Akt Serine Threonine Kinase Regulates Platelet-derived Growth Factor-induced DNA Synthesis in Glomerular Mesangial Cells

REGULATION OF c-fos AND p27kip1 GENE EXPRESSION*

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Proliferation of mesangial cells requires platelet-derived growth factor receptor β (PDGFR)-mediated signal transduction. We have previously shown that activation of phosphatidylinositol (PI) 3-kinase is necessary for PDGFR-induced DNA synthesis in these cells. The mechanism by which PI 3-kinase stimulates DNA synthesis is not known. One target of PI 3-kinase, Akt serine threonine kinase, regulates survival of many cells by inhibiting the actions of certain proapoptotic proteins. In this study, we investigated the role of Akt in PDGFR-induced DNA synthesis in mesangial cells. PDGF increased Akt serine threonine kinase activity in a time- and PI 3-kinase-dependent manner. Expression of dominant negative Akt by adenovirus-mediated gene transfer blocked PDGFR-induced activation of endogenous Akt in mesangial cells, resulting in complete inhibition of DNA synthesis. On the other hand, inhibition of MAPK attenuated PDGFR-induced DNA synthesis only partially. Inhibition of Akt also attenuated PDGFR-induced c-fos gene transcription, with concomitant inhibition of Elk-1-dependent transcription, indicating positive regulation of this early response gene by Akt. To further determine the role of Akt in PDGFR-induced DNA synthesis, we investigated its effect on cyclin-dependent kinase 2 (CDK2). PDGF stimulated CDK2 activity in mesangial cells and decreased the level of p27kip1 cyclin kinase inhibitor protein. Expression of dominant negative Akt increased p27kip1 protein and resulted in inhibition of CDK2 activity. The increase in p27kip1 expression in response to Akt kinase inhibition was due to increased transcription of the p27kip1 gene. p27kip1 transcription similarly was decreased by expression of constitutively active Akt kinase in mesangial cells. These data provide the first evidence that Akt kinase regulates PDGFR-induced DNA synthesis by regulating CDK2 activity and define Akt-mediated inhibition of transcription of p27kip1 as one of the mechanisms for PDGFR-induced DNA synthesis in mesangial cells.

Mesangial cells constitute one-third of the renal glomerular cell population. These cells proliferate in response to growth factor and cytokines during glomerular injury. Platelet-derived growth factor (PDGF) increases proliferation of mesenchymal cells including mesangial cells (1, 2). Mice null for PDGF B-chain or PDGF receptor β (PDGFR) fail to develop mesangial cells, suggesting the requirement of PDGFR-mediated signal transduction in the development and survival of mesangial cells in the kidney (3, 4).

Binding of PDGF to its receptor induces dimerization of the receptor molecules, with concomitant increase in intrinsic tyrosine kinase activity, resulting in autophosphorylation. Tyrosine-phosphorylated PDGFR serves as the docking site for many signal transducing molecules that include proteins with Src homology 2, PTB, and PDZ domains (5, 6). Thus, activated PDGFR induces two major kinase cascades, Ras/Raf/MEK/Erk1/2 type mitogen-activated protein kinase (MAPK) and phosphatidylinositol (PI) 3-kinase (5, 7). MAPK has been shown to be involved in PDGFR-induced mitogenesis in fibroblasts (8). However, the requirement of MAPK in PDGF-induced mesangial cell proliferation has not been tested. The lipid-modifying enzyme PI 3-kinase, which contains Src homology 2 domains, binds directly to the phosphorylated tyrosines 740 and 751 of the PDGFR (2, 5). Mutational analysis of these two tyrosines demonstrated that binding and subsequent activation of PI 3-kinase is necessary for PDGFR-induced DNA synthesis (9). Using pharmacological inhibitors of PI 3-kinase, we have recently shown that PDGFR-induced PI 3-kinase activity regulates proliferation of mesangial cells (10). However, the mechanism by which PI 3-kinase regulates PDGFR-induced DNA synthesis is not known.

One of the downstream targets of PI 3-kinase is the serine/threonine kinase Akt/PKB (Akt) (11). The D3 phosphorylated inositides, produced by the PDGFR-activated PI 3-kinase, bind to the N-terminal pleckstrin homology domain of Akt, leading to its translocation to the plasma membrane (12). Subsequently, two serine threonine kinases, phosphoinositide-dependent kinases 1 and 2, phosphorylate threonine 308 and serine 473 residues of Akt in the activation loop and in the regulatory domain, respectively, leading to full activation (13, 14). In vivo and in vitro studies have suggested that Akt phosphorylates a spectrum of proteins regulating diverse biological activities. Phosphorylation of the proapoptotic proteins Bad, caspase 9, and the forkhead family of transcription factors inhibit their activities (15–17). Thus, phosphorylation and inactivation of proapoptotic proteins by Akt leads to inhibition of...
apoptosis (13). Similarly, phosphorylation of endothelial nitric-oxide synthase by Akt inhibits apoptosis of human vascular endothelial cells (18, 19). These studies have established a role for Akt in promoting cell survival by inhibiting apoptosis. However, the role of Akt in cell proliferation has not been investigated directly. In this study, we show that MAPK partially attenuates PDGF-induced DNA synthesis in mesangial cells. Inhibition of Akt kinase activity, by adenovirus-mediated gene transfer of a dominant negative Akt, blocks PDGF-induced DNA synthesis completely. Inhibition of Akt attenuates c-fos gene transcription by inhibition of Elk-1 transactivation. We also provide the first evidence that Akt regulates PDGF-induced cyclin-dependent kinase 2 (CDK2) activity by modulating p27
\(^{kip}\) cyclin kinase inhibitor (CKI) gene transcription and protein expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture reagents and LipofectAMINE were purchased from Life Technologies, Inc. Aprotinin was obtained from Bayer. Phenylmethylsulfonyl fluoride, Nonidet P-40, sodium orthovanadate, and antibody against actin were purchased from Sigma. Recombinant PDGF-BB was obtained from R & D Systems. Akt antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Histone H1 and H2B were purchased from Roche Molecular Biochemicals. Anti-Erk1/2 MAPK, anti-CDK2, and anti-p27
\(^{kip}\) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-HA antibody was purchased from Babco. The dual luciferase assay kit was obtained from Promega. The dominant negative pCMV6-HA-Akt (K179M) and constitutively active pCMV6-Myr-Akt-HA expression vectors were kind gifts from Dr. Thomas Franke (Harvard University). The adenovirus vector expressing a HA-tagged dominant negative Akt, in which the two activating amino acid residues threonine 308 and serine 473 were changed to alanine, was kindly provided by Dr. Kenneth Walsh (Tufts University School of Medicine). This vector has been described previously (20). p27G1,1609 reporter plasmid, in which luciferase CDNA is driven by the p27
\(^{kip}\) promoter, was a kind gift of Dr. B. M. T. Burginger (The Netherlands).

**Cell Culture and Adenovirus Infection**—Harlan Sprague-Dawley rat glomerular mesangial cells were grown in RPMI 1604 medium with 16% fetal bovine serum to confluence as described before (21, 22). The cells were made quiescent by incubating in serum-free RPMI for 24 h. The cells were serum-free medium were infected with 100 MOI of Ad-DN Akt coding for dominant negative Akt gene at room temperature for 1 h. Medium was changed with fresh serum-free medium for another 24 h before stimulation with PDGF.

**DNA Synthesis**—DNA synthesis was determined as incorporation of \(^{3}H\)-thymidine into trichloroacetic acid-insoluble material as described (10, 21–24). Immunoprecipitation, MAPK, Akt Kinase, CDK2 Kinase Assay, and Immuno blotting—Cells were lysed in radioimmune precipitation buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na$_{3}$VO$_{4}$, 1 mM phenylmethylsulfonyl fluoride, 0.05% aprotinin, and 1% Nonidet P-40) buffer at 4 °C for 30 min. The lysates were cleared at 10,000 × g for 30 min at 4 °C. Protein was determined in the supernatant using the BCA method (25). Equal amounts of protein were immunoprecipitated with anti-MAPK or anti-Akt or anti-CDK2 antibody as described above (10, 21–24). Washed immunobeads were resuspended either in MAPK assay buffer (10 mM HEPES, pH 7.4, 10 mM MgCl$_{2}$, 0.5 mM diithiothreitol and 0.5 mM Na$_{3}$VO$_{4}$) (10) or Akt assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl$_{2}$, 25 mM \(\beta\)-glycerophosphate, 2 mM diithiothreitol, 1 mM Na$_{3}$VO$_{4}$, and 6 μM ATP (25) or CDK2 assay buffer (Tris-HCl, pH 7.5, 4 mM MgCl$_{2}$, and 25 μM ATP) (26). Myelin basic protein (MBP), histone H2B and histone H1 were used as substrates for MAPK, Akt, and CDK2, respectively. For MAPK, the reaction was started with 1 μCi of \([γ-^{32}P]\)ATP. For Akt and CDK2, reactions were started by the addition of 10 μCi of \([γ-^{32}P]\)ATP and incubated at 30 °C for 30 min. 4× SDS sample buffer was added to stop the reaction. Phosphorylated MBP or histone H2B or histone H1 was separated by 15% SDS-polyacrylamide gel electrophoresis and autoradiography. Immunoblotting of lysates was performed using appropriate antibodies as described previously (21, 22, 25, 26).

**Transient Transfection and Luciferase Assay**—Transient transfection of firefly luciferase reporter plasmid along with vector, dominant negative Akt (pCMV6-HA-Akt K179M), or constitutively active Akt (pCMV6-Myr-Akt-HA) expression vector was performed as described (21, 22). To correct for transfection efficiency, a cytomegalovirus-\(\alpha\)-galactosidase plasmid was also included. For Elk-1 transactivation, the GAL-4 DNA binding domain-Elk-1 transactivation domain fusion protein was co-transfected with 5× GAL-4 DNA binding element-driven firefly luciferase reporter plasmid (21, 22). The transiently transfected mesangial cells were harvested for another 24 h. PDGF at a concentration of 10 ng/ml was added for 12 h before harvesting. Luciferase activity in the lysates was determined using a dual luciferase assay kit as described (21, 22).

**RESULTS**

MAPK Partially Regulates PDGF-induced DNA Synthesis in Mesangial Cells—PDGF is the most potent mitogen for cultured mesangial cells (1). One of the earliest downstream targets of PDGFR-mediated signaling is the enzyme MAPK (5, 7). To investigate the contribution of MAPK to PDGF-induced mitogenesis, we studied activation of this enzyme in rat mesangial cells. MAPK immunoprecipitates from lysates of mesangial cells were assayed for kinase activity using myelin basic protein as substrate. PDGF increased MAPK activity in a time-dependent manner (Fig. 1A). Maximum activity was achieved at 5 min (Fig. 1, compare lane 2 with lane 1). To test the requirement of MAPK in PDGF-induced DNA synthesis, we used a pharmacological inhibitor (PD098059) of MEK, the upstream kinase that activates the MAPK. Mesangial cells were treated with PD098059 followed by stimulation with PDGF. Immune complex kinase assay of the MAPK immunoprecipitates showed a dose-dependent inhibition of MAPK activity induced by PDGF (Fig. 1B). At 25 μM, PD098059 completely abolished PDGF-induced MAPK activity (Fig. 1B, compare lane 6 with lane 2). In contrast, this same concentration of PD098059 resulted in only 47% inhibition of PDGF-induced DNA synthesis in mesangial cells (Fig. 1C). These data indicate that although MAPK partially regulates PDGF-mediated DNA synthesis, it is not the predominant enzyme cascade that regulates PDGF-induced mitogenesis in mesangial cells.

**Akt Regulates PDGF-induced DNA Synthesis in Mesangial Cells**—We previously demonstrated that PI 3-kinase partially regulates PDGF-induced MAPK activity in mesangial cells (10). However, inhibition of PI 3-kinase by pharmacological inhibitors completely blocked PDGF-induced DNA synthesis in these cells (10). These data indicate that PI 3-kinase is the predominant enzyme that regulates PDGF-induced mitogenesis in mesangial cells. To elucidate the signaling pathways necessary for PI 3-kinase to exert its mitogenic effect, we analyzed activation of Akt, the downstream target of PDGF. Akt activity was determined in an immune complex kinase assay using histone H2B as substrate. PDGF increased Akt activity in a time-dependent manner, with maximum activity achieved at 5 min (Fig. 2A). To determine the requirement of PI 3-kinase in this activation, mesangial cells were incubated with Ly 294002 or wortmannin, two pharmacological inhibitors of PI 3-kinase, prior to the incubation with PDGF. Both inhibitors blocked PDGF-induced Akt activity in mesangial cells (Fig. 2, B and C). These data confirm the previous observation that PDGF-induced Akt activity is PI 3-kinase-dependent (11).

The critical target of PI 3-kinase required for PDGF induction of DNA synthesis is not yet known. Akt, the immediate downstream target of PI 3-kinase, has been shown to regulate the survival of many cells by inhibiting apoptosis (13–19). However, its effect on DNA synthesis has not been investigated. To elucidate the role of Akt in DNA synthesis, we used an adenovirus vector (Ad-DN Akt) expressing a dominant negative version of HA-tagged Akt serine threonine kinase (20). Infection of mesangial cells with Ad-DN Akt followed by immunoblot analysis of the lysates with anti-HA antibody showed abundant expression of dominant negative Akt in a time-dependent man-
complex kinase assay with Akt antibody as described above. The lysates were used in an immune complex kinase assay in the presence of MBP as substrate and \([\gamma-\text{32P}]\)ATP as described under “Experimental Procedures.” Phosphorylated MBP was separated by 15% SDS gel. The bottom panel shows the immunoblot analysis of the same samples with MAPK antibody. B, quiescent mesangial cells were incubated with indicated concentrations of PD098059 for 1 h before stimulation with PDGF BB for 5 min. The lysates were used in an immune complex kinase assay and analyzed as described for A. The bottom panel shows the immunoblot analysis of the same samples with MAPK antibody. C, quiescent mesangial cells were treated with 25 \(\mu\)M PD098059 for 1 h. PDGF BB was added to these cells for 24 h. During the last 4 h of PDGF stimulation, 1 \(\mu\)Ci/ml \([\text{3H}]\)thymidine was added as described (10, 21–24). Results are means \(\pm\) S.E. of quadruplicate determinations.

**Fig. 1.** MAPK regulates PDGF-induced DNA synthesis in mesangial cells. A, quiescent mesangial cells were incubated with 10 ng/ml PDGF BB for different periods of time. Equal amounts of cleared cell lysates were immunoprecipitated with MAPK antibody followed by immune complex kinase assay in the presence of MBP as substrate and \([\gamma-\text{32P}]\)ATP as described under “Experimental Procedures.” Phosphorylated histone H2B was separated by 15% SDS gel. The bottom panel shows the immunoblot analysis of the same samples with MAPK antibody. B, quiescent mesangial cells were incubated with indicated concentrations of PD098059 for 1 h before stimulation with PDGF BB for 5 min. The lysates were used in an immune complex kinase assay and analyzed as described for A. The bottom panel shows the immunoblot analysis of the same samples with MAPK antibody. C, quiescent mesangial cells were treated with 25 \(\mu\)M PD098059 for 1 h. PDGF BB was added to these cells for 24 h. During the last 4 h of PDGF stimulation, 1 \(\mu\)Ci/ml \([\text{3H}]\)thymidine was added as described (10, 21–24). Results are means \(\pm\) S.E. of quadruplicate determinations.

**Fig. 2.** Effect of PI 3-kinase inhibitors on PDGF-induced Akt kinase activity. A, quiescent mesangial cells were incubated with 10 ng/ml PDGF BB for different periods of time. Equal amounts of cleared cell lysates were immunoprecipitated with Akt antibody followed by immune complex kinase assay in the presence of histone H2B as substrate and \([\gamma-\text{32P}]\)ATP as described under “Experimental Procedures.” Phosphorylated histone H2B was separated by 15% SDS gel. The bottom panel shows the immunoblot analysis of the same samples with Akt antibody. B and C, quiescent mesangial cells were incubated with 25 \(\mu\)M Ly 294002 (B) or with 250 \(\mu\)M wortmannin (WMN) (C) before stimulation with PDGF BB for 5 min. The lysates were used in an immune complex kinase assay with Akt antibody as described above. The bottom panels show the immunoblot analysis with Akt antibody.

The transcription of one such gene, \(c-fos\), follows activation of PDGFR (21). It has previously been shown that PI 3-kinase regulates \(c-fos\) gene transcription (29). Since Akt serine threonine kinase activity is regulated by PI 3-kinase (27, 28), we next tested the effect of Ad-DN Akt on PDGF-induced DNA synthesis in mesangial cells. Twenty-four hours after infection, mesangial cells were incubated with PDGF, and \([\text{3H}]\)thymidine incorporation was used as a measure of DNA synthesis. Ad-DN Akt inhibited PDGF-induced DNA synthesis completely (Fig. 3C). Note that the basal activity was also inhibited, indicating that Akt is required for the basal level of DNA synthesis in mesangial cells.

One of the established biological functions of Akt is inhibition of apoptosis (13–17). Inhibition of endogenous Akt kinase activity by dominant negative Akt may therefore result in apoptosis of mesangial cells. The cells did not show any gross morphological changes of apoptosis, however, up to 3 days after Ad-DN Akt infection. Furthermore, the viability of the mesangial cells was tested by trypan blue exclusion analysis. 98% of the cells infected with either Ad-\(\beta\)-gal or Ad-DN Akt were viable 3 days postinfection. From these results, we conclude that Akt regulates PDGF-induced DNA synthesis in mesangial cells rather than indirectly affecting DNA synthesis by promoting apoptosis.

**Akt Kinase Activity Modulates \(c-fos\) Gene Transcription—** Growth factor-mediated DNA synthesis requires \(de novo\) gene transcription. Early response genes are the targets of many mitogens (27, 28). The transcription of one such gene, \(c-fos\), follows activation of PDGFR (21). It has previously been shown that PI 3-kinase regulates \(c-fos\) gene transcription (29). Since Akt serine threonine kinase activity is regulated by PI 3-kinase in mesangial cells (Fig. 2, B and C), we investigated the effect of dominant negative Akt expression on \(c-fos\) gene transcription. A reporter construct (Fos-Luc), in which luciferase cDNA is driven by a 550-base pair \(c-fos\) promoter, was co-transfected in mesangial cells along with a dominant negative Akt expression vector (21). As expected, PDGF increased reporter gene expression in mesangial cells transfected with Fos-Luc (Fig.

**Fig. 3.** Effect of dominant negative Akt expression on PDGF-induced Akt activation and \(c-fos\) gene transcription. A, quiescent mesangial cells were transfected with Fos-Luc reporter plasmid, a control adenovirus, Ad-\(\beta\)-gal, or with Ad-DN Akt for 24 h before incubation with PDGF BB. Immune complex kinase assays performed with Akt immunoprecipitates showed that expression of dominant negative Akt inhibited the endogenous Akt activity induced by PDGF (Fig. 3B; compare lane 4 with lane 2). These data indicate that Ad-DN Akt can be used as a tool to block Akt-dependent biological activity. Therefore, we next tested the effect of Ad-DN Akt on PDGF-induced DNA synthesis in mesangial cells. Twenty-four hours after infection, mesangial cells were incubated with PDGF, and \([\text{3H}]\)thymidine incorporation was used as a measure of DNA synthesis. Ad-DN Akt inhibited PDGF-induced DNA synthesis completely (Fig. 3C). Note that the basal activity was also inhibited, indicating that Akt is required for the basal level of DNA synthesis in mesangial cells.

**Fig. 4.** Effect of PI 3-kinase inhibitors on PDGF-induced Akt kinase activity. A, quiescent mesangial cells were incubated with 10 ng/ml PDGF BB for different periods of time. Equal amounts of cleared cell lysates were immunoprecipitated with Akt antibody followed by immune complex kinase assay in the presence of histone H2B as substrate and \([\gamma-\text{32P}]\)ATP as described under “Experimental Procedures.” Phosphorylated histone H2B was separated by 15% SDS gel. The bottom panel shows the immunoblot analysis of the same samples with Akt antibody. B and C, quiescent mesangial cells were incubated with 25 \(\mu\)M Ly 294002 (B) or with 250 \(\mu\)M wortmannin (WMN) (C) before stimulation with PDGF BB for 5 min. The lysates were used in an immune complex kinase assay with Akt antibody as described above. The bottom panels show the immunoblot analysis with Akt antibody.

Another, with maximum protein expression at 24 h postinfection (Fig. 3A). We next investigated the effect of dominant negative Akt expression on PDGF-induced Akt activity. Mesangial cells were infected with a control adenovirus, Ad-\(\beta\)-gal, which expresses the enzyme \(\beta\)-D-thiogalactosidase, or with Ad-DN Akt for 24 h before incubation with PDGF BB. Immune complex kinase assays performed with Akt immunoprecipitates showed that expression of dominant negative Akt inhibited the endogenous Akt activity induced by PDGF (Fig. 3B; compare lane 4 with lane 2). These data indicate that Ad-DN Akt can be used as a tool to block Akt-dependent biological activity. Therefore, we next tested the effect of Ad-DN Akt on PDGF-induced DNA synthesis in mesangial cells. Twenty-four hours after infection, mesangial cells were incubated with PDGF, and \([\text{3H}]\)thymidine incorporation was used as a measure of DNA synthesis. Ad-DN Akt inhibited PDGF-induced DNA synthesis completely (Fig. 3C). Note that the basal activity was also inhibited, indicating that Akt is required for the basal level of DNA synthesis in mesangial cells.
Co-transfection of dominant negative Akt expression vector inhibited PDGF-induced reporter gene expression significantly (Fig. 4A). These data indicate that Akt regulates PDGF-induced c-fos gene transcription. We have recently shown that in mesangial cells, PDGF-induced c-fos gene transcription is induced by Elk-1 transcription factor, which forms a ternary complex with serum response factor in the serum response element of c-fos promoter (21, 27). Therefore, we studied the effect of dominant negative Akt kinase on Elk-1 transactivation. For this, we used a mammalian one-hybrid system in which transfected mesangial cells with an expression vector encoding the Elk-1 transactivation domain fused to GAL-4 DNA binding domain and a firefly luciferase reporter plasmid under the control of GAL-4 DNA element. As expected, PDGF stimulated Elk-1-dependent expression of the reporter gene, suggesting activation of the Elk-1 transcription factor by PDGF (Fig. 4B). Cotransfection of dominant negative Akt construct with the Elk-1 fusion plasmid and the reporter plasmid resulted in inhibition of Elk-1-dependent reporter gene expression by PDGF (Fig. 4B). These data indicate that Akt kinase may regulate PDGF-induced c-fos gene transcription by modulating the Elk-1 transcription factor.

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FIG. 3. Expression of dominant negative HA-Akt blocks PDGF-induced Akt activity and PDGF-induced DNA synthesis. A, expression of Akt in Ad-DN Akt-infected mesangial cells. Quiescent mesangial cells were infected with Ad-DN Akt at 100 MOI for different periods of time. 20 μg of cell lysates were immunoblotted with HA antibody. The bottom panel shows immunoblot analysis of the same lysates with actin antibody. B, expression of dominant negative Akt inhibits endogenous Akt kinase activity. Quiescent mesangial cells were infected with Ad-β-gal or Ad-DN Akt (100 MOI) for 24 h before the addition of PDGF. An immune complex kinase assay of Akt immunoprecipitate was performed as described in the legend to Fig. 1. The bottom panel shows the immunoblot analysis of the lysates with anti-HA antibody to demonstrate the expression of dominant negative Akt. C, effect of dominant negative Akt on PDGF-induced DNA synthesis. Serum-deprived transfected cells were incubated with PDGF, and [3H]thymidine incorporation was determined as described in the legend to Fig. 1. Results are means ± S.E. of quadruplicate determinations. A representative of four independent experiments is shown.

FIG. 4. A, Effect of dominant negative Akt on PDGF-induced c-fos gene transcription. Mesangial cells were transfected with Fos-Luc reporter plasmid, in which the firefly luciferase cDNA is driven by c-fos promoter (19) and cytomegalovirus-Renilla plasmid along with vector or dominant negative Akt expression plasmid (DN Akt) as described under “Experimental Procedures.” Serum-deprived transfected cells were treated with PDGF and the luciferase activity in the cell lysate was determined as described under “Experimental Procedures.” B, effect of dominant negative Akt on PDGF-induced Elk-1-dependent reporter transcription. Mesangial cells were transfected with GAL-4 luciferase reporter plasmid, GAL-4-Elk-1 transactivation domain fusion plasmid and cytomegalovirus-Renilla plasmid as described under “Experimental Procedures.” Serum-deprived transfected cells were incubated with PDGF, and the luciferase activity was determined in the cell lysate as described under “Experimental Procedures.”
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FIG. 5. Effect of dominant negative Akt on PDGF-induced CDK2 activity and p27kip1 protein expression. A, dominant negative Akt blocks PDGF-induced CDK2 activity. Quiescent mesangial cells were infected with Ad-β-gal or Ad-DN Akt (100 MOI) for 24 h before the addition of PDGF. The lysates were immunoprecipitated with CDK2 antibody and the immunobeads were used in an immune complex kinase assay in the presence of histone H1 as substrate and [γ-32P]ATP as described under “Experimental Procedures.” The middle panel shows immunoblot analysis of the lysates with anti-HA antibody to demonstrate the expression of dominant negative Akt. The bottom panel shows immunoblot analysis with anti-CDK2 antibody. B, effect of dominant negative Akt on p27kip1 protein expression. Quiescent mesangial cells were infected with Ad-DN Akt for different periods of time. 20 μg of lysates were immunoblotted with p27kip1 antibody. The middle and lower panels show immunoblot analysis of the same lysates with anti-HA and actin antibodies, respectively. C, effect of Ad-DN Akt on PDGF-induced p27kip1 expression. Quiescent mesangial cells were infected with Ad-β-gal or Ad-DN Akt (100 MOI) for 24 h before the addition of PDGF for 24 h. 20 μg of cell lysates were immunoblotted with p27kip1 antibody. The middle and lower panels are immunoblot analyses of the same lysates with anti-HA and anti-actin antibodies as above.

required for mitogen-induced DNA synthesis (30–32). Since inhibition of Akt kinase activity blocked PDGF-induced DNA synthesis in mesangial cells (Fig. 3C), we investigated the effect of dominant negative Akt expression on CDK2 activity. PDGF-stimulated CDK2 activity was determined by an immunocomplex kinase assay using histone H1 as substrate (Fig. 5A, compare lane 2 with lane 1). Infection of mesangial cells with Ad-DN Akt for 24 h prior to PDGF addition inhibited PDGF-induced CDK2 activity (Fig. 5A, compare lane 4 with lane 2). These data indicate that the decrease in DNA synthesis observed by inhibition of Akt kinase activity (Fig. 3C) may be due to CDK2 inhibition.

FIG. 6. Akt Regulates p27kip1 Transcription—The relative amount of p27kip1 in the cells modulates the G1/S transition. In fact, a 3-fold increase in p27kip1 protein level is sufficient to inhibit the CDK2 activity, which regulates DNA synthesis (31). To address the mechanism of increased p27kip1 expression by dominant negative Akt kinase, we studied p27kip1 gene transcription. A reporter plasmid containing the luciferase gene driven by p27kip1 promoter was used in transient transfection assays. Treatment of transiently transfected mesangial cells with PDGF decreased reporter gene expression (Fig. 6A). Cotransfection of a dominant negative Akt expression plasmid stimulated both basal and PDGF-induced p27kip1 transcription. Similarly, when myristoylated Akt, a constitutively active version of the enzyme, was cotransfected with the reporter plasmid, the basal as well as PDGF-induced p27kip1 transcription was inhibited (Fig. 6B). These data indicate that in mesangial cells, Akt serine threonine kinase regulates p27kip1 gene transcription. This may be one of the mechanisms for high protein level observed when the Akt kinase activity was inhibited by dominant negative Akt expression (Fig. 5, B and C).

DISCUSSION

These studies represent the first direct demonstration that Akt serine threonine kinase activity is required for PDGF-induced DNA synthesis and c-fos gene transcription in glomerular mesangial cells. Our data also provide the first evidence

Note that the amount of p27kip1 in the presence of PDGF in mesangial cells expressing dominant negative Akt was significantly higher than that in the control and in PDGF-treated cells (compare lane 4 with lanes 2 and 1). Taken together, the data presented thus far indicate that PDGF-induced reduction in p27kip1 protein expression results in DNA synthesis in mesangial cells, whereas a significant increase in p27kip1 protein level by inhibition of Akt kinase results in attenuation of PDGF-induced DNA synthesis.

requirements for mitogen-induced DNA synthesis (30–32). Since inhibition of Akt kinase activity blocked PDGF-induced DNA synthesis in mesangial cells (Fig. 3C), we investigated the effect of dominant negative Akt expression on CDK2 activity. PDGF-stimulated CDK2 activity was determined by an immunocomplex kinase assay using histone H1 as substrate (Fig. 5A, compare lane 2 with lane 1). Infection of mesangial cells with Ad-DN Akt for 24 h prior to PDGF addition inhibited PDGF-induced CDK2 activity (Fig. 5A, compare lane 4 with lane 2). These data indicate that the decrease in DNA synthesis observed by inhibition of Akt kinase activity (Fig. 3C) may be due to CDK2 inhibition.

A family of proteins called CKIs regulates cyclin-dependent kinase activity (30, 31). Among the CKIs, p27kip1 protein level is regulated by mitogens (33–36). p27kip1 also inhibits the activity of CDK2 in vitro and in vivo (30). Therefore, we explored whether Akt kinase regulates p27kip1 expression. Quiescent mesangial cells were infected with Ad-DN Akt, and the lysates were analyzed by immunoblotting with anti-p27kip1 antibody. Quiescent mesangial cells expressed appreciable amounts of p27kip1 (Fig. 5, B and C, upper panel, lane 1). Expression of dominant negative Akt for 24 and 48 h increased p27kip1 protein expression (Fig. 5B, upper and middle panels, lanes 2 and 3). By 72 h postinfection of Ad-DN Akt, significant amounts of p27kip1 accumulated (data not shown). These data indicate that inhibition of even basal Akt activity in quiescent mesangial cells increases p27kip1 protein abundance. Next, we tested the effect of dominant negative Akt expression on PDGF regulation of p27kip1 expression. Quiescent mesangial cells were infected with Ad-DN Akt before incubation with PDGF. Immunoblot analysis of the lysates showed that PDGF alone decreased p27kip1 protein expression (Fig. 5C, compare lane 2 with lane 1). Expression of dominant negative Akt (Fig. 5C, middle panel) significantly increased p27kip1 protein abundance (Fig. 5C, upper panel, compare lane 3 with lane 1). However, p27kip1 protein expression was reduced in the presence of PDGF as compared with PDGF-ununtreated control in the cells expressing dominant negative Akt (Fig. 5C, compare lane 4 with lane 3).

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DISCUSSION

These studies represent the first direct demonstration that Akt serine threonine kinase activity is required for PDGF-induced DNA synthesis and c-fos gene transcription in glomerular mesangial cells. Our data also provide the first evidence
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FIG. 6. Effect of Akt kinase on PDGF-induced p27kip1 transcription. Mesangial cells were transfected with p1609p27lac and cytomegalovirus-Renilla plasmid along with vector or dominant negative Akt expression construct (A) or constitutively active Myr-Akt expression plasmid (B) as described under “Experimental Procedures.” Serum-deprived transfected cells were treated with PDGF, and the luciferase activity in the cell lysate was determined as described under “Experimental Procedures.”

that Akt regulates PDGF-induced CDK2 activity by modulating the expression of the p27kip1 cyclin kinase inhibitor.

Accumulating studies in human and experimental animals suggest that PDGF plays an important role in proliferative and inflammatory glomerular diseases (37–39). In fact, increased expression of PDGF B-chain and PDGFR has been associated with experimental mesangial proliferative glomerulonephritis, and administration of antibody against PDGF B-chain blocks proliferation of mesangial cells, leading to amelioration of the disease (37, 38, 40). Increased activity of PDGFR in glomerular disease in adult animals is reminiscent of the requirement for PDGFR in mesangial cell development during embryogenesis (3, 4). PDGF stimulates pleotrophic effects in glomerular mesangial cells, including activation of phospholipase C, PLA2, MAPK, and PI 3-kinase (5, 7, 10, 41). In the present study, we show that MAPK partially regulates PDGF-induced mitogenesis in these cells (Fig. 1). We reported previously that PI 3-kinase activity is required for PDGF-induced DNA synthesis in mesangial cells (10). Although MAPK is partially regulated by PI 3-kinase in mesangial cells (10), activation of the former does not fully account for PDGF-induced DNA synthesis (Fig. 1). These data indicate that two signal transduction pathways, MAPK and PI 3-kinase, initiated by PDGF act together to induce the DNA synthesis in mesangial cells. It is possible that part of the effect of PI 3-kinase on PDGF-induced DNA synthesis may involve MAPK. However, PI 3-kinase plays the predominant role in PDGF-induced mitogenesis (10). We now have demonstrated that PI 3-kinase employs its downstream target, Akt serine threonine kinase, to elicit this biological response (Fig. 2 and Fig. 3).

Akt kinase activity is stimulated by a variety of extracellular signals including growth factors and cytokines, T-cell receptor activation and integrin engagement, hyperosmolarity, hypoxia, and heat shock (42–49). Many direct substrates of Akt have been identified that have divergent biological functions. Known substrates of Akt include glycogen synthase kinase 3, mTOR, insulin receptor substrate 1, BAD, caspase 9, endothelial nitric-oxide synthase, 6-phosphofructico-2-kinase, InsB kinase, Raf-1 protein kinase, Rac 1, phosphodiesterase 3B, BRCA1, and forkhead transcription factors (15–18, 50–55). Although this broad group of proteins has diverse biological responses, the role of Akt in relation to inhibition of apoptosis has been studied most extensively. These results are based on the observation that phosphorylation of the proapoptotic Bcl-2-family protein BAD leads to its quenching by 14-3-3 protein in cytosol, resulting in release of antiapoptotic Bcl-2 (15). However, recent results in different cell types indicate that phosphorylation of BAD by Akt is not sufficient to inhibit apoptosis (56–59). Another mechanism of cell survival is inhibition of caspase 9 activity following its phosphorylation by Akt (16). However, recently Akt phosphorylation of caspase 9 has been shown to be species-dependent. For instance, rat caspase 9 is not phosphorylated by Akt due to lack of a consensus phosphorylation site (60). These data argue against Akt-induced inhibition of caspase 9 as the major mechanism of cell survival. They are also consistent with our observation in rat mesangial cells that expression of dominant negative Akt, which blocks the endogenous Akt serine threonine kinase activity, does not affect cell viability. Since Akt can repress the genes that induce apoptosis via phosphorylation of the forkhead family transcription factors (17), overexpression of dominant negative Akt may induce apoptosis by derepressing the transcription of these genes. This could explain why we observed inhibition of PDGF-stimulated DNA synthesis in mesangial cells expressing dominant negative Akt (Fig. 3C). Activation of the caspases constitutes the common pathway that leads to apoptosis (61). In the caspase cascade, the final executor protease is caspase 3. Therefore, we measured caspase 3 activity in lysates of mesangial cells using a fluorogenic peptide substrate. No difference in caspase activity was observed between mock- and Ad-DN Akt-infected cells (data not shown), indicating that overexpression of dominant negative Akt in mesangial cells does not induce apoptosis. Therefore, we conclude that the inhibition of PDGF-induced DNA synthesis we observed in the presence of dominant negative Akt expression was not due to increased apoptosis of mesanginal cells.

One biological response to PDGF and many other growth factors is stimulation of early response genes, the activities of which are thought to be required for mitogenesis (27, 28). It was reported earlier that PI 3-kinase regulates c-fos gene expression (29). Since Akt is a downstream target of PI 3-kinase, it may be a candidate as the kinase that regulates the PDGF-induced c-fos gene expression. Indeed, our data show that Akt activity modulates the PDGF-induced c-fos gene transcription in mesangial cells (Fig. 4A). We showed previously that PDGF-stimulated c-fos gene transcription is mediated by the ETS domain transcription factor Elk-1 in mesangial cells (21). Now we provide the first evidence that Elk-1-dependent transcription is regulated by Akt kinase (Fig. 4B), suggesting that Akt regulation of c-fos gene expression may be due to Elk-1 transactivation.

Activation of cyclin D1 and cyclin E-dependent kinases during early through late phase of G_{1} is required for cells to enter into S phase (30, 31). Recent experiments, where cyclin D1 deficiency was functionally rescued by knocking in cyclin E, suggest that activation of CDK2, the only target of cyclin E, is necessary for the progression through the restriction point into S phase (32). Therefore, we chose this kinase in our study as the mediator of PDGF-induced DNA synthesis. We show that PDGF increased CDK2 activity in mesangial cells (Fig. 5A). In
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Akt, a serine/threonine kinase, has been implicated in the regulation of cell proliferation, apoptosis, and cell survival. The role of Akt in the biology of mesangial cells and other kidney cells remains to be explored. Although Akt-dependent phosphorylation of proapoptotic proteins leads to inhibition of apoptosis is well established in many cells, our data provide the first direct evidence that Akt regulates PDGF-induced DNA synthesis in mesangial cells. Increased mesangial cell proliferation by PDGF activation is a prominent feature in glomerular injury. Akt may prove to be an effective target to prevent mesangioproliferative disorders.

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