EGF stimulates mesangial cell mitogenesis via PI3-kinase-mediated MAPK-dependent and AKT kinase-independent manner: involvement of c-fos and p27Kip1

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EGF stimulates mesangial cell mitogenesis via PI3 kinase-mediated MAPK-dependent and AKT kinase-independent manner: involvement of c-fos and p27Kip1. Am J Physiol Renal Physiol 289: F72–F82, 2005. First published February 8, 2005; doi:10.1152/ajprenal.00277.2004.—Epidermal growth factor (EGF) is a potent mitogen for mesangial cells. The mechanism by which EGF induces DNA synthesis is not precisely understood. We investigated the role of phosphatidylinositol (PI3)-kinase in regulating mitogenesis. EGF increased PI3-kinase activity resulting in stimulation of PDK-1 and Akt kinase activities. Blocking of PI3-kinase activity using LY-294002 or adenoviral expression of PTEN, which dephosphorylates PI3,4,5-tris-phosphate and thus inactivates PI3-kinase signaling, significantly inhibits EGF-induced DNA synthesis. Expression of dominant-negative Akt kinase, however, had no effect on DNA synthesis. But it inhibited EGF-induced phosphorylation of FoxO3a transcription factor, thus demonstrating its functional consequences. These data indicate that EGF increases the DNA synthesis in a PI3-kinase-dependent but Akt-independent manner. In addition to activating PI3-kinase signaling, EGF increased Erk1/2 MAPK activity, leading to transcriptional activation of its nuclear target Elk-1 and resulting in c-fos expression. Inhibition of MAPK activity by MEK inhibitor U-0126 abolished EGF-induced DNA synthesis. Because EGF activates PI3-kinase, which also regulates DNA synthesis, the effect of PI3-kinase on MAPK activity was also examined. Inhibition of PI3-kinase signaling blocked EGF-induced MAPK activity as well as Elk-1-dependent reporter transcription and c-fos gene transcription. To further determine the mechanism of EGF-induced DNA synthesis, we investigated the effect of EGF on the cyclin-dependent kinase inhibitor p27Kip1. EGF reduced the expression of p27Kip1. Inhibition of PI3-kinase action or MAPK activity abolished the reduction in p27Kip1 expression induced by EGF. These data provide the evidence that a linear signal transduction pathway involving PI3-kinase-dependent MAPK regulates EGF-induced DNA synthesis in mesangial cells by regulating c-fos and p27Kip1 expression.

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dependent but Akt kinase-independent manner. We show that EGF-stimulated PI3-kinase regulates MAPK activity. Finally, we demonstrate PI3-kinase regulates EGF-induced c-fos gene transcription and p27Kip1 cyclin kinase inhibitor expression, thus providing a mechanism of mitogenesis.

MATERIALS AND METHODS

Materials. Recombinant EGF was obtained from R&D Systems. Nonidet P-40, phenylmethylsulfonil fluoride, phosphatidylinositol, and actin antibody were purchased from Sigma. Aprotinin was obtained from Bayer. AG-1478 was purchased from Calbiochem. PI3-kinase inhibitor LY-294002 and MEK inhibitor U-0126 were obtained from Alexis. Histone H2B was purchased from Roche Molecular Biochemicals. Phospho-Erk1/2 MAPK, phospho-MEK1/2, and phospho-PDK-1 antibodies were obtained from Cell Signaling. Anti-phosphotyrosine antibody 4G10 and Akt antibody were purchased from UBI. MAPK, c-fos, PTEN, and p27Kip1 antibodies and protein G plus agarose beads were obtained from Santa Cruz Biotechnology. Gal-4-Erk-1 fusion plasmid and the GAL-4-luciferase reporter construct were described before (22). Dominant-negative Erk2 was provided by Dr. T. W. Sturgill, University of Virginia. pSRkoAp85 containing dominant-negative p85 subunit was a kind gift of Dr. W. Ogawa, Kobe University, Japan. pSGL PTEN containing wild-type PTEN was a gift from Dr. W. Sellers, Dana-Farber Cancer Institute, Boston, MA. The adenovirus vector containing PTEN was provided by Dr. R. Parsons, Department of Pathology and Medicine, Columbia University, New York, NY. Dual luciferase assay kit was purchased from Promega.

Cell culture and adenovirus infection. Rat glomerular mesangial cells were grown in RPMI 1640 medium containing 17% fetal bovine serum as described (16, 20, 22). The cells were made quiescent by incubating in serum-free RPMI 1640 medium for 48 h. Cells were infected in PBS with Ad PTEN or Ad dominant negative (DN) Akt at multiplicities of infection (moi) of 1 h at room temperature following by addition of serum-free medium (17, 18, 23, 33). Experiments were carried out 24 h postinfection, and 100 ng/ml EGF was followed by addition of serum-free medium (17, 18, 23, 33). PI3-kinase assay. DNA synthesis, DNA synthesis was determined as incorporation of [3H]thymidine into trichloroacetic acid-insoluble material as described (16, 19, 21, 22). First, the time course of EGF-induced DNA synthesis was established and compared with that in response to PDGF (supplemental Fig. S1A and S1B; supplemental figures S1–S4 were accessed at http://ajprenal.physiology.org/cgi/content/full/289/7/R9262/C1). It is obvious that the time course of DNA synthesis is significantly similar initiating at 12 h and sustained till 24 h after growth factor stimulation. For the present study, we used 20 h of EGF stimulation to determine the incorporation of [3H]thymidine. For all experiments, absolute values of [3H]thymidine incorporation are shown. Variation in [3H]thymidine incorporation among different experiments may be due to changes in specific activity of the radiolabel.

**RESULTS**

Tyrosine phosphorylation is necessary for EGF-induced DNA synthesis. We and others showed previously that EGF is a mitogen for mesangial cells (20, 35). For biological activity of EGF, its receptor tyrosine kinase activity is necessary. However, in fibroblasts mutational analysis of the receptor in which all five tyrosine residues, which undergo autophosphorylation, were changed to phenylalanine, revealed that this mutant receptor is capable of inducing DNA synthesis (10). This observation questions the necessity for tyrosine phosphorylation of the receptor for mitogenesis (10). To determine the requirement of the EGFR tyrosine kinase activity for DNA synthesis in mesangial cells, we examined the effect of EGF on tyrosine phosphorylation. EGF increased tyrosine phosphorylation of proteins, including the 170-kDa EGFR, in a time-dependent manner (Fig. 1A). To determine the requirement of EGFR tyrosine kinase activity for DNA synthesis, we employed AG-1478, a specific EGFR tyrosine kinase inhibitor. AG-1478 inhibited EGF-induced DNA synthesis in a dose-dependent manner with half-maximal inhibitory concentration of 70 nM (Fig. 1B). At 100 nM concentration, AG-1478 completely blocked EGF-induced DNA synthesis. This concentration of AG-1478 also abolished EGFR tyrosine phosphorylation (data not shown). These data indicate that EGFR tyrosine kinase activity is essential for DNA synthesis in mesangial cells.

**EGF-induced PI3-kinase activity regulates DNA synthesis.** The role of PI3 kinase in EGF-induced DNA synthesis is controversial (10). The mechanism of activation of PI3-kinase requires association of p85 SH2 domains with the autophosphorylated tyrosine kinase receptors. Because EGFR does not contain the consensus Y-X-X-M motif that binds SH2 domains of p85 subunit of PI3-kinase, the kinase may be recruited to the tyrosine-phosphorylated EGFR by other adaptor proteins. These adaptors directly interact with the receptor, thus recruiting the lipid kinase in a signaling complex (45). To
Fig. 1. Epidermal growth factor (EGF) induces tyrosine phosphorylation and DNA synthesis in mesangial cells. A: equal amounts of lysates from EGF-stimulated mesangial cells were immunoblotted with anti-phosphotyrosine (top) and anti-actin (bottom) antibodies. B: quiescent mesangial cells were incubated with AG-1478 for 1 h followed by incubation with EGF. [.H]Thymidine incorporation assay was performed as a measure of DNA synthesis (21, 22). Means of triplicate determinations ± SE are shown. *P < 0.05 vs. control. **P < 0.05 vs. EGF treated.

examine activation of PI3-kinase in response to EGF in a signaling complex, anti-phosphotyrosine immunoprecipitates were employed in an immunocomplex kinase assay. EGF increased PI3-kinase activity within 5 min, which was sustained for 15 min (Fig. 2A). To examine the involvement of PI3 kinase in DNA synthesis, mesangial cells were incubated with different concentrations of LY-294002 followed by treatment with EGF. EGF-induced DNA synthesis was significantly inhibited by LY-294002 in a dose-dependent manner, with a half-maximal inhibitory concentration of 6.0 µM (Fig. 2B). EGF-induced PI3-kinase activity was readily inhibited by preincubation of cells with LY-294002 (Fig. 2C, compare lane 4 with lane 2).

To confirm the above observation, we used the tumor suppressor protein PTEN, which dephosphorylates the D3 phosphate of the PI3-kinase product PI 3,4,5-tris-phosphate (PIP3) and results in inhibition of signaling induced by the lipid kinase. To express PTEN in mesangial cells, we used an adenovirus vector, which expressed the protein in a time-dependent manner (Fig. 3A). To examine the functional consequence of PTEN, we tested its effect on activation of PDK-1, which is a direct target of PIP3 (49). Lysates of mesangial cells incubated with EGF were immunoblotted with a phospho-PDK-1-specific antibody that recognizes only the activated form of this kinase. EGF increased PDK-1 phosphorylation in a time-dependent manner (Fig. 3B). Expression of PTEN prevented EGF-induced activation of PDK-1, indicating that PTEN was acting as the bona fide PIP3 phosphatase (Fig. 3C), which should inhibit downstream signaling of PI3-kinase (49). Consequently, expression of PTEN abolished EGF-induced DNA synthesis (Fig. 3D). These data indicate that PI3-kinase activation is essential for EGF-induced DNA synthesis in mesangial cells.

EGF-stimulated Akt serine threonine kinase is not required for DNA synthesis. Akt is one of the downstream targets of PI3-kinase (15, 16). Akt kinase activity is involved in growth factor-induced cell survival as well as proliferation (9). We recently showed that Akt activity is necessary for PDGF-induced DNA synthesis in mesangial cells (16). However, contradictory results exist about EGF-induced Akt activation. For example, in tumor cells EGF activates Akt kinase activity and this activation subserves the anti-apoptotic function of the kinase. However, in fibroblasts, EGF does not activate Akt kinase activity (15). Therefore, in mesangial cells, first we examined activation of Akt in response to EGF using an immunocomplex kinase assay. EGF time dependently increased Akt kinase activity (Fig. 4A). Treatment of mesangial cells with the PI3-kinase inhibitor LY-294002 blocked EGF-induced Akt kinase activity, indicating that Akt is a downstream target of PI3-kinase in these cells (Fig. 4B). To address the role of Akt in DNA synthesis, we used an adenovirus vector to express dominant-negative Akt kinase (Ad DN Akt), which blocks the endogenous Akt activity in response to various growth factors in mesangial cells (16, 23). Ad DN Akt-infected mesangial cells were stimulated with EGF. Expression of dominant-negative Akt inhibited EGF-induced Akt activity (Fig. 4C). To investigate the functional consequence of Akt inhibition, we examined the phosphorylation of Akt substrate FoxO3a transcription factor (23). EGF increased the phosphorylation of FoxO3a (Fig. 4D, compare lane 2 with lane 1). Expression of dominant-negative Akt abolished EGF-induced phosphorylation of FoxO3a (Fig. 4D, compare lane 4 with lane 2), indicating that DN Akt is functionally active in these cells. However, expression of DN Akt did not have any effect on EGF-induced DNA synthesis (Fig. 4E). These data indicate that PI3-kinase regulates EGF-induced DNA synthesis independently of Akt kinase activity.

PI3-kinase regulates MAPK activity in mesangial cells. Stimulation of EGFR is associated with activation of MAPK, which has been linked to the proliferative effect of EGF in many tumor cells (41). However, our results show that PI3-kinase regulates EGF-induced DNA synthesis in mesangial cells (Figs. 2B and 3D). These results prompted us to examine MAPK as a downstream effector of PI3-kinase. Immunoblot analysis of lysates of mesangial cells with a phospho-Erk1/2-specific antibody that specifically recognizes the activated form of Erk1/2 type MAPK showed a time-dependent increase in activation of MAPK in response to EGF (Fig. 5A). Incubation of mesangial cells with the MEK inhibitor U-0126 inhibited EGF-dependent MAPK activity and resulted in attenuation of DNA synthesis (Fig. 5, B and C). To examine whether PI3-kinase regulates MAPK activity, lysates of mesangial cells
pretreated with the PI3-kinase inhibitor LY-294002 and incubated with EGF were immunoblotted with phospho-Erk-1/2 antibody. Inhibition of PI3-kinase abolished EGF-induced MAPK activation (Fig. 5D). To confirm this observation, Ad PTEN-infected mesangial cells were incubated with EGF followed by immunoblot analysis of the cell lysates with a phospho-Erk antibody. Expression of PTEN abolished EGF-induced MAPK activation (Fig. 5E). Because MAPK is activated by its upstream kinase MEK, to study the mechanism of PI3-kinase action on MAPK, effect of the PI3-kinase inhibitor LY-294002 on activation of MEK was examined. EGF increased activation of MEK as determined by the phospho-MEK immunoblotting using an antibody that specifically recognizes the activated form of this kinase (Fig. 5F, compare lane 2 with lane 1). LY-294002 abolished EGF-induced activation of MEK (Fig. 5F, compare lane 4 with lane 2). These data demonstrate that inhibition of PI3-kinase signaling abolishes EGF-induced MAPK activation by inactivating its upstream kinase MEK.

**PI3-kinase regulates EGF-induced c-fos transcription.** The mitogenic action of EGF initiated by MAPK involves phosphorylation of transcription factors that induce de novo gene expression. One of the targets of MAPK is the ETS domain transcription factor Elk-1. COOH-terminal serine phosphorylation of Elk-1 is necessary for its ability to transactivate target genes (52). Because PI3-kinase regulates MAPK activity, to examine the effect of the lipid kinase on Elk-1 activation, we first studied MAPK regulation of this transcription factor. We cotransfected into mesangial cells expression vectors encoding the Elk-1 COOH-terminal transactivation domain fused to the GAL-4 DNA binding domain (GAL-4 dbd-Elk-1) with a firefly luciferase reporter plasmid under the control of a GAL-4 DNA element. EGF stimulated transcription of the reporter gene (Fig. 6A). EGF did not affect the reporter transcription induced by the empty GAL-4 DNA binding domain vector (Supplemental Fig. S2). These data demonstrate the specificity of the Elk-1 COOH-terminal transactivation domain used in this assay. Expression of dominant-negative Erk-2 along with
GAL-4 dbd-Elk1 abolished EGF-induced reporter transcription (Fig. 6A). Cotransfection of a deletion mutant of the regulatory subunit of PI3-kinase, which acts as a dominant negative, also blocked EGF-induced Elk-1-dependent transcription of the reporter gene (Fig. 6B). Similarly, expression of PIP3 phosphatase PTEN, a protein inhibitor of PI3-kinase signaling, attenuated EGF-induced DNA synthesis. Quiescent mesangial cells were infected with Ad GFP or Ad PTEN followed by incubation with EGF. DNA synthesis was measured as described in Fig. 1. *P < 0.05 vs. control. **P < 0.005 vs. EGF treated.

Fig. 3. Expression of PTEN blocks EGF-induced DNA synthesis. A: expression of PTEN in mesangial cells. Lysates of mesangial cells infected with Ad PTEN for different periods of time were immunoblotted with anti-PTEN (top) and anti-actin (bottom) antibodies. B: effect of EGF on activation of PDK-1. Lysates of mesangial cells incubated with EGF for different periods of time were immunoblotted with phospho-PDK-1 (top) and anti-PDK-1 antibodies. C: expression of PTEN inhibits PDK-1 activation. Ad GFP or Ad PTEN-infected mesangial cells were stimulated with EGF for 5 min and the cell lysates were immunoblotted with anti-phospho-PDK-1 (top), anti-PTEN (middle), and anti-actin (bottom) antibodies. D: expression of PTEN inhibits EGF-induced DNA synthesis. Quiescent mesangial cells were infected with Ad GFP or Ad PTEN followed by incubation with EGF. DNA synthesis was measured as described in Fig. 1. *P < 0.05 vs. control. **P < 0.005 vs. EGF treated.

PI3-kinase regulates EGF-induced cyclin kinase inhibitor p27Kip1 expression. Growth factor induced DNA synthesis involves regulation of cyclin-dependent kinase (CDK) activity by cyclin kinase inhibitors (CKIs) to induce cell cycle progression from G1 to S phase. p27Kip1 inhibits the activity of CDK2 in vitro and in vivo. We explored the role of PI3-kinase in regulation of p27Kip1 expression. Quiescent mesangial cells expressed significant amounts of p27Kip1 protein, as expected with their reduced basal DNA synthesis. Incubation of mesangial cells with EGF decreased the level of p27Kip1 protein, as expected with their reduced basal DNA synthesis. Incubation of mesangial cells with EGF decreased the level of p27Kip1 protein, as expected with their reduced basal DNA synthesis. Incubation of mesangial cells with EGF decreased the level of p27Kip1 protein, as expected with their reduced basal DNA synthesis.

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significant effect on the p27Kip1 compared with the baseline (Fig. 8B, compare lane 3 with lane 1). However, LY-294002 significantly reversed the inhibitory effect of EGF on p27Kip1 level (Fig. 8B, compare lane 4 with lane 2 and the quantitation below). To confirm this observation, PTEN was expressed in mesangial cells to inhibit PI3-kinase signaling. Expression of PTEN significantly reversed the EGF-induced reduction of p27Kip1 expression (Fig. 8C, compare lane 4 with lane 2). Because we established above that PI3-kinase regulates EGF-induced MAPK in mesangial cells (Fig. 5D), the MEK inhibitor U-0126 was used to inhibit MAPK and p27Kip1 expression was examined. Inhibition of MAPK significantly blocked the reduction of p27Kip1 expression in the presence of EGF (Fig. 8D, compare lane 4 with lane 2). These data conclusively demonstrate that PI3-kinase and MAPK signaling regulate EGF-induced expression of p27Kip1 in mesangial cells.

**DISCUSSION**

Previous reports suggested that EGF receptor signaling plays an important role in proliferative glomerular injury. In the present study, we show that PI3-kinase regulates EGF-induced DNA synthesis in mesangial cells in Akt kinase-independent manner. We provide evidence that PI3-kinase targets MAPK, which in turn stimulates EGF-mediated DNA synthesis concomitant with Elk-1-dependent c-fos expression. Furthermore, we demonstrate that PI3-kinase stimulates EGF-induced mitogenesis of mesangial cells by downregulating the cyclin kinase inhibitor p27Kip1 and that PI3-kinase and MAPK regulate p27Kip1 expression.

PI3-kinase plays an important role in growth factor-induced proliferation of cells including mesangial cells (21). However, its role in EGF-induced DNA synthesis is controversial. In the present study, we demonstrated that EGF stimulates PI3-kinase activity in a tyrosine-phosphorylated...
protein fraction in mesangial cells (Fig. 2A). LY-294002 inhibits EGF-induced DNA synthesis, suggesting involvement of PI3-kinase in mitogenesis (Fig. 2). PIP_3 is the biologically active lipid product of PI3-kinase, which activates PDK-1 and plays an important role in cell proliferation. To support this notion, we demonstrated that EGF stimulated PDK-1 (Fig. 3B). Furthermore, we provide evidence that PTEN, which is a PIP_3 phosphatase, blocks EGF-induced PDK-1 activation (Fig. 3C).

One of the targets of PI3-kinase and PDK-1 is the serine threonine kinase Akt (15, 16, 49). A variety of extracellular signals including many growth factors and cytokines, integrin engagement, T-cell receptor engagement, hypoxia, hyperosmolality, and heat shock, all stimulate Akt kinase activity (9). Conflicting results exist about EGF-induced Akt activation. For example, in tumor cells EGF induces Akt kinase activity and this activation is attributed to Akt’s antiapoptotic function (5, 9). On the other hand, in fibroblasts, EGF does not activate Akt kinase activity (15). Our data in this study show that EGF activates Akt kinase activity in mesangial cells in a PI3-kinase-sensitive manner (Fig. 4, A and B). Surprisingly, the EGF-induced increase in Akt kinase activity is not necessary for DNA synthesis (Fig. 4E). Our results indicate that in mesangial cells, EGF receptor tyrosine kinase activation utilizes PI3-kinase activity to regulate DNA synthesis by regulating signaling molecules other than Akt kinase.

We reported previously that EGF stimulates MAPK activity in mesangial cells resulting in DNA synthesis (20). Several studies including our own demonstrated that PI3-kinase inhibitors blocked MAPK activity by some stimuli such as thrombin, insulin, IGF-1, PDGF, and lysophosphatidic acid (7, 21, 26). But a previous report showed that PI3-kinase did not have any effect on EGF-induced MAPK activity (26). Recently, it has been shown that the basal activity of PI3-kinase may regulate EGF-induced MAPK activation, in the absence of PI3-kinase activation in response to EGF (51). Furthermore, in Cos-7 cells overexpression of the constitutively active catalytic subunit of PI3-kinase did not increase MAPK activity, sug-

Fig. 5. PI3-kinase-dependent MAPK regulates EGF-induced DNA synthesis in mesangial cells. A: effect of EGF on MAPK activity. Lysates of mesangial cells incubated with EGF for different periods of time were immunoblotted with anti-phospho-Erk (top) and anti-Erk1/2 (bottom) antibodies. B: effect of MEK inhibitor U-0126 on EGF-induced MAPK activity. Quiescent mesangial cells were incubated with 5 μM U-0126 for 1 h before incubation with EGF. The lysates were immunoblotted with phospho-Erk (top) and anti-Erk1/2 antibodies. C: inhibition of MAPK blocks EGF-induced DNA synthesis. Quiescent mesangial cells were incubated with 5 μM U-0126 for 1 h before incubation with EGF and DNA synthesis was measured as described in Fig. 1. Means of triplicate determinations ± SE are shown. *P < 0.05 vs. control. **P < 0.05 vs. EGF treated. D: inhibition of PI3 kinase blocks EGF-induced MAPK activation. Lysates of mesangial cells incubated with 10 μM LY-294002 before stimulation with EGF were immunoblotted with phospho-Erk (top) and anti-Erk1/2 (bottom) antibodies. E: expression of PTEN blocks EGF-induced MAPK activation. Lysates of mesangial cells infected with Ad GFP or Ad PTEN followed by incubation with EGF were immunoblotted with phospho-Erk (top), anti-PTEN (middle), and Erk1/2 (bottom). F: inhibition of PI3-kinase blocks EGF-induced activation of MEK. Lysates of mesangial cells incubated with 10 μM LY-294002 followed by EGF were immunoblotted with anti-phospho-MEK antibody that recognizes the activated form of the kinase. Bottom: anti-actin immunoblot.
gesting that increased PI3-kinase activity is not sufficient for MAPK activation (51). Furthermore, and similar to our results in mesangial cells, in hepatocytes, EGF stimulates DNA synthesis by increased PI3-kinase activity. However, this increase in PI3-kinase activity was shown to block activation of MAPK, suggesting a negative regulatory role of PI3-kinase on MAPK.

In contrast to these results, our results demonstrate that PI3-kinase activity in response to EGF regulates MAPK in mesangial cells (Fig. 5D). This observation is also confirmed by our data demonstrating that the PIP3 phosphatase PTEN downregu-

Fig. 6. MAPK and PI3-kinase regulate EGF-induced Elk-1 transactivation. Effect of dominant-negative MAPK (A), dominant-negative PI3-kinase (B), and PTEN (C) expression on EGF-induced Elk-1-mediated transcription. Mesangial cells were transfected with the GAL-4-luciferase reporter plasmid, GAL-4-Erk1 fusion plasmid, and Renilla luciferase construct along with vector alone or dominant-negative Erk2 (DN Erk2; A) or dominant-negative PI3-kinase (deletion mutant of p85 regulatory subunit, DN p85; B) or PTEN (C) expression vectors as described in MATERIALS AND METHODS (16, 18, 20, 22, 23). Serum-starved cells were incubated with EGF and the luciferase activity was measured in the cell lysate as described in MATERIALS AND METHODS. D: dominant-negative PI3-kinase does not affect constitutively active MEK-induced Elk-1 transactivation. Mesangial cells were transfected with GAL-4-luciferase reporter plasmid, GAL-4-Erk1 fusion plasmid, and Renilla luciferase construct along with vector alone or dominant-negative p85 subunit of PI3-kinase (DN p85). Lysates were assayed as described in MATERIALS AND METHODS. Means of triplicate determinations ± SE are shown. *P < 0.05 vs. control. **P < 0.05 vs. EGF treated.

Fig. 7. PI3-kinase regulates c-fos expression. A: inhibition of PI3-kinase blocks EGF-induced c-fos transcription. A reporter plasmid in which the luciferase cDNA is driven by c-fos promoter was transiently transfected with Renilla luciferase plasmid into mesangial cells. Serum-deprived transfected cells were treated with 10 μM LY-294002 followed by incubation with EGF. Luciferase activity was measured as described in MATERIALS AND METHODS. B: expression of dominant-negative PI3-kinase blocks EGF-induced c-fos transcription. A vector or dominant-negative PI3-kinase (DN p85) was cotransfected with the fos promoter-driven luciferase reporter plasmid into mesangial cells as described in A. Serum-deprived cells were treated with EGF and luciferase activity in the cell lysates was determined as described in MATERIALS AND METHODS (16, 18, 20, 23). C: expression of PTEN inhibits c-fos transcription. Same reporter plasmids as in A and B were transfected into mesangial cells with vector or with pSGL PTEN, which codes for PTEN protein. Serum-deprived cells were incubated with EGF and luciferase activity in the cell lysates was measured. Means of triplicate determinations ± SE are shown *P < 0.05 vs. control. **P < 0.05 vs. EGF treated.
lates EGF-induced MAPK activity (Fig. 5E). Furthermore, as one of the mechanisms, we found that PI3-kinase regulates MAPK activating kinase MEK (Fig. 5F). Because PI3-kinase/PDK-1 target Akt does not play any role in EGF-induced DNA synthesis, it is possible that other target of these kinases may regulate MAPK activity to finally regulate the DNA synthesis. In fact, many isoforms of PKCs including those, which are regulated by diacylglycerol, are targets of PI3-kinase/PDK-1 (13, 32, 38). PKC regulates MAPK activity in response to many growth factors including EGF (3, 54). We examined the role of PKC on EGF-induced MAPK activation by phospho-Erk immunoblotting. Downregulation of PKC by chronic phorbol ester treatment partially inhibited EGF-induced MAPK activation (Supplemental Fig. S4, compare lane 4 with lane 2). These data indicate that PKC, which may be target of PI3-kinase/PDK-1 in EGF signal transduction, regulates MAPK activation in mesangial cells. We demonstrated that MAPK is essential for EGF-induced DNA synthesis (Fig. 5C). However, a very small contribution of EGF-induced DNA synthesis seems MAPK-independent (Fig. 5C). Now, we provide evidence that PI3 kinase also regulates mitogenesis in response to EGF.

The positive regulatory role of PI3-kinase on MAPK indicates that PI3-kinase should modulate transcriptional targets of MAPK necessary for the mitogenic effect of EGF. In mammalian cells, one of the downstream targets of MAPK is the ETS domain containing transcription factor Elk-1. We showed here that expression of dominant-negative MAPK blocks EGF-induced transactivation of Elk-1 in mesangial cells (Fig. 6A). Furthermore, our data demonstrate that PI3-kinase regulates Elk-1-dependent transcription of reporter genes (Fig. 6, B and C). These data provide the first evidence that PI3-kinase regulates EGF-induced Elk-1 transactivation. One of the downstream targets of Elk-1 is the early response gene c-fos (50, 52). Mutation of the phosphorylation sites in the COOH-terminal domain of Elk-1 inhibits c-fos gene expression, in-
cating that activation of MAPK is essential for c-fos expression (34). Our data demonstrate that PI3-kinase-dependent MAPK activation (Fig. 5, D and E) regulates Elk-1 transactivation in mesangial cells (Fig. 6). In accordance with these results, we show that PI3-kinase regulates c-fos expression (Fig. 7). It should be noted that inhibition of PI3-kinase also attenuated the basal c-fos transcription (Fig. 7). These results suggest that the lipid kinase also plays a central role in basal expression of c-fos gene as basal PI3-kinase activity has been shown previously to regulate certain biological activity (51). Together, these data indicate the presence of a linear relationship between PI3-kinase and MAPK to regulate c-fos expression possibly by utilizing Elk-1 transcription factor.

Mitogenesis induced by growth factors is regulated by the concerted action of cyclin-dependent kinases and CKIs (47). Increase in the abundance of the CKI p27Kip1 correlates with different biological functions. Apoptosis was decreased in hematopoietic cells isolated from p27Kip1 heterozygous and null mice (31). On the other hand, mesangial cells and fibroblasts isolated from p27Kip1 heterozygous and nullizigous mice showed increased apoptosis compared with wild-type cells (27). Studies in different cell types revealed that quiescent state of the cells in G1 phase is always associated with abundant p27Kip1 expression (1, 6, 53). Administration of EGF to rats reduces hepatic accumulation of p27Kip1 (2). Our results in mesangial cells demonstrating that EGF reduces the level of p27Kip1 also confirm this observation (Fig. 8A). We provided evidence that inhibition of PI3-kinase activity in mesangial cells blocks EGF action by increasing p27Kip1 protein level (Fig. 8, B and C). These data indicate that PI 3 kinase negatively regulates expression of p27Kip1. Furthermore, our data demonstrate a temporal correlation between reduced p27Kip1 abundance and increased DNA synthesis in response to EGF. PI3-kinase reverses this correlation to inhibit EGF-induced mitogenesis.

p27Kip1 abundance is mainly regulated by protein degradation via proteasomal pathways (37). Activated MAPK phosphoraylates p27Kip1, which then cannot associate with and inhibit the CDK2 activity necessary for G1/S transition (30). On the other hand, MAPK-phosphorylated p27Kip1 serves as a target for 26S proteasome for degradation (30). In fact, inhibition of MAPK activity blocked EGF-induced reduction in the p27Kip1 level (Fig. 8D). Thus it is possible that reduced expression of p27Kip1 protein in the presence of EGF may result from increased phosphorylation by activated MAPK followed by proteasomal degradation.

In summary, we investigated the role of PI 3 kinase in EGF-induced mitogenesis in mesangial cells. Our data provide evidence that PI3-kinase regulates EFG-induced MAPK activity. This cross talk of signaling pathways increases transactivation of Elk-1 transcription factor to finally modulate c-fos gene expression. Additionally, our data demonstrate that PI3-kinase-dependent MAPK regulates EGF-induced DNA synthesis in mesangial cells. Furthermore, regulation of p27Kip1 by the PI3-kinase/MAPK axis contributes to the effect of EFG on DNA synthesis. Because proliferation of mesangial cells is a major pathology in glomerulonephritis, selective therapeutic targeting of PI3-kinase may prove to be effective in preventing this disease.

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