibitor of G₁ cyclin-Cdk protein kinase activity, is related to p21. *Cell*. 78:67–74.
- Tsuda, T., Y. Kawahara, Y. Ishida, M. Koide, K. Shii, and M. Yokoyama. 1992. Angiotensin II stimulates two myelin basic protein/microtubule-associated protein 2 kinases in cultured vascular smooth muscle cells. *Circ. Res.* 71:620–630.
- van den Heuvel, S., and E. Harlow. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science*. 262:2050–2054.
- van Kleef, E.M., J.F.M. Smits, J.G.R. De Mey, J.P.M. Cleutjens, D.M. Lombardi, S.M. Schwartz, and M.J.A.P. Daemen. 1992. α 1-Adrenoreceptor blockade reduces the angiotensin II-induced vascular smooth muscle cell DNA synthesis in the rat thoracic aorta and carotid artery. *Circ. Res.* 70: 1122–1127.
- Vlach, J., S. Hennecke, and B. Amati. 1997. Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27^{Kip1}. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:5334–5344.
- Weber, J.D., W. Hu, S.C. Jefcoat, Jr., D.M. Raben, and J.J. Baldassare. 1997. Ras-stimulated extracellular signal-related kinase 1 and RhoA activities coordinate platelet-derived growth factor-induced G₁ progression through the independent regulation of cyclin D1 and p27^{Kip1}. *J. Biol. Chem.* 272:32966–32971.
- Wei, G.L., K. Krasinski, M. Kearney, J.M. Isner, K. Walsh, and V. Andrés. 1997. Temporally and spatially coordinated expression of cell cycle regulatory factors after angioplasty. *Circ. Res.* 80:418–426.
- Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell*. 81:323–330.
- Winston, J., F. Dong, and W.J. Pledger. 1996. Differential modulation of G₁ cyclins and the CDK inhibitor p27^{Kip1} by platelet-derived growth factor and plasma factors in density arrested fibroblasts. *J. Biol. Chem.* 271:11253–11260.
- Xiong, Y., G.J. Hannon, H. Zang, D. Casso, R. Kobayasi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature*. 366:701–704.
- Zhang, Y., and S.C. Lin. 1997. Molecular characterization of the cyclin-dependent kinase inhibitor p27 promoter. *Biochim. Biophys. Acta*. 1353:307–317.