Vascular endothelial growth factor (VEGF) increases protein synthesis and induces hypertrophy in renal tubular epithelial cells (Senthil, D., Choudhury, G. G., McLaurin, C., and Kasinath, B. S. (2003) *Kidney Int. 64*, 468–479). We examined the role of Erk1/2 MAP kinase in protein synthesis induced by VEGF. VEGF stimulated Erk phosphorylation that was required for induction of protein synthesis. VEGF-induced Erk activation was not dependent on phosphoinositide (PI) 3-kinase activation but required sequential phosphorylation of type 2 VEGF receptor, PLCγ and c-Src, as demonstrated by inhibitors SU1498, U73122, and PP1, respectively. c-Src phosphorylation was inhibited by U73122, indicating it was downstream of phospholipase (PLC)γ. Studies with PP1/2 showed that phosphorylation of c-Src was required for tyrosine phosphorylation of Raf-1, an upstream regulator of Erk. VEGF also stimulated phosphorylation of Pyk-2; VEGF-induced phosphorylation of Pyk-2, c-Src and Raf-1 could be abolished by BAPTA/AM, demonstrating requirement for induction of intracellular calcium currents. We examined the downstream events following the phosphorylation of Erk. VEGF stimulated phosphorylation of Mnk1 and eIF4E and induced Mnk1 to shift from the cytoplasm to the nucleus upon phosphorylation. VEGF-induced phosphorylation of Mnk1 and eIF4E required phosphorylation of PLCγ, c-Src, and Erk. Expression of dominant negative Mnk1 abrogated eIF4E phosphorylation and protein synthesis induced by VEGF. VEGF-stimulated protein synthesis could be blocked by inhibition of PLCγ by a chemical inhibitor or expression of a dominant negative construct. Our data demonstrate that VEGF-stimulated protein synthesis is Erk-dependent and requires the activation of VEGF receptor 2, PLCγ, c-Src, Raf, and Erk pathway. VEGF also stimulates Erk-dependent phosphorylation of Mnk1 and eIF4E, crucial events in the initiation phase of protein translation. The response of a cell to injurious stimuli includes increase in cell protein synthesis (hypertrophy), cell division (hyperplasia) or apoptosis. Of these processes, the mechanisms underlying cell hypertrophy have not been well understood. Regulation of cellular protein synthesis occurs at the initiation phase of protein translation (1). One of the main events in initiation phase is phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1, 15–20 kDa) (2). Phosphorylation of this repressor protein results in dissociation of eIF4E-eIF4E-BP1 complex, releasing the eIF4E to bind to the mRNA cap and promote translation initiation (3). An increase in eIF4E phosphorylation is commonly seen during the initiation phase and has been attributed to Erk1/2-type MAP kinase (4). However, Erk is not the direct kinase for eIF4E; it activates Mnk1/2 kinase, which, in turn, phosphorylates Ser-209 on eIF4E (5, 6). Agonists that induce Mnk1 phosphorylation have not been well studied. We examined whether VEGF regulates this important kinase.

Functional effects of VEGF as an angiogenic agent and as a key regulator of endothelial cell functions have been well documented. Our recent observations have shown that VEGF increases protein synthesis and induces hypertrophy in renal proximal tubular epithelial (MCT) cells, suggesting a role for VEGF in non-angiogenic processes in non-endothelial cells (7). Previously, we have also shown that VEGF stimulation of protein synthesis and hypertrophy in MCT cells recruit PI 3-kinase-Akt axis to induce phosphorylation of 4E-BP1 (7). In that study, the role of Erk in VEGF-induced protein synthesis was not examined. Increase in Erk phosphorylation in the renal cortex of mice with type 2 diabetes occurs simultaneously with the onset of kidney hypertrophy that mostly involves renal tubular epithelial cells (8). Coinciding with Erk phosphorylation and kidney hypertrophy, VEGF expression in the kidney is increased (7), and anti-VEGF antibody reverses kidney enlargement (9). However, whether VEGF recruits Erk pathway in regulation of protein synthesis and renal cell hypertrophy is not known. Also unknown is the identity of upstream kinases that regulate Erk activity in response to VEGF in MCT cells. In this study, we tested the hypothesis that PLCγ, an
important kinase that binds to type 2 VEGF receptor (VEGFR2), plays a regulatory role in VEGF-induced Erk activation and protein synthesis. Downstream of Erk, we also studied whether VEGF regulated Mnk1 phosphorylation and its target, eIF4E.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium, penicillin and streptomycin were purchased from Invitrogen. Fetal bovine serum was purchased from Hydline Laboratories, Inc. VEGF-165 recombinant protein and anti-actin antibody were purchased from Sigma. Anti-phosphotyrosine antibody 4G10 was purchased from Upstate, Inc. (Charlottesville, Virginia). Antibody against Mnk1 and anti v-Src antibody were bought from Santa Cruz Biotechnology (Santa Cruz, CA) and Oncogene Research Products (San Diego, CA), respectively. All other antibodies were purchased from Cell Signaling (Beverly, MA). \(^{35}S\) Labeled methionine was purchased from PerkinElmer Life Sciences. ECL chemiluminescent substrate reagent was from Pierce Biotechnology. Inc. PP1 and PP2 analog, SU1498, LY294002, and BAPTA/AM were purchased from Calbiochem-EMD Biosciences. As PP1 is no longer available we employed PP2 as an inhibitor of c-Src. U73122 was from Biomol International (Plymouth Meeting, PA), and U0126 was purchased from Promega.

**Cell Culture**—SV-40-immortalized murine proximal tubular epithelial (MCT) cells (kindly provided by Dr. Eric Neilson, Vanderbilt University, Nashville, TN) were grown in Dulbecco’s modified Eagle’s medium containing 7% fetal bovine serum, 5 mmol/liter glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mm glutamine (7). MCT cells express in vivo properties of proximal tubular epithelial cells (10). The cells were grown to 90% confluence and then growth-arrested for 18 h in serum-free Dulbecco’s modified Eagle’s medium before experiments.

**Immunoblotting**—Equal amounts of protein from control and VEGF-treated cells were separated by SDS-PAGE and transferred to a nitrocellulose membrane (7). After transfer overnight at 4 °C, the membrane was blocked in Tris-buffered saline, pH 7.2, containing 0.1% Tween 20 (TBST) for 1 h. The membrane was then washed and probed with primary antibody for 3 h. After extensive washing in TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories, Inc, West Grove, PA). Proteins were visualized by chemiluminescence using ECL reagent. The images of the bands were scanned by reflectance scanning densitometry, and the intensity of the bands was quantified using NIH Image software.

**Protein Synthesis Measurement**—Serum-starved cells were incubated with 20 ng/ml of VEGF-165 in the presence of 10 µCi/ml \(^{35}S\) Methionine for 2 h with or without the respective inhibitors (11). Cells

**FIG. 1.** A, VEGF stimulates Erk phosphorylation. Serum-deprived cells were incubated with VEGF (20 ng/ml). Equal amounts of protein from the cell lysates were fractionated on a 12.5% gel. Immunoblotting was performed using a 1:2000 dilution of an antibody that specifically detects phospho-Erk. The blots were stripped and reprobed with an Erk antibody to assess loading. A representative blot from more than three experiments is shown. *, \(p < 0.01\) VEGF versus control. B, VEGF stimulation of protein synthesis is Erk-dependent. Quiescent MCT cells were incubated with or without VEGF (20 ng/ml) simultaneously in the presence of \(^{35}S\) methionine for 2 h with or without preincubation with 25 µM U0126, a MEK inhibitor, for 30 min. Incorporation of radiolabel into trichloroacetic acid-precipitable protein was taken as a measure of protein synthesis and expressed as percentage of control. Composite data from three experiments is shown in a graph. *, \(p < 0.01\) VEGF versus control. **, \(p < 0.001\) VEGF versus control. C, VEGF-induced Erk activation is independent of PI 3-kinase activation. MCT cells were incubated with or without VEGF (20 ng/ml) for 10 min with or without preincubation with LY294002 (25 µM) or U0126 (25 µM) for 30 min. Equal amounts of cell lysates (100 µg) were immunoprecipitated with an anti-Erk antibody. Erk activity was determined in the immunoprecipitates by an in vitro kinase assay using myelin basic protein (MBP) as the substrate. The samples were fractionated by 15% SDS-PAGE, and phosphorylation of myelin basic protein was visualized by autoradiography. Immunoblotting with total Erk antibody was done to assess loading, and band intensities were measured by densitometry. A representative blot from three individual experiments is shown. The graph presents composite densitometric data from three experiments with controls expressed as 100%. *, \(p < 0.001\) VEGF or VEGF + LY294002 versus control; **, \(p < 0.001\) VEGF + U0126 versus VEGF, determined by ANOVA.
FIG. 2. A, VEGF-induced tyrosine phosphorylation of proteins is dependent on VEGFR2 tyrosine phosphorylation. Serum-starved MCT cells were incubated with VEGF (20 ng/ml) for 10 min with or without preincubation with SU1498, the selective VEGFR2 inhibitor. Equal amounts of cell lysates were resolved on SDS-PAGE and immunoblotted with phosphotyrosine antibody. A representative blot from three independent experiments is shown. The bottom panel shows immunoblot analysis of the same samples with anti-actin antibody. B and C, VEGF stimulates phosphorylation of PLCγ (B) and c-Src kinase (C). Serum-starved MCT cells were incubated with VEGF (20 ng/ml) for the time periods indicated. Equal amounts of cell lysates were resolved on SDS-PAGE and immunoblotted with phosphospecific antibodies for PLCγ and c-Src, and loading was assessed by immunoblotting with antibodies against PLCγ and Src, respectively. A representative blot from three independent experiments is shown. D and E, VEGF-induced phosphorylation of PLCγ and c-Src is mediated through VEGFR2. Cells were incubated with or without VEGF (10 ng/ml) for 10 min after preincubation for 30 min with or without 10 μM SU1498, a VEGFR2-selective inhibitor. Equal amounts of cell lysates were fractionated on SDS-PAGE and immunoblotted with the phosphospecific antibodies for PLCγ and c-Src, and loading was assessed by immunoblotting with antibodies against PLCγ and Src, respectively. A representative blot from three independent experiments is shown. F, c-Src phosphorylation is PLCγ-dependent. Cells were pretreated with or without U73122 (5 μM), a PLC inhibitor, prior to incubation for 10 min with or without VEGF 20 ng/ml. A Western blot analysis was performed with the cell lysates using phospho-Src antibody. The lower panel represents immunoblot analyses of the same samples with Src antibody to assess loading. A representative blot from three independent experiments is shown.

were then washed in phosphate-buffered saline and lysed in radiolabeled protein precipitation assay buffer. An equal amount of protein (20 μg) was spotted onto the 3-mm filter paper (Whatman). Filters were washed three times by boiling for 1 min in 10% trichloroacetic acid containing 0.1 g/liter methionine before determining radioactivity.

Erk1/2 MAP kinase (Erk) activity assay—Equal amounts of cell lysates (200 μg) were immunoprecipitated with Erk1/2 antibody (12). Protein A-agarose beads were added and incubated at 4 °C for 1 h. The beads were then washed, and the Erk kinase assay was performed in kinase assay buffer in the presence of 50 μM of cold ATP, 1 μCi of [γ-32P]ATP, and 5 μg of myelin basic protein at 30 °C for 30 min. The reaction was arrested by the addition of an equal volume of 2× sample buffer. Phosphorylated bands were detected by SDS-PAGE followed by autoradiography. In some experiments, immunoblotting with antiphospho-Erk antibody was done to assess Erk phosphorylation.

Immunofluorescence Microscopy—Cells were seeded in 8-well chamber slides. Semiconfluent cells were serum-starved for 18 h and treated with VEGF (20 ng/ml) for different time periods. Cells were washed with phosphate-buffered saline, fixed, and incubated with rabbit anti-phospho-Mnk antibody followed by staining with Cy5-conjugated donkey anti-rabbit secondary antibody (Chemicon International, Temecula, CA). Phospho-Mnk1 was visualized with a confocal laser microscopy system (Olympus Fluoview 500). The confocal images were analyzed using Fluoview software to determine the localization of phospho-Mnk1.

For quantification of phospho-Mnk1 nuclear localization, cells were grown in chamber slides, with individual chambers representing one time point. Cells were viewed under uniform magnification (40×). For each time point, the number of cells showing nuclear localization of phospho-Mnk1/total number of cells in a field of fixed area containing a minimum of 150 cells was counted and converted to percentage. Finally, these values were compared with that of value at time 0 (control). The experiment was performed three times, and composite data were converted into graphic form and analyzed by analysis of variance (ANOVA).

Transfection Studies—Plasmid constructs containing dominant negative mutants of Src and PLCγ (PLCζ) were employed for transient transfection in MCT cells using Lipofectamine and lipo-plus reagent (Invitrogen), as described earlier (13). PCR 295 expression vector containing dominant negative c-Src was kindly provided by Dr. T. Yoneda, University of Texas Health Science Center, San Antonio, TX. The kinase inactivation mutation in c-Src consisted of Lys295–Met295. PLCζ is a dominant negative fragment of PLCγ gene which encodes the Z region (PCI region, PLC inhibitor region) containing the SH2 and SH3 domains (amino acids 517–901) of this enzyme (14). The pXφPLCζ expression vector was driven by SV40 early promoter. pEBG-3X eukaryotic expression vector containing dominant-interfering Mnk1 mutant (pEBG-T2A2 containing T197A/T202A mutants) was a kind gift from Dr. J. Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA).

Statistics—All values are expressed as mean ± S.E. obtained from at least three independent experiments. Statistical analysis was performed using one-way ANOVA for comparison between multiple groups; p values of p < 0.05 were considered significant.

RESULTS

VEGF-induced Protein Synthesis Is Erk-dependent in MCT Cells—We investigated whether VEGF induced Erk phosho-
rylation and whether VEGF-induced protein synthesis was Erk-dependent because Erk occupies a central position in transducing signals from the extracellular milieu to the nucleus. VEGF promoted Erk phosphorylation within 2 min that lasted nearly 15 min with peak phosphorylation at 10 min (Fig. 1A). VEGF significantly stimulated protein synthesis in MCT cells that was abrogated by preincubation of cells with U0126, an inhibitor of MEK, the direct upstream kinase of Erk (Fig. 1B). VEGF induction of protein synthesis is, thus, dependent on the stimulation of Erk.

We initiated the search for upstream regulators of VEGF-induced Erk activation. We started with PI 3-kinase as insulin- and insulin-like growth factor 1-induced Erk activation in MCT cells is PI 3-kinase-dependent (12, 13). We have previously shown that VEGF induces PI 3-kinase activity in these cells (7). Preincubation with LY294002, a selective inhibitor of PI 3-kinase, did not affect VEGF-induced Erk activity, although it was abolished by U0126 (Fig. 1C). These observations suggest that VEGF-induced Erk activation is independent of PI 3-kinase but dependent on MEK activation.

VEGF stimulated tyrosine phosphorylation of PLCγ and c-Src rapidly, within 5 min (Fig. 2, B and C). VEGFR2 is believed to mediate most of the biological effects of VEGF. Pretreatment of cells with SU1498 abolished VEGF-induced PLCγ and c-Src phosphorylation (Fig. 2, D and E), suggesting involvement of VEGFR2. Next, we examined the sequence of phosphorylation of PLCγ and c-Src by VEGF. U73122, a PLC inhibitor, blocked phosphorylation of c-Src, demonstrating that the latter is downstream of PLCγ in VEGFR2 signaling events in MCT cells (Fig. 2F).

We investigated whether PLCγ plays a role in regulating VEGF-induced Erk activation. VEGF-stimulated Erk activation was inhibited by U-73122, the PLC inhibitor (Fig. 3A). This was further confirmed by expressing dominant negative constructs of PLCγ (PLCz). VEGF-stimulated Erk activation was abolished in cells transfected with PLCz but not in control cells transfected with vector alone (Fig. 3B). Although it appears as though expression of PLCz reduced Erk activity only upon addition of VEGF, densitometric analysis of bands from three experiments did not reveal a significant difference in band intensity between VEGF+PLCz and untreated control groups (Fig. 3B, bars 1 versus 4).

We next examined the role of c-Src in VEGF induction of Erk. PP1, a c-Src inhibitor, abolished VEGF-induced Erk phosphorylation (Fig. 4A). Further confirmation was obtained by employing a kinase-inactive dominant negative construct of c-Src (Fig. 4B). Raf is an upstream kinase in Erk pathway (17, 18). VEGF stimulated Raf phosphorylation at Tyr334 and Tyr331 within 5 min that lasted for nearly 15 min (Fig. 4C). This was completely inhibited by PP2, a c-Src inhibitor (Fig. 4D), indicating Tyr334/Tyr331 phosphorylation of Raf was c-Src-dependent. The Src inhibitor, at this concentration, did not affect VEGF-induced PLCγ phosphorylation, which is VEGFR2-dependent (See supplemental Fig. S1).

VEGF induces phosphorylation of Pyk2, c-Src, and Erk in a Calcium-dependent Manner—VEGF increases intracellular...
calcium transients through VEGFR2 (19) that correlate with phosphorylation of PLCγ (20). Activation of PLCγ has been linked to the production of inositol-1,4,5-trisphosphate and diacylglycerol, leading to increased intracellular calcium mobilization and PKC activation, respectively. A read-out of calcium signaling is the phosphorylation of Pyk2, a proline-rich, Ca2+-dependent non-receptor tyrosine kinase. Pyk2 phosphorylation was studied using a phosphospecific antibody that detects phosphorylation on Tyr402. Incubation with VEGF rapidly induced phosphorylation of Pyk2 that lasted for nearly 30 min (Fig. 5A). We next examined the role of Ca2+ in Erk activation induced by VEGF. Chelation of intracellular Ca2+ by BAPTA/AM abolished VEGF-induced phosphorylation of Pyk2, Src, and Erk (Fig. 5, B–D), confirming that Ca2+ transients are required for VEGF-induced phosphorylation of Erk and its upstream regulators.

The data described above established the upstream regulators of VEGF-induced Erk activation along the lines of VEGFR2, PLCγ, Pyk2/c-Src, Raf, Erk. We next sought to gain insight into the possible mechanism downstream of Erk activation related to events of regulatory importance in protein synthesis.

**VEGF Induces Phosphorylation of Mnk1**—As discussed before, phosphorylation of 4E-BP1 releases eIF4E from the dimeric complex. 4E-BP1 phosphorylation is required for VEGF-induced protein synthesis and hypertrophy in MCT cells; this process is PI 3-kinase- and Akt-dependent (7). Following dissolution of eIF4E/4E-BP1 complex, free eIF4E binds to a scaffolding protein eIF4G and is phosphorylated by MAP kinase-integrating kinases (Mnk1/2) that also bind eIF4G at the C terminus (21). Mnk1, a 52-kDa protein, is activated by phosphorylation at Thr197 by Erk. As Erk regulation of protein synthesis could involve Mnk and eIF4E, we examined whether VEGF regulates their phosphorylation. VEGF rapidly stimulated phosphorylation of Mnk1 within 5 min and the effect lasted for nearly 30 min (Fig. 6A). These data suggest that VEGF activates a downstream target of Erk. Mnk1 shuttles between the cytoplasm and the nucleus (22); however, agonists that stimulate nuclear shift of Mnk1 have not been extensively identified. Therefore, we examined whether VEGF regulates the shift of Mnk1 into the nucleus. As seen in Fig. 6B, we observed increased immunofluorescence corresponding to Mnk1 phosphorylation in the cytoplasm within 2 min with perinuclear localization at 5 min. Nuclear translocation was observed at 15 min, coinciding with maximum phosphorylation of the protein (Fig. 6A). Fig. 6A also shows the scoring for nuclear localization of phospho-Mnk1. There was a progressive increase in nuclear immunofluorescence that peaked at 15 min...
following treatment of the cells with VEGF ($p < 0.01$ at 5 and 15 min). To our knowledge, this is the first demonstration of VEGF regulation of Mnk1 phosphorylation and its localization in the cell. We examined the upstream regulators of VEGF-induced Mnk1 phosphorylation. U73122, the PLC inhibitor, PP2, the c-Src inhibitor, and U0126, the MEK inhibitor, all abolished VEGF-stimulated Mnk1 phosphorylation (Fig. 7), identifying PLC, c-Src, and MEK (immediately upstream of Erk) as kinases that control Mnk1 phosphorylation.

**VEGF Induces Phosphorylation of eIF4E**—Our next aim was to test whether VEGF induced phosphorylation of eIF4E, the Mnk1 substrate. VEGF stimulated the phosphorylation of eIF4E robustly within 5 min, and the effect lasted for about 30 min (Fig. 8A). U73122, PP2, and U0126 completely inhibited VEGF-induced phosphorylation of eIF4E (Fig. 8, B–D). Thus, VEGF-induced phosphorylation of eIF4E is regulated via the PLC, c-Src, and Erk axis.

**Dominant Negative Mnk1 Expression Inhibits eIF4E Phosphorylation and Protein Synthesis Induced by VEGF**—We next investigated the involvement of Mnk1 in VEGF-stimulated protein synthesis by expressing dominant negative Mnk1 in which the Thr197 and Thr202 are mutated to Ala. Both VEGF-induced eIF4E phosphorylation and protein synthesis were abrogated by expression of mutant Mnk1 (Fig. 9). These data demonstrate that VEGF-induced protein synthesis requires Mnk1 phosphorylation.

**PLCγ Activation Is Required for VEGF-induced Enhanced Protein Synthesis**—We have established the role of PLCγ in activation of Erk to increase phosphorylation of eIF4E, a regulator of protein synthesis initiation. We directly examined whether PLCγ activation, the proximal event in Erk activation, is required for VEGF induction of protein synthesis. VEGF-induced protein synthesis in MCT cells was completely abrogated when the cells were pretreated with U73122 (Fig. 10A). Further confirmation was obtained employing cells transfected to express PLCz, the dominant negative construct of PLCγ (Fig. 10B). Fig. 11 summarizes the proposed sequence of signaling events induced by VEGF leading to protein synthesis in MCT cells.

**DISCUSSION**

In this report we demonstrated that VEGF augments protein synthesis in an Erk phosphorylation-dependent manner. Our data show that VEGF-induced Erk phosphorylation is regulated by sequential phosphorylation of VEGFR2, PLCγ, and c-Src. We reported for the first time that VEGF stimulation of Erk phosphorylation, in turn, results in phosphorylation of Mnk1 and its substrate eIF4E. The phosphorylation of Mnk1 is associated with its shift from the cytoplasm to the nucleus. Our results provide new evidence for a role for PLCγ in VEGF-induced eIF4E phosphorylation and protein synthesis.

VEGF stimulation of protein synthesis in MCT cells is mediated via activation of VEGFR2 (7). Pathways regulating phosphorylation of 4E-BP1 and eIF4E seem to diverge downstream of VEGFR2, the former being dependent on PI 3-kinase-Akt activation (7) whereas, as reported here, eIF4E phosphorylation requires Erk phosphorylation. As Erk phosphorylation was independent of PI 3-kinase, our observations suggest distinct roles for PI 3-kinase and Erk pathways in the regulation of crucial events that control initiation phase of protein translation. PLCγ phosphorylation by VEGFR2 is the most proximal event identified in eIF4E phosphorylation in this study. PLCγ binds to Tyr1175 in the cytoplasmic domain of VEGFR2 (23). Its role in the regulation of protein translation has not been studied in depth. U73122, an inhibitor of PLC, has been shown to abolish cardiac myocyte hypertrophy induced by norepineph-
rino (24), although it appears to be not required in angiotensin II induction of protein synthesis in cardiac fibroblasts (25). Thus, the involvement of PLC$_{\gamma}$ in protein synthesis appears to be cell- and agonist-specific. In several previous studies, the involvement of PLC$_{\gamma}$ was not directly studied but suspected by the use of its inhibitor, and changes in eIF4E were not ad-
FIG. 8. VEGF stimulates eIF4E phosphorylation that is dependent on PLCγ, c-Src, and Erk. A, serum-deprived MCT cells were stimulated with VEGF 20 ng/ml. The lysates were fractionated by SDS-PAGE and immunoblotted with a phosphospecific antibody for eIF4E. Loading was assessed by probing with eIF4E antibody (lower panel). A representative blot from three experiments is shown for each kinase. B–D, quiescent cells were subjected to pretreatment with or without U73122 (5 μM), a PLC inhibitor (B), PP2 analog (10 μM), a Src inhibitor (C) and U0126 (25 μM), a MEK inhibitor (D), prior to incubation with or without VEGF 20 ng/ml for 10 min. Equal amounts of cell lysates were run on SDS-PAGE and immunoblotted with phosphospecific antibody for eIF4E. The lower panels of B, C, and D show the blots probed with eIF4E antibody to assess loading. Representative blots from three experiments with each kinase inhibitor are shown.

FIG. 9. Mnk1 mediates eIF4E phosphorylation and is needed for VEGF-induced protein synthesis. A, MCT cells were transfected with a plasmid carrying dominant negative (DN)-Mnk1 (pEBG-T2/A2) or empty vector and allowed to grow for 48 h. Phosphorylation of eIF4E was assessed by immunoblotting as described in the legend to Fig. 8. A representative blot from three experiments is shown. B, MCT cells were transfected with dominant negative Mnk1 or empty vector as described in A, treated with or without VEGF prior to addition of [35S]methionine, and incubated for a total of 2 h. Equal amounts of protein from the lysates were taken for the estimation of 35S label incorporation into trichloroacetic acid-precipitable protein and expressed as percentage of control (Mean ± S.E.). Data from three experiments are shown in a graph. *, p < 0.01 for VEGF versus vector-transfected control; **, p < 0.01 for VEGF versus VEGF-treated dominant negative Mnk1-expressing cells, determined by ANOVA.

FIG. 10. VEGF-stimulated protein synthesis is dependent on phosphorylation of PLCγ. A, quiescent cells were stimulated with VEGF simultaneously in the presence of [35S]methionine (Met.) for 2 h with or without preincubation with 5 μM U73122, a PLC inhibitor, for 30 min. Incorporation of radiolabel into trichloroacetic acid-precipitable protein was taken as a measure of protein synthesis and expressed as percentage of control (Mean ± S.E.). Composite data from three experiments are shown in a graph. *, p < 0.01 VEGF versus control; **, p < 0.01 for VEGF + U73122 versus VEGF, determined by ANOVA. B, MCT cells transfected with empty plasmid vector or with PLCz, a plasmid carrying a dominant negative construct of PLCγ, were incubated with VEGF in the presence of [35S]methionine for 2 h. Protein synthesis was measured as in A. The values are expressed as the percentage of control from an average of three individual experiments. *, p < 0.01 VEGF versus control; **, p < 0.01 for VEGF + U73122 versus VEGF, determined by ANOVA.
Phosphorylation on Ser209, the physiologically important site in the m7 cap of the mRNA. Erk has been implicated in eIF4E phosphorylation, which results in the dissolution of its heterodimeric complex that promotes protein synthesis. 4E-BP1 is phosphorylated, by eIF4E, the cap-binding protein (36). In response to signals that promote translation of most capped mRNAs in eukaryotes is facilitated, which is regarded as the rate-limiting step (35). In general, the expression of an eIF4E phosphorylation mutant resulted in stunted development, suggesting that phosphorylation of eIF4E facilitates cell protein synthesis (40). However, opposing views have recently been expressed. The phosphorylation of eIF4E is thought to cause electrostatic repulsion from phosphate groups on the mRNA cap and inhibit its cap binding (41, 42). The precise timing of eIF4E phosphorylation relative to mRNA cap binding is not known. If phosphorylation of eIF4E were to occur after the binding of the factor to the mRNA cap, anionic repulsion could serve to disengage eIF4E from the mRNA. The association and dissociation reactions between eIF4E and mRNA are known to be rapid (43), and could be facilitated by eIF4E phosphorylation. Although present in cytoplasm, a significant fraction of cellular eIF4E is present in the nucleus and may participate in nuclear protein translation (44) and export of select mRNAs, e.g., cyclin D1, from the nucleus (45, 46). Role of eIF4E phosphorylation in these processes is not yet known.

The stimulation of protein synthesis in renal epithelial (MCT) cells by VEGF has implications for kidney hypertrophy, which may occur physiologically, after removal of one kidney (9), or pathologically in the early phase of diabetes (7, 47). Neutralizing antibodies against VEGF ameliorate early renal dysfunction in experimental diabetes including renal hypertrophy (9, 48). These data implicate VEGF in the pathogenesis of kidney hypertrophy in diabetes that mostly involves renal epithelial cells (49). VEGF could have constructive effects on the kidney in a different setting. In thrombotic glomerular disease, VEGF administration helps restore renal architecture (50). Knowledge of signaling intermediaries that regulate VEGF-induction of protein synthesis in renal cells should help facilitate modulation of this process to advantage in renal disease states.

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Phospholipase Cγ-Erk Axis in Vascular Endothelial Growth Factor-induced Eukaryotic Initiation Factor 4E Phosphorylation and Protein Synthesis in Renal Epithelial Cells

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