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Platelet-derived growth factor induces p21/WAF1 promoter in vascular smooth muscle cells via activation of an Sp1 site

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Abstract Many studies suggested that cyclin-dependent kinase inhibitor (CDKI) p21 acts as a universal inhibitor of cyclin/CDK catalytic activity. This protein has also been shown to be a component of active cyclin/CDK complexes. In addition, it has recently been suggested that p21 serves as an assembly factor in platelet-derived growth factor (PDGF)-stimulated vascular smooth muscle cells (VSMC). However, little is known concerning the molecular mechanisms by which PDGF induces p21 gene expression in VSMC. In this report we demonstrate that PDGF induces the p21 expression at both the mRNA and protein levels. This increase in p21 gene expression was due to activation of the p21 promoter by PDGF. Through both deletion and mutation analysis of the p21 promoter, we defined a 10-bp sequence that is required for the activation of the p21 promoter by PDGF. In addition, gel shift and supershift assays demonstrated that this PDGF-responsive element binds specifically to the transcription factor Sp1. These results demonstrate that Sp1 mediates PDGF-induced p21 gene expression in VSMC. Moreover, immunoblot and immonoprecipitation analysis showed that the level of hyperphosphorylated retinoblastoma protein (Rb) is increased and the protein is physically associated with Sp1 in PDGF-treated cells, indicating that phosphorylated Rb may play a role in regulating Sp1 to activate p21 expression.

Key words: Platelet-derived growth factor; p21 promoter; Vascular smooth muscle cell; Sp1; Retinoblastoma protein

1. Introduction

Cell cycle transitions are controlled by the action of the cyclin-dependent kinases (CDKs) and their activating subunits, the cyclins [1–3]. Arterial vascular smooth muscle cells (VSMC) are normally quiescent, proliferate at low indices ($<0.05\%$), and stay in the G0/G1 phase of the cell cycle [4]. After vascular injury, VSMC are stimulated to divide in response to mitogens, and they exit the G1 phase and enter the S phase. Cyclin D-CDK4 and cyclin E-CDK2 predominantly act sequentially during the G1/S transition and are required for cell cycle progression in this period [5]. CDK activity is tightly regulated by a combination of mechanisms, including changes in the cyclin or CDK levels and phosphorylation at positively and negatively regulating amino acid residues. The kinase activity of these cyclins/CDK complexes can be negatively regulated by CDK-inhibitory proteins, including p21 and p27 [5,6]. Most in vivo studies suggest that the inhibitory effect of p21 is largely exerted during the G1 phase of the cell cycle, with preferential binding to CDK4- and CDK2-containing complexes, and that it either inhibits their kinase activities or prevents their activation by CDK-activating kinase (CAK) leading to G1 arrest [7]. In addition, the regulation of p21 is largely dependent on the presence of functional p53, a transcriptional regulator that mediates cell cycle arrest after DNA damage and in senescence [8,9]. However, the expression of p21 in a variety of tissues from p53 null mice suggests that it is also regulated by a p53-independent mechanism [10]. Recently, there has appeared data implicating p21 in positive effects on cyclin/CDK activation [11,12]. In addition, it has been suggested that in Platelet-derived growth factor (PDGF)-stimulated VSMC, p21 functions as an assembly factor for cyclin D1/CDK4 but not cyclin E/CDK2 [13,14]. In a previous study in this laboratory, immunoblot analysis showed that p21 protein is increased in response to tumor necrosis factor-α stimulation in VSMC [15]. In this report, we investigate the mechanism of PDGF-induced transcriptional activation of p21. We show here that PDGF activates the p21 promoter by stimulating the transcription factor Sp1, which binds adjacent to the TATA box of the p21 promoter. Furthermore, we demonstrate that Sp1 is physically associated with retinoblastoma protein (Rb) in PDGF-treated VSMC.

2. Materials and methods

2.1. Materials

PDGF-BB was obtained from R&D Systems. The polyclonal antibodies to Sp1 and Rb were obtained from Santa Cruz.

2.2. Cell culture

Human aortic smooth muscle cells were purchased from Bio-Whittaker (San Diego, CA, USA) and cultured in smooth muscle cell growth medium containing 10% fetal bovine serum, 2 ng/ml of human basic fibroblast growth factor, 0.5 ng/ml of human epidermal growth factor, 50 μg/ml of gentamicin, 50 μl/ml of amphotericin B, and 5 μg/ml of bovine insulin.

2.3. Immunoblot

Growth-arrested VSMC were treated with PDGF for the specified

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time periods at 37°C. Cell lysates were prepared, and immunoblotting was performed as described previously [15].

2.4. RNA isolation and Northern blot analysis

Total RNA was isolated from cells grown in a 100-mm-diameter dish to 80% confluency using an Isogen RNA isolation kit (Nippon gene), and 20 μg of total RNA per lane was examined by Northern blot analysis. The p21*WAF1/Cip1* cDNA for a probe was obtained from a pCEP-WAF1 plasmid (a kind gift from Dr. Bert Vogelstein) by digesting with *Not*I. Northern blot analysis was performed by following standard methods [16].

2.5. Creation of p21 promoter reporter constructs

The human $p21^{waf1}$ promoter construct, WWW-luc (p21P), was a gift from Dr. B. Vogelstein [17]. p21P Δ2.3 and p21P smaΔ1 were described by Datto et al. [18]. The construction procedures of p21P 93-S and its mutated constructs were also described in detail by Datto et al. [18].

2.6. Transient transfection

Each plasmid was transfected into VSMC using the Superfect reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. To assess promoter activity, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris–phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Firefly luciferase activities were standardized for β-galactosidase activity.

2.7. Stable transfectants

VSMC were transfected with either p21P93-S or p21P93-S Mut#2, together with the pSV2neo vector to provide a selection marker, using the Superfect reagent (Qiagen) according to the manufacturer's instructions. Stable cell clones were selected by adding 400 μg/ml G418 in culture medium. After two weeks of selection, G418-resistant clones were isolated with cloning rings.

2.8. Electrophoretic mobility shift assays

Nuclear extracts were prepared essentially as previously described [15]. Binding reactions (20 μl) containing 2 μg poly(dI-dC), 10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 mM DTT, 2.5 mM EDTA, 7 mM $MgCl_2$, 4% glycerol, and 4–6 μg nuclear extract were incubated on ice for 30 min with 20 000 cpm of ^{32}P-labelled oligonucleotide, corresponding to the bases −71 through −86 in either the wild-type p21 promoter, the mutant promoter construct p21P93-S mut#2.2, or the mutant p21P93-S mut#2.3 (71–86 wt, GGTCCCGCCTCCTTGA and TCAAGGAGGCGGGACC; 71–86 mut#2.2, GGTCCCGGATCCTTGA and TCAAGGATCCGGGACC; 71–86 mut#2.3, GGTCCCGCCGGCTTGA and TCAAGCCGGCGGGACC). Complexes were separated on 6% non-denaturing polyacrylamide gels. The gels were subsequently dried and autoradiographed [15].

For Sp1 supershift assay, Sp1-specific polyclonal antibody was added to the binding reaction prior to the addition of radiolabeled probe.

2.9. Immunoprecipitations

Nuclear extracts of cells were precleared with 30 μl of 1:1 slurry of protein G-Sepharose™-radioimmune precipitation assay buffer for 1 h with bi-directional rotation at 4°C. Ten μl of Sp1 rabbit polyclonal IgG (Santa Cruz Biotechnology) was incubated with the precleared lysates overnight at 4°C with gentle shaking. After isolation of immune complexes with protein G-Sepharose, the pellets were washed twice and resolubilized in loading buffer, boiled for 5 min, and subjected to Western blot analysis.

3. Results and discussion

To determine whether PDGF affects p21 expression in VSMC, we measured, first, the levels of p21 protein and mRNA expression in the absence and presence of PDGF. In agreement with previous reports [13,14], PDGF treatment induced p21 mRNA expression in a time-dependent manner as determined by Northern blot analysis, and consequently increased p21 protein levels (Fig. 1A,B). Because PDGF drastically induced the p21 mRNA expression, we then investigated whether PDGF could stimulate the activity of the p21 gene promoter in cells transiently transfected by WWP-Luc, which contains the 2.4-kb 5′ flanking region of the human p21 gene and includes the p53 binding site located at 2.3 kb, driving the expression of a luciferase reporter gene. As shown in Fig. 2A, the luciferase activity was increased by treatment with 10 ng/ml PDGF. These results demonstrate that PDGF induces the expressions of p21 protein and mRNA, and also activates its promoter activity.

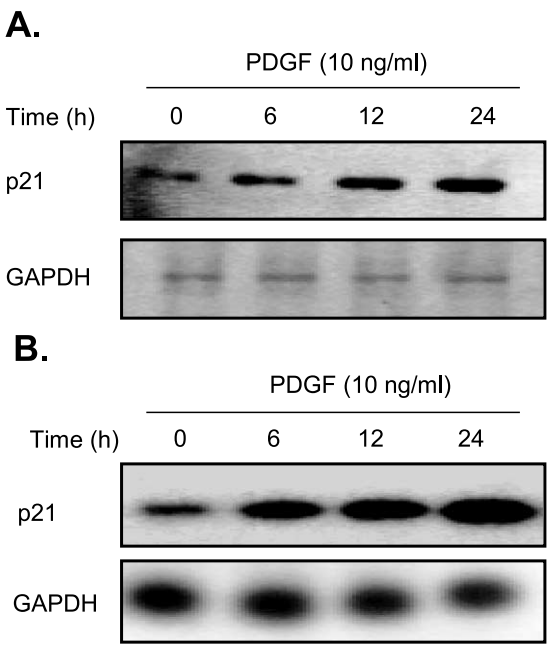

Fig. 1. Increased p21 expression and transcriptional activity in VSMC after PDGF treatment. A: VSMC were incubated in the absence or presence of PDGF for various times and cellular lysates were prepared and analyzed by immunoblotting using p21-specific antibody. Blotted membranes were reprobed with anti-GAPDH. B: VSMC were plated in the absence or presence of PDGF for various times and total RNA was extracted and analyzed by Northern blotting. GAPDH was used as control.

To identify the DNA elements activated by PDGF in the p21 promoter, we determined the regions of the p21 promoter responsible for the activation of p21 promoter by PDGF. To this end, a series of progressive 5′ promoter deletion mutants of the p21 promoter were tested (Fig. 2B). Deletion mutant p21pΔ2.3 which does not contain p53 binding sites (Fig. 2A), exhibited a modestly reduced PDGF response but did not change the extent of PDGF induction relative to basal or PDGF-induced activity. This result indicated that PDGF is capable of inducing p21 independently of the p53 pathway. Further deletion up to −93 bp did not affect the PDGF-induced activity, although the basal activity of the promoter diminished. However, the minimal promoter construct, p21Psma Δ1, which contains only 61 bp proximal to the transcriptional initiation site, repressed the PDGF-induced promoter activity. Results from these experiments suggest that PDGF induces the p21 promoter through a −33-bp region between −93 and −61.

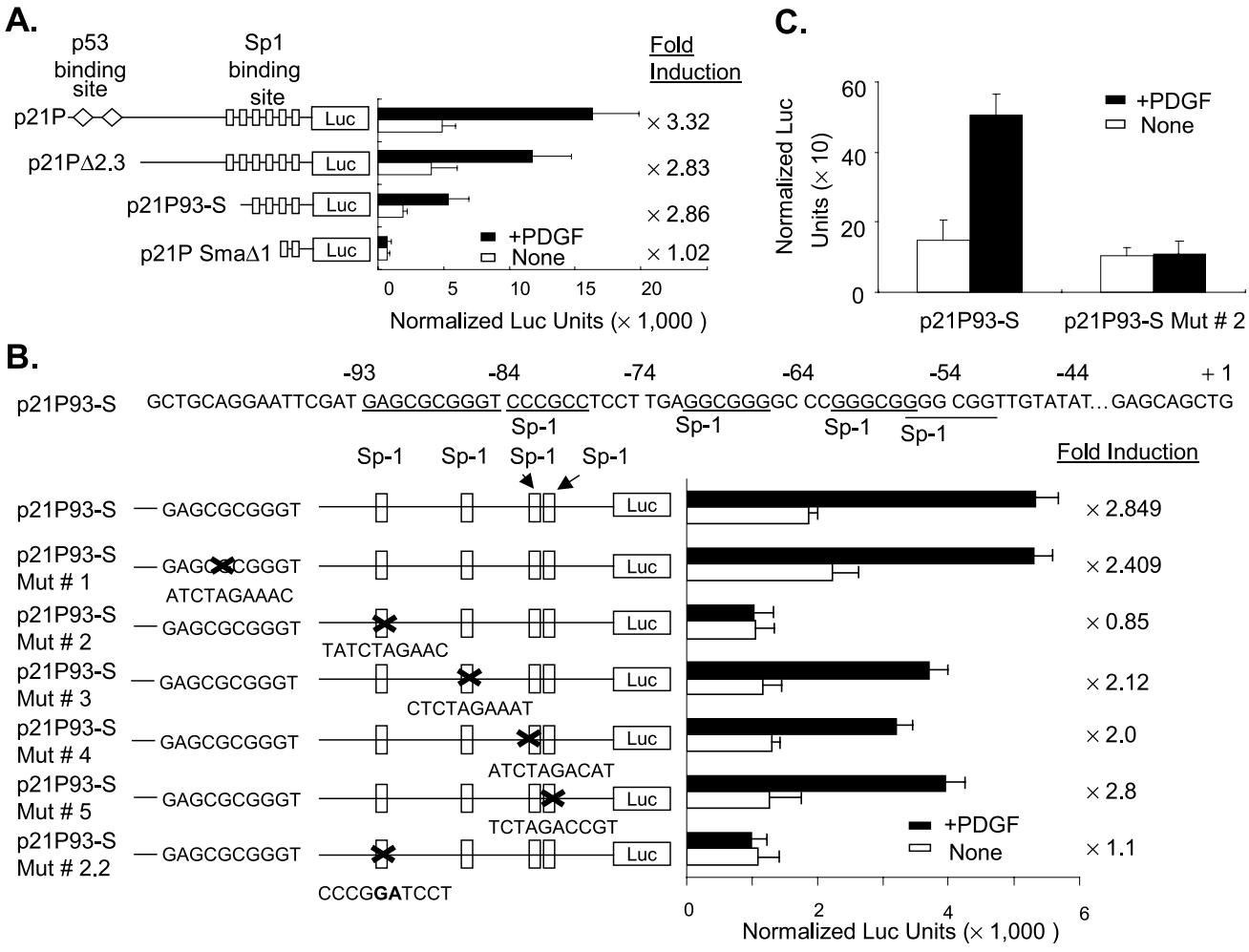

Fig. 2. Analysis of the p21 promoter. A: 5′ deletion analysis of the p21 promoter in VSMC. VSMC were transfected with the indicated p21 promoter reporter constructs, incubated for 24 h with or without PDGF (10 ng/ml), and assayed for luciferase activity. The fold induction was determined by comparing luciferase activity in transfected cells treated with PDGF (closed bar) and in transfected cells without PDGF (open bar). B: Mutational analysis of the p21 promoter in response to PDGF on VSMC. VSMC transfected with the indicated constructs were incubated with or without PDGF and then assayed for luciferase activity. The fold induction by PDGF (closed bar) relative to an untreated control (open bar) is shown. The bars represent the average results of two separate transfections in three independent experiments. C: Analysis of p21P93-S mut#2 in stably transfected cells. VSMC stably transfected with either p21P93-S or p21P93-S Mut#2 promoter fragment inserted into a luciferase reporter plasmid were treated with PDGF. Luciferase activity of the cell lysates was measured. The bars represent the average results of two separate transfections in three independent experiments.

To further define the PDGF-responsive elements, we used a panel of p21 promoters point-mutated between bases −93 and −44. The p21P93S mut#1, p21P93S mut#3, p21P93S mut#4, and p21P93S mut#5 did not affect the PDGF-induced p21 promoter activity compared with that of p21P93S although some constructs reduced the basal levels of activities (Fig. 2B). However, p21P93S mut#2 had almost completely lost the ability to be activated by PDGF. We next examined whether the Sp1 binding site between −74 and −83 is involved in the transcriptional activation by PDGF. To this end, an additional mutant construct, p21P93S mut#2.2, was created. The p21P93S mut#2.2 has a mutation of bases −78 and −79, from CC to AG (Fig. 2B). This mutant construct significantly reduced both the ability of the promoter to be induced by PDGF and the basal promoter activity. These results were confirmed by establishing stable cell lines. In parallel with the transient assay, the cell lines expressing p21P93S mut#2 lost both basal and PDGF-induced activity compared with those of p21P93S-transfected cells (Fig. 2C). Taken together, these results demonstrate that the Sp1 site located between −74 and −83 relative to the transcription start site is the main PDGF-responsive element.

To determine if any proteins could be detected that specifically interact with the PDGF-responsive element, electrophoretic mobility shift assays were performed with the wild-type PDGF-responsive element sequence and the mutant PDGF-responsive elements created in p21P93S mut#2 and p21P93S mut#2.2. Nuclear extracts were prepared from both PDGF-treated and untreated VSMC. Gel shift assays performed with these extracts and ^{32}P-end-labeled double-stranded oligonucleotide probe containing the PDGF-responsive element consensus sequence between −74 and −83 sites revealed a specific band of retarded mobility (Fig. 3A). The binding of this retarded protein could be competed away by an excess of unlabeled PDGF-responsive element DNA (Fig. 3B). Oligonucleotides mut#2 and mut#2.2 failed to bind with nuclear factors derived from PDGF-stimulated VSMC (Fig. 3A). These results indicate that the PDGF-mediated p21 expression

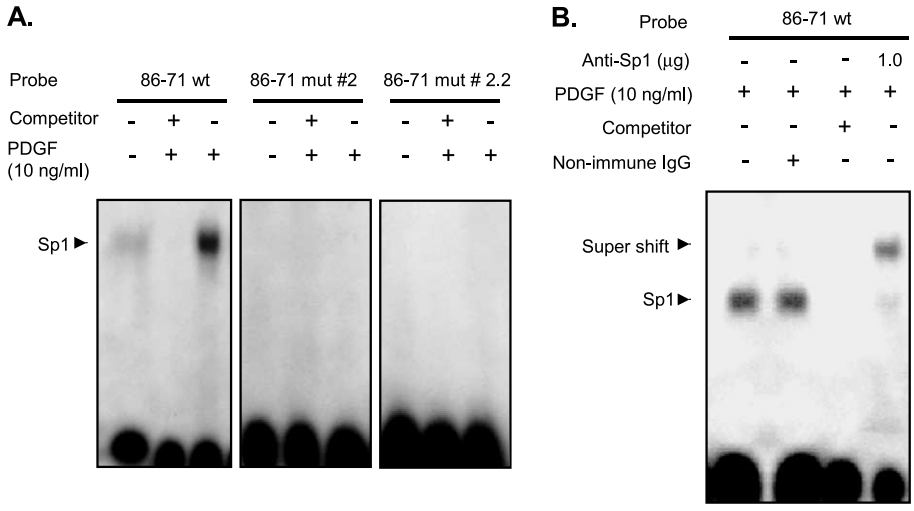

Fig. 3. Electrophoretic mobility shift assays. A: DNA probes corresponding to the bases −71 through −86 of the wild-type p21 promoter, p21P93-S mut#2, or p21P93-S mut#2.2, were end-labeled and incubated with nuclear extracts from PDGF-treated and untreated VSMC. Assays were performed in the presence or absence of a 100-fold excess of unlabeled DNA probe as a specific competitor. B: Supershift analysis of Sp1 site binding protein by anti-Sp1 antibody. Protein samples were prepared from VSMC after treatment with PDGF for 24 h. Polyclonal antibody against Sp1 was added to the binding reaction and incubated for 20 min at room temperature before addition of a labelled DNA probe corresponding to the bases −71 through −86 of the wild-type p21 promoter. Preimmune IgG was used as a control in lane 2, and lane 3 shows competition by unlabeled probe.

is absolutely dependent on the presence of intact Sp1 binding site within the p21 promoter region.

Sp1 is one member of a family of factors that binds to the Sp1 DNA site. To elucidate whether the retarded bands represent the binding of Sp1, a supershift assay was performed by preincubating the nuclear extracts with anti-Sp1 antibody (Fig. 3B). In the presence of anti-Sp1 antibody, the formation of specific DNA–protein complex was almost completely abrogated, and a supershift band appeared in PDGF-treated VSMC. These results indicate that Sp1 may be responsible for PDGF-activated transcription.

Changes in the binding of Sp1 after PDGF stimulation could be due to changes in Sp1 abundance. We therefore evaluated Sp1 protein abundance in quiescent versus PDGF-treated cells. Immunoblot analysis of total cell protein extract showed no difference in Sp1 protein abundance after PDGF stimulation (Fig. 4A). As is also shown, the relative abundance of the bands corresponding to the 95- and 105-kDa species of Sp1 is also unchanged after PDGF treatment. The results indicate that there may be some preexisting factors modulating p21 gene expression. Previous reports have shown using transient transfection assays that Rb is able to regulate transcription of c-fos, c-myc, and TGF-β1 promoters in either a positive or negative manner, dependent on the cell type [19–21]. The Rb control elements in these promoters have been defined and found to interact with Sp1 [19–21]. In this study, we demonstrate that Sp1 is involved in PDGF-induced p21 gene expression. In order to determine the relationship between Rb and p21 in PDGF-treated cells, we investigated the level and phosphorylation status of Rb. As shown in Fig. 4A, hyperphosphorylated Rb increased in PDGF-treated VSMC, indicating that phosphorylated Rb may play an important role in regulating Sp1 to activate p21 expression. To investigate the possibility of a physical interaction between Rb and Sp1, an immunoprecipitation assay was performed. Nuclear extracts were incubated with anti-Sp1 antibody in the primary immunoprecipitation; then the washed immunopreci-

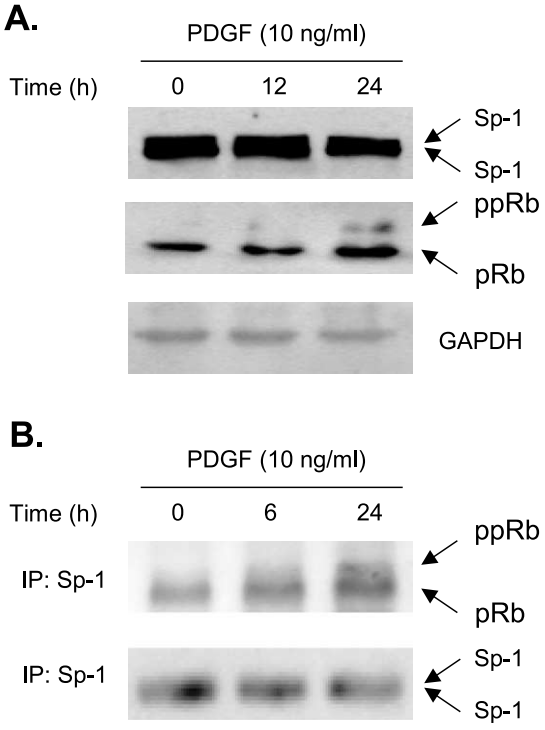

Fig. 4. Sp1 co-immunoprecipitates with Rb. A: Immunoblot analysis of Sp1 and Rb protein. Total cell proteins were isolated from quiescent or PDGF-treated cells and used for immunoblot analysis as described in Section 2. The arrows labeled 'Sp-1' indicate bands corresponding to the 95- and 105-kDa species of Sp1. pRb and ppRb indicate underphosphorylated forms and hyperphosphorylated forms of pRb, respectively. B: Immunoprecipitation using Sp1 antibody. Twenty μg of cell nuclear extract was immunoprecipitated with anti-Sp1 antibody. The washed immunoprecipitates were subjected to immunoblot using anti-Sp1 or anti-Rb antibody.

pitates were subjected to Western blotting using anti-Sp1 or anti-Rb antibody. Fig. 4B revealed the increased formation of a physical complex between Rb and Sp1 protein in PDGF-treated VSMC compared with the control. Our data from immunoblot and immunoprecipitation analysis indicate that the two proteins, phosphorylated Rb and Sp1, form part of the same complex and cooperatively may activate p21 expression, although dissection of potential regulatory interactions between Rb and Sp1 binding sites after PDGF stimulation will require additional investigation.

Collectively, our results demonstrate that PDGF induces p21 expression through activating the transcription factor Sp1 in VSMC. Moreover, we show here that phosphorylated Rb may play a role in regulating Sp1 to activate p21 expression. While much work in the area of inhibition of VSMC proliferation has been concentrated on the elements of the MAPK/ERK and other cytoplasmic kinase cascades and their inhibitors, these findings may have broad implications for our understanding of the normal cell cycle and the subversion of control pathways in VSMC. These studies represent the initial steps toward defining the signaling pathways involved in PDGF-mediated transcriptional activation of p21 in VSMC proliferation. We believe that a major importance of our work lies in its relevance and potential applications to diseases involving aberrant VSMC proliferation, such as atherosclerosis and angioplasty restenosis.

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