Regulation of protein synthesis by IGF-I in proximal tubular epithelial cells

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1Division of Nephrology, Department of Medicine, University of Texas Health Science Center, South Texas Veterans Health Care System, San Antonio, Texas 78229-3900; and 2Geriatrics Research and Education Center, South Texas Veterans Health Care System, San Antonio, Texas 78229-3900; and 3Department of Biochemistry, McGill University, Montreal, Quebec, Canada H3Z 2Z3

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Senthil, Duraisamy, Goutam Ghosh Choudhury, Hanna E. Abboud, Nahum Sonenberg, and Balakuntalam S. Kasinath. Regulation of protein synthesis by IGF-I in proximal tubular epithelial cells. Am J Physiol Renal Physiol 283: F1226–F1236, 2002.—Protein synthesis is required for renal hypertrophy, and proximal tubular epithelial cells are an important cell type involved in this process. We examined IGF-I regulation of protein synthesis in murine proximal tubular epithelial (MCT) cells. We focused on initial events in protein translation and the signaling events involved. Translation of capped mRNAs is under the control of eukaryotic initiation factor 4E (eIF4E). In the resting cell, eIF4E is normally kept in an inactive state by binding to 4E-BP1, its binding protein. Phosphorylation of 4E-BP1 results in dissociation of the eIF4E-4E-BP1 complex allowing eIF4E to initiate peptide synthesis. IGF-I stimulated protein synthesis, augmented phosphorylation of 4E-BP1 and promoted the dissociation of eIF4E from 4E-BP1. IGF-I stimulated the activities of phosphatidylinositol 3-kinase, Akt, and ERK1/2-type MAPK in MCT cells. IGF-I-induced phosphorylation of 4E-BP1, dissociation of the 4E-BP1-eIF4E complex, and increase in protein synthesis required activation of both PI 3-kinase and ERK pathways. Furthermore, ERK activity by IGF-I was also PI 3-kinase dependent. Transfection with the Thr37,46→Ala37,46 mutant of 4E-BP1 showed that phosphorylation of Thr37,46 residues was required for IGF-I induction of protein synthesis in MCT cells. Our observations reveal the importance of initial events in protein translation in IGF-I-induced protein synthesis in MCT cells and identify the regulatory signaling pathways involved.

phosphatidylinositol 3-kinase; mitogen-activated protein kinase; eukaryotic initiation factor 4E; eIF4E binding protein

GROWTH FACTORS HAVE BEEN IMPLICATED in cell proliferation, hypertrophy, and matrix accumulation. Among several growth factors affecting the kidney, IGF-I has been studied in the context of renal parenchymal changes associated with physiological compensatory renal growth (2, 21, 53) as well as pathological states such as diabetic renal disease. IGF-I transgenic mice manifest renal hypertrophy, demonstrating its growth-inducing property (48). Although circulating IGF-I regulates functions of the kidney, renal parenchymal cells serve as an additional local source for the growth factor and its regulatory binding proteins (1). Because circulating IGF-I levels are reported to be low in mice with type 1 diabetes (19), the renal IGF-I system has been implicated in the development of diabetes-induced renal hypertrophy (18). In diabetic nephropathy, glomerular and tubular growth precedes glomerulosclerosis (10, 14). A rise in renal tissue concentration of IGF-I precedes a rapid increase in renal size and in glomerular filtration in type 1 diabetes, implicating the growth factor in diabetes-induced renal hypertrophy (5, 19, 20). Proximal tubular epithelial cells are the dominant cell type in renal cortex and participate in renal hypertrophy in the aforementioned conditions. Because they possess the IGF-I receptor (47), it is likely that IGF-I regulates metabolic pathways involved in the growth of this cell type, including protein synthesis.

There is abundant evidence that IGF-I promotes protein synthesis (6, 9, 22), a requirement for induction of renal growth. Increased synthesis of proteins may involve an increase in the rate of transcriptional events and/or augmented efficiency in the rate of translation of their mRNA transcripts. Protein translation has not been well studied in the regulation of protein synthesis in renal tissue or cells, particularly in the context of exposure to IGF-I. A key step in the regulation of protein translation is the initiation phase, which is critically controlled by an mRNA cap binding protein, eukaryotic initiation factor 4E (eIF4E) (26). eIF4E has a great avidity for the methylated cap of mRNA chains destined for translation. In the resting state, eIF4E is held in an inactive complex by one of its three binding proteins, 4E-BP1–4E-BP3. Phosphorylation of 4E-BP results in dissolution of the eIF4E-4E-BP complex,

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freeing eIF4E to promote the initiation phase of protein translation (39, 46). Free eIF4E combines with a scaffolding protein (eIF4G). The binding sites for eIF4E on 4E-BP1 and eIF4G share a common sequence, and the two proteins compete for eIF4E (41). The ATP-dependent helicase eIF4A combines with eIF4E and eIF4G to form the eIF4F complex that binds to the m⁷G cap of mRNA. Phosphorylation of eIF4E may be required for optimal activity, because it increases the affinity of the protein for the m⁷G cap of mRNA (45). The efficiency of the 43S ribosome to scan for the first AUG is impeded if there are secondary structures in the 5′-untranslated region of mRNA. The helicase activity of eIF4A has been suggested to resolve the complexities in the 5′-untranslated region and facilitate detection of the first methionine codon by the ribosome (26).

In the present study, our purpose was to examine IGF-1 regulation of protein synthesis in proximal tubular epithelial cells by focusing on initial events in protein translation involving phosphorylation of 4E-BP1 and the signaling pathways involved.

**EXPERIMENTAL METHODS**

**Cell culture.** SV40-transformed murine proximal tubular epithelial (MCT) cells were kindly provided by Dr. Eric Neilson (Vanderbilt University, Nashville, TN). Cells were grown in DMEM containing 7% FBS, 5 mM glucose, and no insulin (7). These cells retain properties of proximal tubular epithelial cells in vivo (30). Confluent monolayers of cells were serum-deprived in DMEM for 18 h before the experiment.

**Analysis of 4E-BP1 phosphorylation.** We have recently described the assay in detail (52). Quiescent serum-deprived MCT cells were incubated with or without IGF-1 at the stated concentrations and for the indicated times at 37°C. Cells were washed twice with PBS, collected in 500 μl of lysis buffer [(in mM) 50 Tris-HCl, pH 7.4, 150 KCl, 1 DTT, 1 EDTA, 50 β-glycerophosphate, pH 7.5, 50 NaF, 0.1 sodium orthovanadate, 1 EGTA, 2 benzamidine, and 1 PMSF, as well as 1 μg/ml aprotinin and 1 μg/ml leupeptin], and lysed by three freeze-thaw cycles. Cell debris was removed by centrifugation at 12,000 rpm for 5 min, and the concentration of protein was measured with a Bio-Rad protein assay. Equal amounts of lysate proteins (300 μg) were boiled for 7 min and then cooled on ice before centrifugation at 12,000 rpm for 5 min at 4°C. Heat-soluble proteins were precipitated by addition of TCA to a final concentration of 15%. After 3-h incubation at 4°C, the protein precipitates were centrifuged for 10 min, the supernatant was removed, and the remaining TCA was extracted with diethyl ether. The final pellets were suspended in Laemmli sample buffer. Samples were boiled for 5 min, and proteins were separated on a 15% acrylamide gel followed by electrophoresis to a nitrocellulose membrane. The membrane was rinsed with Tris-buffered saline containing 0.1% Tween-20 (TBST) and blocked with 2% nonfat milk powder in TBST. After rinsing with TBST buffer, membranes were incubated with anti-4E-BP1 antibody (40 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-phospho-4E-BP1 antibody (1:2,000 dilution; New England Biolabs, Beverly, MA). Antibody against phospho-4E-BP1 reacts only with 4E-BP1 phosphorylated on Thr⁷⁰ residue. The membranes were washed twice with TBST buffer, followed by incubation with appropriate peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected by using a chemiluminescence reagent kit (Amersham Pharmacia Biotech, Piscataway, NJ).

**Tyrosine phosphorylation of insulin receptor substrates** (52). Serum-starved control and IGF-1-treated MCT cells were homogenized in RIPA buffer [(in mM) 20 Tris, pH 7.5, 150 sodium chloride, 5 EDTA, 0.1 sodium orthovanadate, and 1 PMSF, as well as 0.1% aprotinin and 1% Nonidet P-40]. Equal amounts of homogenates (1 mg) were immunoprecipitated with a specific antibody against insulin receptor substrate (IRS)-1 or IRS-2 (Upstate Biotechnology, Lake Placid, NY). The immunoprecipitates were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membrane overnight. After blocking in 5% nonfat dry milk, the membrane was washed three times in TBST and probed with a 1:2,000 dilution of a mouse monoclonal antibody against phosphotyrosine (Upstate Biotechnology). The latter was detected by an antibody against mouse IgG that was linked to horseradish peroxidase. The reactive bands were detected by chemiluminescence. The membrane was stripped and immunoblotted with the antibody against IRS-1 and IRS-2 to assess loading of samples and to ascertain the identity of the immunoprecipitated band.

**Phosphatidylinositol 3-kinase assay.** The method for phosphatidylinositol 3-kinase analysis employed in our laboratory has been recently described (52). Control and IGF-1-treated MCT cells were homogenized in the RIPA buffer. Equal amounts of homogenates (300 μg) were immunoprecipitated by using anti-phosphotyrosine antibody (Upstate Biotechnology) as previously described (7). The immunobeads were incubated with 10 μg PI for 10 min at 25°C in the PI 3-kinase assay buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 mM EGTA. After the addition of 20 mM magnesium chloride and 5 μCi of [γ-³²P]ATP to the assay mixture, the reactants were further incubated for 10 min at 25°C. Reaction was stopped by the addition of a mixture of chloroform/methanol and 11.6 N HCl at a ratio of 50:100:1. The reactants were extracted with 100 μl of chloroform, and the organic layer was washed with methanol and 1 N HCl in a 1:1 ratio. The reaction products were dried, resuspended in chloroform, and separated by thin-layer chromatography by using the solvent chloroform/methanol-28% ammonium hydroxide-water in a ratio of 129:114:15:21. The spots were visualized by autoradiography. LY-294002 was employed as an inhibitor of PI 3-kinase. Preliminary experiments evaluating different doses and duration of incubation showed that 30-min preincubation of cells with 50 μM concentration of the inhibitor gave optimal reduction in the kinase activity.

**Akt/PKB assay.** Equal amounts of MCT cell lysates from control and IGF-1-treated cells were separated on 15% SDS-PAGE, followed by Western blotting with a phospho-Akt/PKB (Ser473) antibody (Cell Signaling Technology, Beverly, MA). The membrane was stripped and immunoblotted with the antibody against Akt to assess loading of samples.

**Interaction between eIF4E and 4E-BP1.** m⁷GTP-Sepharose chromatography was performed as described previously (17); 25 μl of a 50:50 slurry of m⁷GTP-Sepharose CL-6B (Amersham Pharmacia Biotech) was added to 300 μg of protein. The lysates were then rotated for 1 h at 4°C. The m⁷GTP-Sepharose was pelleted by centrifugation at 1,000 g for 2 min. The beads were then washed three times in RIPA buffer. For SDS-PAGE, proteins were removed from the m⁷GTP matrix by boiling it in SDS loading buffer. Proteins were separated on a 15% acrylamide gel followed by electrophoresis to a nitrocellulose membrane. The membrane was rinsed with TBST and blocked with 2% nonfat milk powder in TBST. After rinsing with TBST buffer, membranes were
incubated with anti-4E-BP1 antibody (40 ng/ml) or antibody against eIF4E (Santa Cruz Biotechnology). The membranes were washed twice with TBST buffer followed by incubation with appropriate peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected by using a chemiluminescence reagent kit (Amersham Pharmacia Biotech).

ERK1/2-type MAPK assay. Equal amounts (100 µg) of MCT cell lysates from control and IGF-I-treated cells were immunoprecipitated by incubating with anti-ERK1 antibody (Santa Cruz Biotechnology) at 4°C for 3 h. Protein A-Sepharose beads were added and incubated at 4°C for 1 h. The immunobeads were washed three times in RIPA buffer and twice in PBS. Beads were incubated with the substrate myelin basic protein (MBP), 25 µM cold ATP, and 1 µCi of [γ-32P]ATP in MAPK buffer (in mM) 10 HEPES, pH 7.4, 10 magnesium chloride, 0.5 DTT, and 0.5 sodium orthovanadate for 30 min at 30°C. The reaction was stopped by addition of sample buffer, and the reaction mixture was boiled for 5 min and subjected to SDS-PAGE employing a 15% gel. The gel was dried and exposed to Kodak Biomax MR X-ray film with a transilluminating screen. Western blotting with anti-ERK1 antibody was performed on an aliquot of assay lysates to assess loading. U0126 and PD-98059 were employed as ERK inhibitors. Preliminary experiments evaluating different doses and duration of incubation showed that 30-min preincubation of cells with 50 µM U0126 or 100 µM PD-98059 gave optimal reduction in the kinase activity.

Protein synthesis assay. Serum-starved cells were preincubated with LY-294002 (50 µM) or U0126 (50 µM), and 15 min after stimulation with IGF-I, the cells were incubated with [35S]methionine at 10 Ci/ml for 30 min. The cells were then washed in PBS and lysed in RIPA buffer. Twenty micrograms of protein were spotted onto 3MM paper (Whatman International, Maidstone, UK). The filters were then washed by boiling them for 1 min in 10% TCA containing 0.1 g/l methionine. This was repeated three times. The filters were then dried and immersed in scintillation fluid before determining radioactivity by scintillation counting (34, 52).

Stable transfection with 4E-BP1 mutant. The plasmid pACTAG-BP1 Thr37,46→Ala37,46 contains 4E-BP1 mutated at Thr37 and Thr46 phosphorylation sites. The mutant does not undergo phosphorylation at these sites and remains bound to eIF4E. For control, we employed a plasmid vector that does not contain a 4E-BP1 insert. Plasmids (up to 5 µg) were mixed with 250 µl of OPTI-MEM medium and 25 µl LipofectAMINE and incubated for 15 min at room temperature, followed by addition of 750 µl of OPTI-MEM medium. Cell layers grown in 10-cm dishes were washed in serum-free medium, and the DNA-LipofectAMINE mixture was added. After 6 h at 37°C, serum-supplemented medium containing neomycin was added. Cells resistant to neomycin are cloned and propagated. As the mutant protein was tagged with hemagglutinin (HA), efficiency of transfection was monitored by a monoclonal anti-HA antibody (Boehringer Mannheim). The effectiveness of the plasmids has been recently demonstrated (24).

Infection of MCT cells with adenovirus. Replication-defective adenovirus vector carrying mouse Akt mutated at its phosphorylation sites (T308A, S473A) (23) was used to study the Akt involvement in IGF-I-induced hypertrophy. The dominant-negative (DN) Akt carries an HA tag. Cells were infected in serum-free medium for 24 h and infected with a multiplicity of infection (MOI) of 70 of adenovirus carrying DN Akt construct (AdDNAkt) at room temperature for 1 h, as previously described (12). Preliminary experiments showed that maximal HA expression occurred at this MOI. Control cells were infected with the same MOI of adenovirus carrying green fluorescent protein (AdGFP). After incubation for 24 h in serum-free medium, cells were incubated with IGF-I for 24 h. Hypertrophy was measured by the amount of protein (µg) per 1 × 106 cells and expressed relative to control, taken as 100 percent.

Statistics. Data from a minimum of three experiments were expressed as means ± SE and analyzed by ANOVA for comparison among multiple groups. *P values of <0.05 were considered significant.

RESULTS

IGF-I induces phosphorylation of 4E-BP1 in murine MCT cells. In preliminary experiments, we observed that IGF-I (50 ng/ml) significantly increased de novo protein synthesis. We evaluated an early step in the initiation phase of protein translation, i.e., regulation of 4E-BP1 phosphorylation. Serum-starved cells were incubated with a range of IGF-I concentrations from 25 to 150 ng/ml for 15 min, and the effect on 4E-BP1 phosphorylation was examined by Western blot employing an antibody that specifically binds to phosphorylated 4E-BP1. Although stimulation of 4E-BP1 phosphorylation was evident at 25 ng/ml, maximal effect was seen at 50 ng/ml, with reduction at higher concentrations (Fig. 1A). Results with anti-phospho-4E-BP1 antibody were confirmed by Western blotting with anti-4E-BP1 antibody. On a Western blot, three bands of 4E-BP1 are evident that migrate differently depending on the state of phosphorylation. The fastest migrating form α is unphosphorylated. The intermediate β-band is phosphorylated on some, but not all, serine and threonine residues, whereas the slowest band γ is the most phosphorylated. There was a time-dependent appearance and progressive increase in intensity of the highly phosphorylated γ-band in cells treated with IGF-I, with maximum intensity at 50 ng/ml (Fig. 1A, bottom). Time dependence of 4E-BP1 phosphorylation was examined over 0–60 min by Western blotting using anti-phospho-4E-BP1 and anti-4E-BP1 antibodies. As shown in Fig. 1B, top, IGF-I (50 ng/ml) increased 4E-BP1 phosphorylation, which peaked at 15 min and persisted for 30 min. This was confirmed with Western blotting using the anti-4E-BP1 antibody (Fig. 1B, bottom). On the basis of the above observations, we employed incubation with IGF-I (50 ng/ml) for 15 min for the rest of the study.

IGF-I stimulates phosphorylation of both IRS-1 and IRS-2 in MCT cells. We examined signaling events involved in IGF-I-induced 4E-BP1 phosphorylation. Two types of receptors mediate the biological actions of IGF-I. The type 1 receptor is a heterotetrameric tyrosine kinase receptor, similar to the insulin receptor (38). The type 2 receptor is a mannose-6 phosphate receptor devoid of tyrosine kinase activity. After binding of IGF-I, the type 1 receptor undergoes stimulation of its intrinsic tyrosine kinase activity, resulting in autophosphorylation and phosphorylation of several downstream intracellular substrates. We examined whether any of the IRS proteins that serve as docking proteins were activated after IGF-I stimulation in
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MCT cells. Cell homogenates were immunoprecipitated with an antibody against IRS-1 or IRS-2 and immunoblotted with an anti-phosphotyrosine antibody. IGF-I increased the tyrosine phosphorylation of IRS-1 by fourfold (Fig. 1C) and IRS-2 by threefold (Fig. 1D), without a change in the amount of the respective proteins. These data demonstrate that both IRS-1 and IRS-2 are recruited by the IGF-I receptor signaling pathway in MCT cells.

IGF-I stimulates PI 3-kinase activity in a time-dependent manner. Insulin stimulation of protein synthesis is dependent on IRS-1 phosphorylation with subsequent activation of PI 3-kinase (43). We examined whether PI 3-kinase was activated in MCT cells after IGF-I stimulation. Serum-starved MCT cells were stimulated with IGF-I for 0–60 min. There was a progressive increase in PI 3-kinase activity that began at 2 min after exposure to IGF-I, peaked at 15 min, and persisted for nearly 60 min (Fig. 2A).

IGF-I induces Akt/PKB activity that is PI 3-kinase dependent. Akt/PKB, a serine/threonine protein kinase, is activated downstream of PI 3-kinase. It binds to the product of PI 3-kinase activity, phosphatidylinositol 3-phosphate, and is activated by sequential phosphorylation by PDK-1 and, possibly, integrin-linked kinase (13, 15). However, recent studies have demonstrated non-PI 3-kinase-dependent pathways of Akt activation in mesangial cells (28). Although phosphorylation of 4E-BP1 has been shown to involve activation of Akt (25), Akt-independent activation has also been recently reported (51). Therefore, we investigated whether Akt/PKB was activated by IGF-I and whether it was PI 3-kinase dependent in MCT cells. Serum-starved MCT cells were stimulated with IGF-I for 15 min with or without preincubation with the PI 3-kinase inhibitor LY-294002. Equal amounts of cell homogenates were immunoblotted with phospho-Akt antibody. IGF-I robustly induced Akt/PKB activity (Fig. 2B, lane 2 vs. lane 1) that was inhibited by LY-294002 (lane 4 vs. lane 2).

IGF-I-induced 4E-BP1 phosphorylation is PI 3-kinase dependent. Having observed that IGF-I induces Akt/PKB, an important regulator of 4E-BP1 phosphorylation (25), and that Akt/PKB activation by IGF-I is PI 3-kinase dependent, we next examined whether activation of PI 3-kinase was necessary for IGF-I regulation of 4E-BP1 phosphorylation. Serum-starved MCT cells were stimulated with IGF-I with or without preincubation with LY-294002. Cell lysates were immunoblotted with anti-phospho-4E-BP1 or anti-4E-BP1 antibodies. IGF-I augmented phosphorylation of 4E-BP1 (Fig. 3A, lane 2 vs. lane 1). The PI 3-kinase inhibitor LY-294002 completely abrogated IGF-I-induced 4E-BP1 phosphorylation (Fig. 3A, lane 4 vs. lane 2). These data show that IGF-I-induced 4E-BP1 phosphorylation is PI 3-kinase dependent.

IGF-I increases dissociation of 4E-BP1 from eIF4E, which is PI 3-kinase dependent. In resting cells, eIF4E is held inactive in a heterodimeric complex with 4E-BP1. Phosphorylation of 4E-BP1 leads to a reduction in its affinity for eIF4E and dissociation of the heterodimeric complex, releasing eIF4E to combine with eIF4G, a step that precedes initiation of mRNA translation. We examined whether IGF-I increases dissociation of 4E-BP1 from eIF4E, which is PI 3-kinase-dependent. Having observed that IGF-I induces Akt/PKB, an important regulator of 4E-BP1 phosphorylation (25), and that Akt/PKB activation by IGF-I is PI 3-kinase dependent, we next examined whether activation of PI 3-kinase was necessary for IGF-I regulation of 4E-BP1 phosphorylation. Serum-starved MCT cells were stimulated with IGF-I with or without preincubation with LY-294002. Cell lysates were immunoblotted with anti-phospho-4E-BP1 or anti-4E-BP1 antibodies. IGF-I augmented phosphorylation of 4E-BP1 (Fig. 3A, lane 2 vs. lane 1). The PI 3-kinase inhibitor LY-294002 completely abrogated IGF-I-induced 4E-BP1 phosphorylation (Fig. 3A, lane 4 vs. lane 2). These data show that IGF-I-induced 4E-BP1 phosphorylation is PI 3-kinase dependent.

Fig. 1. IGF-I stimulates phosphorylation of 4E-BP1 in murine proximal tubular epithelial (MCT) cells. A: serum-starved MCT cells were treated with a range of IGF-I concentrations (0–150 ng/ml) for 15 min. Cell lysates were separated on 15% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was immunoblotted with an anti-phosphotyrosine antibody (P-Tyr mAb). Western blotting with anti-IRS-1 (A, top) or anti-IRS-2 (A, bottom) antibodies was done to assess loading. A representative blot from 3 individual experiments is shown. IB, immunoblotted; IP, immunoprecipitated.

B: serum-starved MCT cells were incubated with 50 ng/ml of IGF-I for different durations and processed for immunoblotting as described in A. A representative blot from 3 independent experiments is shown. IGF-I augments phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2. Serum-starved MCT cells were incubated with or without IGF-I (50 ng/ml) for 15 min. Lysates were immunoprecipitated with specific antibodies against IRS-1 (C) or IRS-2 (D) antibodies. Immunoprecipitated proteins were separated on 7.5% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were immunoblotted with monoclonal anti-phospho-4E-BP1 antibody (P-Tyr mAb). Western blotting with anti-IRS-1 (C, bottom) or anti-IRS-2 (D, bottom) antibodies was done to assess loading. A representative blot from 3 independent experiments is shown.
**IGF-I induces ERK1/2 MAPK activity in MCT cells.**

Involvement of ERK1/2 MAPK in 4E-BP1 phosphorylation appears to be specific for the cell type or agonist examined. For instance, 4E-BP1 phosphorylation induced by PGF$_{2\alpha}$ in smooth muscle cells and by insulin in MCT cells is dependent on ERK activation (7, 50). However, in rat skeletal muscle, insulin induction of 4E-BP1 phosphorylation is ERK independent (4). We examined whether ERK kinase had a role in IGF-I regulation of 4E-BP1 phosphorylation. First, we investigated whether IGF-I-stimulated ERK1/2 MAPK activity in MCT cells. Serum-starved MCT cells were stimulated with IGF-I for 0–60 min. Equal amounts of cell lysates were immunoprecipitated with an anti-ERK1 antibody, and the immune complexes were used in an in vitro kinase assay with MBP as a substrate. IGF-I significantly increased the activity of ERK that was evident at 2 min and reached a peak at 15 min. By 60 min, the stimulation had subsided (Fig. 4A). The specificity of IGF-I-induced ERK1/2 MAPK activity was confirmed by employing two dissimilar MEK inhibitors, PD-098059 and U0126. Both MEK inhibitors completely inhibited IGF-I-induced ERK1/2 MAPK activity (Fig. 4B).

![Diagram](http://ajprenal.physiology.org/)

**Fig. 2.** A: IGF-I activates phosphatidylinositol (PI) 3-kinase. Serum-starved MCT cells were incubated with or without IGF-I (50 ng/ml) for 15 min. Equal amounts of protein (300 μg) were immunoprecipitated with an anti-phosphotyrosine antibody, and kinase activity in immune complexes was measured by using PI as a substrate, as described in EXPERIMENTAL METHODS. Phospholipids were separated by thin-layer chromatography and visualized by autoradiography. A representative autoradiograph from 2 individual experiments is shown. PIP, PI 3-phosphate. B: IGF-I induces Akt/PKB activity in MCT cells that is PI 3-kinase dependent. Serum-starved MCT cells were incubated with or without IGF-I (50 ng/ml) for 15 min with or without preincubation for 30 min with LY-294002 (LY; 50 μM). Cell lysates were separated on 15% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was immunoblotted with a phospho-Akt (Ser473) antibody. A representative blot from 3 separate experiments is shown.

**Fig. 3.** A: PI 3-kinase activation is required for IGF-I-induced phosphorylation of 4E-BP1. Serum-starved MCT cells were incubated with or without IGF-I (50 ng/ml) for 15 min with or without preincubation for 30 min with LY-294002 (50 μM). Phosphorylation of 4E-BP1 was determined as described in Fig. 1. A representative blot from 4 individual experiments is shown. B: IGF-I increases dissociation of 4E-BP1 from eIF4E. IGF-I increased the dissociation of 4E-BP1 from eIF4E, resulting in failure to detect 4E-BP1 in association with eIF4E that was bound to m$^7$GTP-Sepharose (Fig. 3B, lane 2 vs. lane 1). Preincubation with LY-294002 completely inhibited the dissociation of 4E-BP1 from eIF4E (Fig. 3B, lane 4 vs. lane 2). Because LY-294002 inhibited 4E-BP1 phosphorylation, 4E-BP1 remained associated with eIF4E in the heterodimeric complex as in the resting cell (Fig. 3B, lane 4 vs. lane 1). The amount of eIF4E in cell lysates undergoing various manipulations was unchanged (Fig. 3B, bottom). These data support a role for PI 3-kinase in IGF-I-induced phosphorylation of 4E-BP1 and for its dissociation from the heterodimeric complex with eIF4E.
IGF-I-induced ERK1/2 MAPK activity is PI 3-kinase dependent. ERK kinase activation with IGF-I may occur via activation of the Ras-Raf-MEK pathway (49). However, it may also follow PI 3-kinase activation, as suggested by our recent observations with insulin (7). We explored whether IGF-I regulation of ERK kinase activity was PI 3-kinase dependent. Serum-starved MCT cells were stimulated with IGF-I with or without preincubation with LY-294002. PI 3-kinase inhibitor greatly reduced IGF-I-induced ERK1/2 MAPK activation (Fig. 5A). These data show that in MCT cells, IGF-I stimulation of ERK is PI 3-kinase dependent.

ERK1/2 MAPK is required for IGF-I-induced 4E-BP1 phosphorylation. We next tested the involvement of ERK kinase in IGF-I-induced 4E-BP1 phosphorylation. Serum-starved MCT cells were stimulated with IGF-I with or without preincubation with U0126. Cell lysates were immunoblotted with anti-phospho-4E-BP1 or anti-4E-BP1 antibodies. Preincubation with the MEK inhibitor completely abolished the IGF-I-induced phosphorylation of 4E-BP1 (Fig. 5B). These data show that ERK activation is required for IGF-I induction of 4E-BP1 phosphorylation.

IGF-I-stimulated protein synthesis is PI 3-kinase and MAPK dependent. Our study suggests that stimulation of 4E-BP1 phosphorylation by IGF-I involves activation of PI 3-kinase and ERK1/2 MAPK. We wished to determine whether activation of these kinases was important in IGF-I-stimulated de novo protein synthesis. Serum-starved cells were preincubated with LY-294002 or U0126 for 30 min followed by stimulation with IGF-I. Cells were labeled with [35S]methionine for 30 min. Incorporation of the label into TCA-precipitable protein was estimated. IGF-I significantly increased de novo protein synthesis (P < 0.001) that was completely inhibited by LY-294002 or U0126 (Fig. 6A) (P < 0.0001 by ANOVA). Because the inhibitors reduced the rate of protein synthesis in control cells not incubated with IGF-I as well, activation of these kinases appears to be important for constitutive protein synthesis. The kinase inhibitors did not affect the viability of the cells as assessed by lactate dehydrogenase activity.
assay (data not shown). These data show that both PI 3-kinase and MAPK pathways are needed for IGF-I-induced de novo protein synthesis. However, these data do not address the requirement of 4E-BP1 phosphorylation for IGF-I-induced protein synthesis. We have demonstrated that IGF-I stimulates protein synthesis in MCT cells by recruiting factors that regulate protein translation. IGF-I increased phosphorylation of 4E-BP1, an early step in the initiation phase of protein translation. Analysis of signaling events showed that activation of PI 3-kinase and MAPK pathways was required for IGF-I stimulation of 4E-BP1 phosphorylation and protein synthesis. Inactivation of Akt was shown to abrogate IGF-I-induced cell hypertrophy. These data suggest Akt/PKB activity is required for IGF-I-induced hypertrophy in MCT cells.

**DISCUSSION**

We have demonstrated that IGF-I stimulates protein synthesis in MCT cells by recruiting factors that regulate protein translation. IGF-I increased phosphorylation of 4E-BP1, an early step in the initiation phase of protein translation. Analysis of signaling events showed that activation of PI 3-kinase and MAPK pathways was required for IGF-I stimulation of 4E-BP1 phosphorylation and protein synthesis. Inactivation of Akt was shown to abrogate IGF-I-induced cell hypertrophy. Protein translation is divided into three phases, i.e., initiation, elongation, and termination. Each of these phases is regulated by distinct factors. The initiation phase is thought to be a rate-controlling step in protein translation (36) and is closely controlled by eIF4E. Dissolution of the eIF4E-4E-BP1 heterodimeric complex occurs by phosphorylation of the binding protein (39, 46). Because regulatory steps in the initiation phase of protein translation involve phosphorylation events, kinases play an important role in control of the process. In our studies on MCT cells, activation of both PI 3-kinase and ERK pathways were required for IGF-I-induced 4E-BP1 phosphorylation. There is consensus regarding the important role played by PI 3-kinase in signaling pathways induced by receptor tyrosine kinases leading to 4E-BP1 phosphorylation (37). However, the involvement of ERK is controversial. Al-
Fig. 6. A: IGF-I-stimulated de novo protein synthesis is PI 3-kinase- and ERK kinase-dependent. Serum-starved cells were pretreated with LY-294002 (50 μM) or U0126 (50 μM) for 30 min and incubated with or without IGF-I (15 min). Cells were then labeled with [35S]methionine for 30 min. Incorporation of the label into TCA-precipitable protein was estimated, and incorporation of [35S]methionine into protein was expressed as percentage of control. Combined data from 3 experiments, with n = 12 at each point, are shown. Data are expressed as means ± SE. *P < 0.001, IGF-I compared with control; **P < 0.0001 IGF-I + LY-294002 and IGF-I + U0126 compared with IGF-I alone by ANOVA. B: transfection of MCT cells with 4E-BP1 phosphorylation mutant. Stable transfection of mouse MCT cells was achieved by LipofectAMINE method. Untransfected control cells (C), control cells transfected with a plasmid pCDNA without a 4E-BP1 insert (P), and cells transfected with Thr37,46→Ala37,46 mutant of 4E-BP1 (M) were tested for the presence of hemagglutinin (HA) tag. Note the presence of HA only in the mutant transfected cells. C: IGF-I-stimulated de novo protein synthesis is dependent on 4E-BP1 phosphorylation. Confluent layers of MCT cells transfected with plasmid without 4E-BP1 insert and MCT cells with Thr37,46→Ala37,46 mutant were incubated with or without IGF-I (50 ng/ml) for 15 min and labeled with [35S]methionine for 30 min. TCA-precipitable radioactivity is shown as percentage relative to control. Note the IGF-I-induced increase in protein synthesis in control cells with empty plasmid (lane 2) compared with control (lane 1); *P < 0.001. Thr37,46→Ala37,46 mutant 4E-BP1 reduced both baseline (lane 3) and IGF-I-induced protein synthesis (lane 4); **P < 0.001, compared with control cells treated with IGF-I by ANOVA. Values are means ± SE; n = 6 at each point from 3 individual experiments. Mut-4E-BP1, 4E-BP1 phosphorylation mutant. D: IGF-I-induced cell hypertrophy is dependent on Akt activation. Replication-defective adenovirus vector carrying mouse Akt mutated at its phosphorylation sites (T308A, S473A) was used to study the Akt involvement in IGF-I-induced hypertrophy. The dominant-negative Akt (DNAkt) carries an HA tag. Cells were quiesced in serum-free medium for 24 h and infected with a multiplicity of infection (MOI) of 70 of HA-tagged adenovirus carrying DNAkt construct (AdDNAkt) at room temperature for 1 h. Control cells were infected with the same MOI of adenovirus carrying green fluorescent protein (AdGFP). After incubation for 24 h in serum-free medium, cells were incubated with IGF-I for 24 h. Hypertrophy was measured by μg protein/10^6cells and expressed relative to control, taken as 100 percent. *P = 0.0004, compared with control cells infected with AdGFP. **P < 0.01. Bottom: immunoblot analysis of the same samples with anti-HA antibody demonstrating DN Akt expression. Values are means ± SE; n = 8 at each point from 2 individual experiments.
though in vitro investigations initially reported ERK induced phosphorylation of Ser64 on 4E-BP1 (32), subsequent reports have disputed its role in the process (37). In 3T3 L1-adipocytes and rat skeletal muscle, 4E-BP1 phosphorylation stimulated by insulin is not subject to ERK regulation (4, 40). However, in MCT cells, vascular smooth muscle cells, and hematopoietic cells, ERK activation is required for 4E-BP1 phosphorylation induced by diverse stimuli (3, 7, 50). Studies reported here also demonstrate recruitment of ERK by IGF-I in stimulation of 4E-BP1 phosphorylation. Previous investigations in rat aortic smooth muscle cells reported that IGF-I-induced 4E-BP1 phosphorylation occurred in the absence of ERK stimulation (29). These data suggest that involvement of ERK in 4E-BP1 phosphorylation is cell-type specific.

Additionally, in MCT cells, we observed that ERK activation by IGF-I is PI 3-kinase dependent, suggesting cross talk between the two signaling pathways. The sites where the PI 3-kinase and ERK pathways interact are unclear. In 32D cells, PKC has been reported to mediate cross talk between the two pathways (42). Downstream of PI 3-kinase, Akt activation is essential for 4E-BP1 phosphorylation (24). Akt activates the mammalian target of rapamycin, which has a direct role in phosphorylating 4E-BP1 (8). More recently, ataxia telangiectasia mutant protein, a serine kinase (54), and cyclin-dependent kinase, cdc2 (33), have been identified as direct kinases for 4E-BP1. Studies are needed to identify the kinases recruited by IGF-I to directly phosphorylate 4E-BP1.

Our studies employing 4E-BP1 Thr37,46→Ala37,46 mutant provide evidence for a regulatory role for 4E-BP1 phosphorylation in protein synthesis stimulated by IGF-I in MCT cells. There are at least six candidate serine and threonine residues in 4E-BP1 that are sites of phosphorylation (54). agonist-induced phosphorylation of these residues is believed to occur in a cascade and may involve distinct kinases phosphorylating specific serine and threonine residues. Among the residues, Thr37,46 have been proposed as initial targets of phosphorylation that may act as priming sites for subsequent phosphorylation of other sites (24). Our data on the mutants demonstrate that phosphorylation of Thr37,46 is essential for both constitutive and IGF-I-induced protein synthesis, although we did not examine whether phosphorylations at Thr37,46 are priming events leading to phosphorylation of other serine threonine sites.

Other signaling pathways, not examined in the present study, are also activated by IGF-I. Activation of p38 MAPK by IGF-I has been implicated in protection against high-glucose-induced apoptosis in neuroblastoma cells or UV-mimetic-induced DNA injury in a cell line derived from NIH-3T3 cells (11, 35). These studies have focused on the role of IGF-I in DNA synthesis. It is unclear whether p38 MAPK has a role in IGF-I-induced protein synthesis in renal cells and whether cross talk between ERK and p38 pathways has a bearing on IGF-I regulation of protein synthesis. This interaction merits investigation in future studies.

The signaling pathway recruited by IGF-I to promote protein synthesis delineated in the present study may not be generalized to other cell types in the kidney. In rat mesangial cells, Gooch et al. (27) have recently shown the involvement of calcineurin phosphatase in cell hypertrophy induced by IGF-I that was independent of the PI 3-kinase pathway. Thus pathways employed by IGF-I to promote protein synthesis may be selective for the cell type in the renal tissue.

It is important to have a deeper understanding of the regulatory steps in IGF-I stimulation of protein synthesis in renal cells, because it may have significance in both physiological and pathological processes that involve renal growth, e.g., compensatory renal hypertrophy and diabetes. Resolution of diabetes-induced renal hypertrophy by ocreotide, a somatostatin analog, and JB3 peptide, an antagonist of the IGF-I receptor, suggests a role for IGF-I in that process (20, 31). Identification of intermediary steps involved in IGF-I induction of protein synthesis may suggest additional potential targets for intervention in diabetic renal disease. Data reported herein identify Akt as one such possible target. Abrogation of Akt activity by employing a dominant-negative construct abolished IGF-I-induced cell hypertrophy. Although these data support an important role for the PI 3-kinase-Akt axis in cell hypertrophy induced by IGF-I, other signaling pathways such as ERK may also be involved. Conversely, IGF-I has been administered to benefit kidney disease, e.g., chronic renal failure (44). In this context, an understanding of signaling pathways may assist in development of strategies to amplify the salutary effects of the growth factor.

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