IGF-I elicits growth of human intestinal smooth muscle cells by activation of PI3K, PDK-1, and p70S6 kinase

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Kuemmerle, John F. IGF-I elicits growth of human intestinal smooth muscle cells by activation of PI3K, PDK-1, and p70S6 kinase. Am J Physiol Gastrointest Liver Physiol 284: G411–G422, 2003. First published November 20, 2002; 10.1152/ajpgi.00310.2002.—Endogenous IGF-I regulates growth of human intestinal smooth muscle cells by jointly activating phosphatidylinositol 3-kinase (PI3K) and ERK1/2. The 70-kDa ribosomal S6 kinase (p70S6 kinase) is a key regulator of cell growth activated by several independently regulated kinases. The present study characterized the role of p70S6 kinase in IGF-I-induced growth of human intestinal smooth muscle cells and identified the mechanisms of p70S6 kinase activation. IGF-I-induced growth elicited via either the PI3K or ERK1/2 pathway required activation of p70S6 kinase. IGF-I elicited concentration-dependent activation of PI3K, 3-phosphoinositide-dependent kinase-1 (PDK-1), and p70S6 kinase that was sequential and followed similar time courses. IGF-I caused time-dependent and concentration-dependent phosphorylation of p70S6 kinase on Thr421/Ser424, Thr389, and Thr229 that paralleled p70S6 kinase activation. p70S6 kinase (Thr421/Ser424) phosphorylation was PI3K dependent and PDK-1 independent, whereas p70S6 kinase (Thr389) and p70S6 kinase (Thr229) phosphorylation and p70S6 kinase activation were PI3K dependent and PDK-1 dependent. IGF-I elicited sequential Akt (Ser308), Akt (Ser473), and mammalian target of rapamycin (Ser2448) phosphorylation; however, transfection of muscle cells with kinase-inactive Akt1(K179M) showed that these events were not required for IGF-I to activate p70S6 kinase and stimulate proliferation of human intestinal muscle cells.

insulin-like growth factor-I; proliferation; protein kinase C; protein kinase B; Akt; extracellular signal-regulated kinase 1/2; and p38 mitogen-activated protein kinase

IGF-I regulates cellular proliferation in two ways: 1) it is mitogenic for many cells and 2) it promotes the increase in cell size that is required for cell division. Binding of IGF-I to the cognate IGF-I receptor stimulates the intrinsic tyrosine kinase activity of the receptor. Mutational analysis has identified several tyrosine phosphorylation sites within the carboxy terminus of the IGF-I receptor β-subunit that confer specificity for the proliferative effects elicited in response to receptor autophosphorylation: Tyr950, Tyr1131, Tyr1135, and Tyr1136. The activated IGF-I receptor tyrosine kinase has several known primary substrates, including the insulin receptor substrate family of proteins, Src-homology domain carboxy terminus, Grb2-associated binder 1, hepatocyte plasma membrane ectoATPase (pp120/HA4), and G2 kDa GAP-associated docking protein (p62DOK). Once phosphorylated, these docking proteins activate downstream intracellular signaling through the phosphatidylinositol 3-kinase (PI3K) or Grb2-SOS pathways that ultimately leads to cellular proliferation. The specific pathways activated by IGF-I and the roles of these pathways in mediating the proliferative response to IGF-I depend on the cell type. We have previously shown that in human intestinal smooth muscle cells, like skeletal muscle cells and vascular smooth muscle cells, IGF-I activates both the ERK1/2 and PI3K pathways that regulate growth (11, 14, 24). The particular importance of IGF-I in regulating the growth of intestinal smooth muscle is demonstrated by the hyperplasia that occurs in both intestinal and vascular smooth muscle tissues of transgenic animals overexpressing an IGF-I cDNA (31, 48). The pathways mediating intestinal smooth muscle proliferation are likely to play an important role in the setting of Crohn’s disease, in which IGF-I expression is up-regulated and may contribute to the hyperplasia of smooth muscle that contributes to the formation of intestinal strictures (53).

Two isoforms of the ribosomal S6K1 are derived from alternative splicing at the amino terminus: p85S6K1αI and p70S6K1αII (35, 36). The p70S6K1αII isofrom (p70S6 kinase) is mainly cytosolic, whereas the p85S6K1αI isoform is predominantly nuclear. p70S6 kinase, a serine/threonine protein kinase, is activated by growth factors and plays a central role in cell growth and proliferation by mediating the phosphorylation of the 40S ribosomal protein, S6, thereby enabling efficient translation of 5’-terminal oligopyrimidine tract mRNAs (5-TOPs). This class of mRNAs encodes for numerous components of the protein synthesis machinery: ribosomal proteins and elongation factors. A second S6K homologue, S6K2, was identified in S6K1-deficient mice that exhibited a phenotype of dimin-

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ished but not absent growth compared with that of wild-type mice (42). In S6K1-deficient mice, S6K2 expression was upregulated and provided partial compensation for the absence of S6K1. S6K2 also exists as two isoforms, p60S6KβIII and p54S6KβII. Both S6K2 isoforms contain a carboxy-terminal nuclear localization signal not found in S6K1 and are therefore localized predominantly in the nucleus.

Activation of p70S6 kinase depends on the sequential phosphorylation of four sets of serine/threonine sites by several independently regulated kinases (49). The initial step in p70S6 kinase activation involves the phosphorylation of serine/threonine residues in the autoinhibitory pseudosubstrate domain (Ser411, Ser418, Ser424, Ser429, and Thr421) and in the catalytic domain extension (Thr390 and Ser394) under the control of proline-directed kinases (7, 30). The result is the release of the autoinhibitory carboxy-terminal tail from the catalytic domain allowing access to Thr229 and Thr389. Phosphorylation of p70S6 kinase on Thr229 and Thr389 is regulated by the lipid products of PI3K, phosphatidylinositol 3,4,5-trisphosphate (PIP₃), and to lesser extent phosphatidylinositol 4,5-bisphosphate (PIP₂), acting both directly on p70S6 kinase and indirectly via 3-phosphoinositide-dependent kinase-1 (PDK-1) (1). Although phosphorylation of p70S6 kinase (Thr389) most closely correlates with p70S6 kinase activity in vivo, considerable positive cooperativity exists with p70S6 kinase (Thr229) phosphorylation (50). Dual phosphorylation of Thr229 and Thr389 fully activates p70S6 kinase.

p70S6 kinase participates in PI3K-dependent regulation of muscle growth by IGF-I in rat and porcine vascular smooth muscle cells (14, 18) and in L6A1 skeletal myoblasts (11). Although several signaling intermediates lying downstream of PI3K have been shown to regulate the activity of p70S6 kinase in other muscle types [PDK-1, Akt, and the mammalian target of rapamycin (mTOR)] it is not known whether IGF-I activates p70S6 kinase in human intestinal smooth muscle cells or the sequence of signaling events emanating from PI3K that regulate p70S6 kinase activity and muscle cell growth.

This study shows that, in human intestinal smooth muscle cells, IGF-I causes phosphorylation of multiple serine/threonine residues on p70S6 kinase that result in its activation. Serine/threonine residues within the autoinhibitory domain are phosphorylated in a PI3K-dependent fashion involving PKC-ζ, p70S6 kinase (Ser308) and p70S6 kinase (Thr229) in the catalytic domain are phosphorylated in a PI3K-dependent, PDK-1-dependent fashion. Whereas IGF-I-induced ERK1/2 and PI3K activation jointly stimulate muscle cell proliferation, active p70S6 kinase is required for growth to occur regardless of the pathway involved.

IGF-I also elicits PI3K-dependent, PDK-1-dependent Akt(Ser308) phosphorylation and subsequent Akt-(Ser473) autophosphorylation that leads to phosphorylation of mTOR on Ser2448. Although mTOR kinase activity is required for stimulation of cell growth, IGF-I-induced, Akt-dependent mTOR(Ser2448) phosphorylation is regulated independently of mTOR kinase activity and is not required for IGF-I-mediated p70S6 kinase activation and proliferation to occur.

METHODS

Culture of isolated smooth muscle cells from normal human jejunum. Muscle cells were isolated and cultured from the circular muscle layer of human jejunum as described previously (21, 22, 24). Briefly, 4- to 5-cm segments of normal jejunum were obtained from patients undergoing surgery for morbid obesity according to a protocol approved by the University Office of Research Subject Protection. After the segments were opened along the mesenteric border, the mucosa was dissected away and the remaining muscle layer was cut into 2 × 2-cm strips. Slices were obtained separately from the circular layer by using a Stadie-Riggs tissue slicer. The slices were incubated overnight at 37°C in 20 ml of DMEM plus 10% fetal bovine serum (DMEM-10) containing 200 U/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamycin, and 2 μg/ml amphotericin B, to which was added 0.0375% collagenase (CLS type II) and 0.1% soybean trypsin inhibitor.

Muscle cells dispersed from the circular layer were harvested by filtration through 500-μm Nitex mesh and centrifuged at 150 g for 5 min. Cells were resuspended and washed twice by centrifugation at 150 g for 5 min. After resuspension in DMEM-10 containing the same antibiotics, the cells were plated at a concentration of 5 × 10⁵ cells/ml as determined by counting in a hemocytometer. Cultures were incubated in a 10% CO₂ environment at 37°C. DMEM-10 medium was replaced every 3 days until the cells reached confluence.

Primary cultures of muscle cells were passaged on reaching confluence by first being washed three times with PBS. After the PBS was removed, cells were treated for 2 min with 0.05% trypsin and 0.53 mM EDTA. The trypsin activity was neutralized by addition of a fourfold excess of DMEM-10. The resulting suspension was centrifuged at 350 g for 10 min at 4°C. The pellet was resuspended in DMEM-10 at a concentration of 2.5 × 10⁶ cells/ml and plated in the appropriate cultureware. The medium was changed after 24 h. All subsequent studies were performed in first-passage cultured cells after 7 days, at which time the cells were confluent. We have previously shown that these cells express a phenotype characteristic of intestinal smooth muscle as determined by immunostaining for smooth muscle markers and expression of γ-entactin. Epithelial cells, endothelial cells, neurons, and interstitial cells of Cajal were not detected in these cultures (21, 46).

[³H]Thymidine incorporation assay. Proliferation of smooth muscle cells in culture was measured by the incorporation of [³H]thymidine as described previously (21, 24, 25). Briefly, the cells were washed free of serum and incubated for 24 h in serum-free DMEM. The quiescent muscle cells were incubated for an additional 24 h with a maximally effective concentration of IGF-I (100 nM) in the presence and absence of various test agents. During the final 4 h of this incubation period, 1 μCi/ml [³H]thymidine was added to the medium. [³H]Thymidine incorporation into the perchloric acid extractable pool was used as a measure of DNA synthesis.

Measurement of p70S6 kinase activity by in vitro kinase assay. The activity of p70S6 kinase was assayed by using an immune complex in vitro kinase assay by modification of the methods of Zhang et al. (52). Briefly, confluent muscle cells were rendered quiescent by incubation in serum-free DMEM for 24 h. Cells were stimulated with IGF-I (100 nM) for the indicated periods of time (0–24 h) in the presence or absence of various inhibitors. The reaction was terminated by wash-
ing the cells in ice-cold PBS. The cells were lysed in buffer consisting of (in mM): 50 Tris (pH 7.5), 1 EDTA, 1 EGTA, 0.5 sodium orthovanadate, 50 NaF, 5 sodium pyrophosphate, 10 sodium glycerol phosphate, and 0.1 PMSF, with 0.1% 2-mercaptoethanol, 1% Triton X-100, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Cell lysates were clarified by centrifugation, and aliquots of the supernatant containing equal amounts of protein (200 μg) were incubated with 4 μg of p70S6 kinase antibody coupled to protein A/G agarose beads for 2 h at 4°C. The protein A/G-agarose-protein immune complexes were washed once with lysis buffer containing 0.5 M NaCl, twice with lysis buffer alone, and finally in assay buffer consisting of (in mM): 20 MOPS (pH 7.2), 25 β-glycerol phosphate, 5 EGTA, 1 sodium orthovanadate, and 1 dithiothreitol. The resulting immune complexes were assayed for p70S6 kinase activity in assay buffer, to which was added (in μM): 15 MgCl₂, 100 ATP, 4 PKC inhibitor, 0.4 PKA inhibitor (PKI), 4 Compound R24571, and 50 S6 substrate peptide (AKRKRSSLRLA). The reaction was initiated by the addition of 10 μCi [γ-32P]ATP and continued for 10 min at 30°C. The reaction was terminated by spotting 25-μl aliquots on P81 phosphocellulose paper. The phosphocellulose paper was washed three times with 0.75% phosphoric acid and one time with acetic acid, dried, transferred to scintillation vials, and counted on a β-scintillation counter. The results were corrected for endogenous substrates by subtraction of values obtained in the absence of added S6 peptide and for background obtained by immunoprecipitation with nonimmune serum. Values were expressed in picomoles of phosphate incorporated into peptide substrate per minute per milligram of protein.

**Measurement of PI3K activity by in vitro kinase assay.** PI3K activity was measured as described previously (24). Briefly, quiescent muscle cells growing in 100-mm dishes were stimulated for 30 min with IGF-I (100 nM) in the presence and absence of various inhibitors. The reaction was terminated by rapidly washing cells with 4°C PBS and by lysis of the cells. Lysis buffer consisted of (in mM): 50 Tris-HCl (pH 7.4), 150 NaCl, 1 Na₂VO₃, 2 EDTA, 1 MgCl₂, and 1 CaCl₂, with 30 nM leupeptin, 1% trasylool (wt/vol), and 1% NP-40 (vol/vol). DNA in the lysate was sheared by passage through a 22-gauge needle, and the lysate was incubated for 20 min at 4°C. Following this incubation, the lysate was centrifuged at 12,000 g for 20 min at 4°C, and aliquots of lysate containing equal amounts of protein were incubated with 25 μl of agarose-conjugated antibody to phosphotyrosine (PY20) with gentle mixing for 2 h at 4°C. The beads were collected by centrifugation at 12,000 g for 5 min at 4°C and washed three times with lysis buffer and two times with kinase assay buffer. Kinase assay buffer consisted of (in mM): 50 Tris-HCl (pH 7.8), 50 NaCl, 2 MgCl₂, and 0.5 EDTA. After the final washing, the beads were resuspended in 30 μl of kinase assay buffer to which 10 μl of sonicated 1 mg/ml phosphatidylinositol was added. The reaction was initiated by the addition of 5 μl of 50 mM ATP containing 0.5 μCi [γ-32P]ATP and was continued for 10 min at 30°C. The reaction was terminated by addition of 0.5 ml of 1 N HCl and 2 ml of chloroform-methanol (2:1, vol/vol). Phospholipids were recovered from the lower organic phase and dried under N₂ gas. The dried phospholipids were dissolved in chloroform and spotted on Silica H gel TLC plates impregnated with 1% potassium oxalate. Chromatograms were developed in chloroform-methanol-28% NH₃-water (70:100:15:25, vol/vol). The plates were dried, and the spots corresponding to authentic PIP₃ were visualized and quantitated by using a Phospho-Imager (Packard Instruments, Meriden, CT). Results are expressed as the increase in [32P] incorporation into PI-3-P above basal values.

**Measurement of PDK-1 activity by in vitro kinase assay.** The activity of PDK-1 was measured by an immune complex in vitro kinase assay according to the methods of Fujita et al. (16). Briefly, quiescent muscle cells were incubated for 30 min with 100 nM IGF-I. The reaction was terminated by washing cells with ice-cold PBS. The cells were lysed in buffer consisting of (in mM): 50 Tris (pH 7.5), 1 EDTA, 1 EGTA, 50 NaF, 1 sodium orthovanadate, 5 sodium pyrophosphate, and 10 sodium β-glycerol phosphate, with 0.1% 2-mercaptoethanol (vol/vol), 0.1% Triton X-100 (vol/vol), and 1 μM Microcystin LR. Following the addition of 50 μl of protein G agarose, the lysates were clarified by centrifugation at 4°C. Protein G-antibody complexes were simultaneously prepared by incubation of 4 μg of anti-PDK-1 antibody (or normal sheep IgG as negative control) with 100 μl of a 50% slurry of protein G agarose beads and 250 μl of lysis buffer overnight at 4°C. Protein G-antibody complexes were washed twice with lysis buffer and twice with PDK-1 assay buffer. PDK-1 assay buffer consisted of (in mM): 50 Tris-HCl (pH 7.5), 0.1 EDTA, 0.1 EGTA, 10 magnesium acetate, and 0.1 ATP, with 1% 2-mercaptoethanol (vol/vol), 2.5 μl PKI, and 1 μl Microcystin LR. The resulting PDK-1 immune complexes were resuspended in 20 μl of PDK-1 assay buffer, and inactive serum- and glucocorticoid-dependent protein kinase (SGK1, Δ1-60, S422D; 500 ng) was added to the immunoprecipitated PDK-1 to be activated by incubation for 30 min at 30°C. The final assay reaction was carried out in a total volume of 50 μl. The reaction was initiated by addition of 4 nmol of Akt/SGK substrate peptide (RPRAATF) and 10 μCi of [γ-32P]ATP and continued for 10 min at 30°C. The reaction was terminated by centrifugation at 14,000 rpm and spotting of 25-μl aliquots on P81 phosphocellulose paper. The phosphocellulose paper was washed three times with 0.75% phosphoric acid and one time with acetic acid, dried, transferred to scintillation vials, and counted on a β-scintillation counter. The results were corrected for endogenous substrates by subtraction of values obtained after immunoprecipitation with normal sheep IgG. Values were expressed in picomoles of phosphate incorporated into peptide substrate per minute per milligram of protein.

**Western blot analysis.** The phosphorylation of PDK-1, Akt, mTOR, PKC-ζ, and p70S6 kinase on specific residues was measured by Western blot analysis by using standard methods (9, 24, 26). Briefly, confluent muscle cells were rendered quiescent by incubation for 24 h in serum-free medium. The cells were stimulated with recombinant human IGF-I for 30 min (the time of peak p70S6 kinase activation). The cells were rapidly washed with ice-cold PBS and lysed in sample buffer. Lysates were boiled for 5 min, and equal amounts of total cell protein were separated with SDS-PAGE under denaturing conditions. After the proteins were electrotransferred to nitrocellulose, the membranes were incubated overnight with a 1:1,000–1:2,000 dilution of antibodies recognizing phosphorylated (activated) signaling intermediates: PDK-1 (Ser417), Akt (Ser473), mTOR (Ser2448), p70S6 kinase (Thr389, p70S6 kinase (Thr422) and p70S6 kinase (Ser424). Bands of interest were visualized with enhanced chemiluminescence. Nitrocellulose membranes were stripped and reblotted to determine levels of total (phosphorylated and nonphosphorylated) pro-
tein using antibodies recognizing PDK-1, Akt, mTOR, PKC-ζ, and p70S6 kinase.

Expression of kinase-inactive Akt. Substitution of methionine for lysine at residue 179 (K179M) results in a kinase-inactive Akt1, which when expressed in cells exerts a dominant-negative effect (43). cDNA for Myc-His-tagged kinase-inactive Akt1/K179M in the pUSEamp(+) expression vector was purified, and muscle cells growing in six-well plates were transiently transfected with 1 μg of pUSEamp(+) Akt1/K179M cDNA or with pUSEamp(+) vector alone as a control by using a LipofectAMINE PLUS reagent kit (Life Technologies). Cells were incubated for 3 h at 37°C with the transfection reagent-DNA complexes. The DNA-containing medium was replaced with DMEM plus 10% FCS. After 48 h incubation, the expression of Akt1/K179M was confirmed by Western blot analysis using an anti-Myc tag antibody, and the kinase-inactive effect of this mutation on IGF-I signaling was confirmed by analysis of Akt(Ser473) phosphorylation.

Measurement of protein content. The protein content of cell lysates was measured by using the Bio-Rad DC protein assay kit according to manufacturer’s directions. Samples were adjusted to provide aliquots of equal protein content before in vitro kinase assay or Western blot analysis.

Statistical analysis. Values given represent the means ± SE of n experiments, where n represents the number of experiments on cells derived from separate primary cultures. Statistical significance was tested by Student’s t-test for either paired or unpaired data as appropriate. Analysis of relative densitometric values was performed using ImageJ 1.26t software (National Institutes of Health, Bethesda, MD). Densitometric values for protein bands of phosphorylated signaling intermediates were reported in arbitrary units above background values and normalized to total signaling intermediate protein levels.

Materials. Recombinant human IGF-I was obtained from Austral Biologicals (San Ramon, CA); collagenase and soybean trypsin inhibitor were obtained from Worthington Biochemical (Freehold, NJ); HEPES was obtained from Research Organics (Cleveland, OH); DMEM and Hank’s balanced salt solution were obtained from Mediatech (Herndon, VA); fetal bovine serum was obtained from Summit Biotechnologies (Fort Collins, CO); [γ-32P]ATP (specific activity 3,000 Ci/mmol), [3H]thymidine (specific activity 6 Ci/mmol), and [3H]thymidine incorporation was not affected (225 ± 25% above basal; vector = 195 ± 15% above basal) (Fig. 1B). The results implied that IGF-I-induced proliferation mediated by the PI3K signaling pathway is PDK-1 dependent and PKC-ζ dependent but does not require Akt activation.

IGF-I activates p70S6 kinase. The ability of growth factors to fully stimulate p70S6 kinase activity involves the sequential phosphorylation of distinct sets of serine/threonine residues within p70S6 kinase. Phosphorylation of serine/threonine residues in the autoinhibitory domain of p70S6 kinase (e.g., Thr421 and Ser424) relieves pseudosubstrate inhibition of the catalytic domain and is a prerequisite for phosphorylation of Thr389 in the activation loop of the catalytic domain and of Thr368 in the carboxy-terminal extension of the catalytic domain (1, 30, 49, 50).

Our initial studies showed that p70S6 kinase activity was required for IGF-I to stimulate growth of human intestinal smooth muscle cells. The ability of IGF-I to activate p70S6 kinase in human intestinal muscle cells was therefore examined in two complementary ways. In the first, the ability of IGF-I to elicit phosphorylation of three specific regions of p70S6 ki-
nase (Thr^{421}/Ser^{424}, Thr^{229}, and Thr^{389}) was examined by Western blot analysis using phosphorylation state-specific antibodies. In the second, the ability of IGF-I to stimulate p70S6 kinase activity was measured by using an immune complex in vitro kinase assay.

Incubation of human intestinal muscle cells with 100 nM IGF-I for increasing periods of time (0–24 h) elicited time-dependent phosphorylation of p70S6 kinase on Thr^{421}/Ser^{424}, Thr^{229}, and Thr^{389} that was rapid, occurring within 5 min, was maximal within 30 min (Thr^{421}/Ser^{424} = 320 ± 25% above basal; Thr^{229} = 220 ± 20% above basal; Thr^{389} = 590 ± 40% above basal) and was sustained at submaximal levels for up to 24 h (Fig. 2A and B). When measured at the 30-min maximum, phosphorylation of Thr^{421}/Ser^{424}, Thr^{229}, and Thr^{389} elicited by IGF-I was also concentration dependent (Fig. 2C).

Incubation of confluent human intestinal smooth muscle cells with IGF-I for increasing periods of time (0–24 h) elicited time-dependent activation of p70S6 kinase. Activation of p70S6 kinase was also rapid, occurring within 5 min, was maximal within 30 min (46.0 ± 2.5 pmol phosphate·min^{-1}·mg protein^{-1} above basal; basal activity = 14.9 ± 0.9 pmol phosphate·min^{-1}·mg protein^{-1}) and was sustained at submaximal levels for up to 24 h (Fig. 3A). When measured at the 30-min maximum, activation of p70S6 kinase by IGF-I (1–100 nM) was concentration dependent (Fig. 3B).

Mechanisms of IGF-I-induced p70S6 kinase activation. The ability of selective inhibitors of PI3K, PDK-1, and PKC-ε to inhibit IGF-I-induced [^3H]thymidine incorporation suggested that these intermediates may participate in the activation of p70S6 kinase. This was also investigated using the two complementary techniques; p70S6 kinase phosphorylation on residues Thr^{421}/Ser^{424}, Thr^{229}, and Thr^{389} was measured by Western blot analysis, and activation of p70S6 kinase was measured by using in vitro kinase assay.

Phosphorylation of p70S6 kinase(Ser^{421}/Thr^{424}) in response to 100 nM IGF-I (320 ± 25% above basal) was inhibited by the PI3K inhibitor LY-294002 (10 nM), the p70S6 kinase inhibitor rapamycin (10 nM), and the
PKC-ζ inhibitor myr-PKC-ζ-PS (15) but was not affected by the PDK-1 inhibitor TPCK (5), MKK1/2 inhibitor U-1026 (1 μM), or p38 MAPK inhibitor SB-203580 (Fig. 4 and Table 1). Phosphorylation of p70S6 kinase(Thr229) by IGF-I (220 ± 20% above basal) was inhibited by the PI3K inhibitor, the PDK-1 inhibitor, the p70S6 kinase inhibitor, and the PKC-ζ inhibitor but was not affected by the MKK1/2 inhibitor or the p38 MAPK inhibitor (Fig. 4 and Table 1). Phosphorylation of p70S6 kinase(Thr229) by IGF-I (590 ± 40% above basal) followed a similar pattern to that observed for p70S6 kinase(Thr229) and was also inhibited by the PI3K inhibitor, the PDK-1 inhibitor, the p70S6 kinase inhibitor, and the PKC-ζ inhibitor but was not affected by the MKK1/2 inhibitor or the p38 MAPK inhibitor (Fig. 4 and Table 1). A similar pattern of inhibition of IGF-I-induced p70S6 kinase(Thr229) and p70S6 kinase(Thr229) phosphorylation but not of p70S6 kinase(Thr421/Ser424) phosphorylation was observed for IGF-I-induced activation of p70S6 kinase. Activation of p70S6 kinase induced by 100 nM IGF-I (46.0 ± 2.5 pmol phosphate/minute/mg protein above basal) was inhibited by the PI3K inhibitor, the PDK-1 inhibitor, the PCK-ζ inhibitor, and the p70S6 kinase inhibitor but was not affected by the MKK1/2 inhibitor or the p38 MAPK inhibitor (Fig. 4 and Table 1). The results implied that PI3K, PDK-1, and PKC-ζ but not ERK1/2 or p38 MAPK participate in IGF-I-induced activation of p70S6 kinase.

PI3K-dependent signaling intermediates activated by IGF-I. The pattern of inhibition of IGF-I-induced p70S6 kinase phosphorylation, p70S6 kinase activation, and growth suggested that PI3K and PDK-1 play essential roles in these processes, as does PKC-ζ, albeit to a lesser extent. The sequence of events initiated by IGF-I was therefore also investigated by using several complementary techniques. Activation of PI3K and PDK-1 in response to IGF-I was measured by using the in vitro kinase assay as had been done for p70S6 kinase. IGF-I-stimulated phosphorylation of signaling intermediates: PDK-1(Ser248), Akt(Ser473), and mTOR(Ser2448) were measured by using phosphospecific antibodies in Western blot analysis. The sequence of signaling events initiated by IGF-I was then determined with the use of selective inhibitors.

We have previously shown that IGF-I elicits time-dependent and concentration-dependent activation of PI3K (24) that was rapid, occurring within 5 min, was...
maximal within 10 min, and was sustained for up to 60 min. The results of the present study show that incubation of muscle cells with 100 nM IGF-I for 30 min increased PI3K activity by 68 ± 90% above basal levels. IGF-I-induced activation of PI3K was inhibited 89 ± 5% by the PI3K inhibitor but was not affected by the PDK-1 inhibitor, the p70S6 kinase inhibitor, or the PKC-ζ inhibitor. The inhibitors of MKK1/2 and p38 MAPK also did not affect IGF-I-stimulated PI3K activity. The results implied that PI3K activation preceded activation of the other kinases (PDK-1, p70S6 kinase, and PKC-ζ).

Incubation of quiescent muscle cells with 100 nM IGF-I for increasing periods of time (0–24 h) elicited time-dependent activation of PDK-1. IGF-I-induced PDK-1 activation was rapid, occurring within 5 min, was maximal within 30 min (6.29 ± 0.10 pmol phosphatidic acid · min⁻¹ · mg protein⁻¹ above basal (basal = 0.54 ± 0.12 pmol phosphatidic acid · min⁻¹ · mg protein⁻¹), and was sustained at submaximal levels for up to 24 h (Fig. 3A). When measured at the 30-min peak, IGF-I-induced activation of PDK-1 was concentration-dependent (Fig. 3B) like that observed for PI3K and p70S6 kinase. The time course of IGF-I-induced activation of PDK-1 was most similar to that of p70S6 kinase and peaked later than PI3K activation induced by IGF-I. IGF-I elicited concentration-dependent activation of all three kinases (PI3K, PDK-1, and p70S6 kinase).

IGF-I (100 nM, 30 min) also increased phosphorylation of PDK-1(Ser241) by 74 ± 7% above basal. Phosphorylation of PDK-1(Ser241) and activation of PDK-1 by IGF-I were abolished by the PI3K inhibitor or by the PDK-1 inhibitor, were partly inhibited by the PKC-ζ inhibitor, but were not affected by the p70S6 kinase inhibitor (Fig. 5 and Table 2). The inhibitors of MKK1/2 and p38 MAPK did not affect IGF-I-stimulated PDK-1 phosphorylation or activity. Together, the results implied that PDK-1 activation occurred downstream of PI3K activation and was partly dependent on PKC-ζ activation. IGF-I-induced activation of p70S6 kinase, in turn, was PI3K dependent and PDK-1 dependent.

IGF-I stimulated the phosphorylation of PKC-ζ/λ (Thr410/Thr403) by 120 ± 20% above basal. IGF-I-induced phosphorylation of PKC-ζ/λ (Thr410/Thr403) was inhibited by the PI3K inhibitor and by the PDK-1 inhibitor but was not affected by the PKC-ζ inhibitor, the p70S6 kinase inhibitor, the MKK1/2 inhibitor, or the p38 MAPK inhibitor (Fig. 5 and Table 2). The ability of the PKC-ζ inhibitor myr-PKC-ζ-PS to block downstream effects mediated by PKC-ζ without affecting phosphorylation of PKC-ζ/λ (Thr410/Thr403) is expected on the basis of its mechanism of action and is consistent with its effects in previous studies (15).

IGF-I also stimulated the phosphorylation of Akt(Ser473) by 370 ± 90% above basal. IGF-I-induced phosphorylation of Akt(Ser473) was inhibited by the PI3K inhibitor and by the PDK-1 inhibitor but was not affected by the inhibitors of p70S6 kinase, PKC-ζ, MKK1/2, or p38 MAPK (Fig. 5 and Table 2). The results implied that IGF-I-induced Akt(Ser473) phosphorylation occurred downstream of PI3K and PDK-1. IGF-I caused an increase in the phosphorylation of mTOR(Ser2448) by 64 ± 8% above basal. IGF-I-induced Table 1. Effect of various inhibitors on IGF-I-induced p70S6 kinase phosphorylation and activation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>p-P70S6 Kinase Thr421/Ser424</th>
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<td>MKK1/2 inhibitor</td>
<td>5 ± 3</td>
<td>7 ± 7</td>
<td>2 ± 3</td>
<td>2 ± 5</td>
</tr>
<tr>
<td>P38 MAPK inhibitor</td>
<td>8 ± 5</td>
<td>7 ± 8</td>
<td>2 ± 1</td>
<td>1 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3–6 experiments. Results are expressed as the percent inhibition of response to 100 nM IGF-I for 30 min in either pmol PO₄ · min⁻¹ · mg protein⁻¹ from kinase assay of p70S6 kinase activity. PI3K, phosphatidylinositol 3-kinase; PDK, phosphoinositide-depndant kinase; MKK, MAP kinase kinase. *P < 0.05 vs. control.

Table 2. Effect of various inhibitors on IGF-I-induced PI3K activation, PDK-1 activation, and phosphorylation of downstream signaling intermediates

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PI3K Activity</th>
<th>PDK-1 Activity</th>
<th>p-PDK-1 Ser241</th>
<th>p-Akt Ser473</th>
<th>p-mTOR Ser2448</th>
<th>p-PKC-ζ/λλ/λ (Thr410/Thr403)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3K inhibitor</td>
<td>89 ± 5</td>
<td>98 ± 2</td>
<td>96 ± 2</td>
<td>94 ± 4</td>
<td>107 ± 10</td>
<td>91 ± 10</td>
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<tr>
<td>PDK-1 inhibitor</td>
<td>−10 ± 4</td>
<td>96 ± 3</td>
<td>93 ± 1</td>
<td>98 ± 1</td>
<td>104 ± 6</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>PKC-ζ inhibitor</td>
<td>0 ± 9</td>
<td>21 ± 10</td>
<td>49 ± 1</td>
<td>2 ± 8</td>
<td>4 ± 3</td>
<td>2 ± 6</td>
</tr>
<tr>
<td>P70S6 kinase inhibitor</td>
<td>5 ± 4</td>
<td>8 ± 4</td>
<td>5 ± 4</td>
<td>5 ± 6</td>
<td>16 ± 10</td>
<td>−10 ± 7</td>
</tr>
<tr>
<td>MKK1/2 inhibitor</td>
<td>−10 ± 8</td>
<td>7 ± 4</td>
<td>3 ± 2</td>
<td>1 ± 5</td>
<td>−2 ± 9</td>
<td>11 ± 10</td>
</tr>
<tr>
<td>P38 MAPK inhibitor</td>
<td>−1 ± 1</td>
<td>6 ± 5</td>
<td>1 ± 11</td>
<td>2 ± 4</td>
<td>−5 ± 10</td>
<td>4 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3–6 experiments. Results are expressed as the % inhibition of response to 100 nM IGF-I for 30 min in either pmol PO₄ · min⁻¹ · mg protein⁻¹ measured by in vitro kinase assay of PI3K or PDK-1 kinase activity or in relative densitometric units from Western blot analysis using phosphorylation state-specific antibodies (see Fig. 5 for representative blots). *P < 0.05.
phosphorylation of mTOR(Ser2448) was inhibited by the PI3K inhibitor and by the PDK-1 inhibitor but was not affected by the PKC-ζ inhibitor, the MKK1/2 inhibitor, or the p38 MAPK inhibitor. The p70S6 kinase inhibitor was not affected by the PI3K inhibitor and by the PDK-1 inhibitor but not by the PKC-ζ inhibitor or the p70S6 kinase inhibitor. Quantitative analysis of these data is provided in Table 2.

**Effects of expression of a kinase-inactive Akt.** Our initial studies showed that, although IGF-I elicited PI3K-dependent and PDK-1-dependent phosphorylation of Akt(Ser473) (Fig. 5 and Table 2), a kinase-inactive Akt mutant did not affect IGF-I-induced, PI3K-dependent proliferation (Fig. 1B). The mechanisms leading to IGF-I-induced activation of Akt and the participation of Akt in IGF-I-induced p70S6 kinase activation were examined further in human intestinal muscle cells transfected with kinase-inactive Akt1(K179M) or empty vector.

The expression of Akt1(K179M) was confirmed by identification of the Myc tag by Western blot analysis (Fig. 6A). The ability of kinase-inactive Akt1(K179M) to block IGF-I-induced Akt(Ser473) phosphorylation was confirmed by incubation of quiescent muscle cells for 30 min with 100 nM IGF-I and measurement of Akt(Ser473) phosphorylation by Western blot analysis. IGF-I-elicited phosphorylation of Akt(Ser473) (vector = 265 ± 31% above basal) was inhibited 87 ± 3% in cells transfected with kinase-inactive Akt1(K179M) (Fig. 6A and B). Interestingly, in contrast to phosphorylation of Akt on Ser473, IGF-I-induced phosphorylation of Akt on Ser308 was not affected by expression of the kinase-inactive Akt1(K179M) mutant [vector = 315 ± 40% above basal; Akt1(K179M) = 360 ± 25% above basal]. The results provide evidence that the phosphorylation of Akt(Ser473) required the intrinsic kinase activity of Akt(Leu179), whereas phosphorylation of Akt(Ser308) occurred upstream of the intrinsic Akt kinase activity resident within Akt(Leu179).

In muscle cells expressing the kinase-inactive Akt1(K179M) mutant, IGF-I retained the ability to elicit phosphorylation of PDK-1(Ser241) by 54 ± 10% above basal (vector alone = 57 ± 8%) and of PKC-ζ/λ(Thr410/Thr403) by 71 ± 16% above basal (vector alone = 72 ± 10%). The ability of IGF-I to increase the phosphorylation of mTOR(Ser2448) (2 ± 2% above basal; vector = 64 ± 14% above basal) was abolished (Fig. 6C). The pattern of inhibition supported further the sequential nature of PI3K-dependent signaling: PI3K-mediated phosphorylation of PDK-1 caused phosphorylation of Akt(Ser308). The results suggested that the intrinsic kinase activity of Akt caused autophosphorylation of Ser473 and resulted in the subsequent downstream phosphorylation of mTOR on Ser2448.

In cells transiently transfected with kinase-inactive Akt1(K179M) gene, IGF-I retained the ability to phosphorylate p70S6 kinase on Thr421/Ser424 (390 ± 40% above basal; vector = 340 ± 50% above basal), Thr229 (210 ± 40% above basal; vector = 240 ± 40% above basal), and Thr389 (560 ± 80% above basal; vector = 580 ± 30% above basal) and to increase p70S6 kinase activity by 25.6 ± 1.5 pmol phosphate$_4^-$ min$^{-1}$ mg protein$^{-1}$ above basal (vector = 23.6 ± 2.7 pmol PO$_4^-$ min$^{-1}$ mg protein$^{-1}$ above basal; Fig. 6D). The results implied that, although IGF-I elicits phosphorylation of Akt and downstream Akt-dependent phosphorylation of mTOR(Ser2448), these events are not required for IGF-I-induced p70S6 kinase activation, similar to the Akt-independent nature of IGF-I-induced [H]thymidine incorporation.

**DISCUSSION**

IGF-I initiates a sequence of signaling events that culminates in cellular proliferation. A key component of this process is the activation of p70S6 kinase. p70S6 kinase regulates phosphorylation of the 40S ribosomal protein S6, a process that enables efficient translation of 5-TOPs and progression through the G1 phase of the cell cycle. We have previously shown that IGF-I stimulates proliferation of human intestinal smooth muscle cells (22, 24) by activating both PI3K and ERK1/2. In these cells, these two pathways are distinct and jointly mediate the effects of IGF-I on growth. Phosphorylation of PI3K by IGF-I results in the production of PIP$_3$ (24). PIP$_3$, the major lipid product of activated PI3K (and to a lesser extent PIP$_2$), regulates both the localization, by stimulating plasma membrane association, and the activation, by facilitating phosphorylation, of downstream signaling molecules such as PDK-1, Akt,
and PKC-ζ that leads to p70S6 kinase phosphorylation and activation.

In the present study, the PI3K-dependent signaling events activated by IGF-I that regulate p70S6 kinase activity and cell proliferation have been elucidated. IGF-I causes the sequential phosphorylation of PI3K, PDK-1, Akt, and mTOR. PI3K and PDK-1 activation mediate IGF-I-induced p70S6 kinase activation and regulate proliferation. Akt activation via PDK-1, although sufficient to modestly increase the levels of mTOR(Ser2448) phosphorylation, is not required for IGF-I-induced p70S6 kinase activation or growth to occur. Moreover, mTOR kinase activity is required for IGF-I-induced p70S6 kinase activation or growth to occur. Moreover, mTOR kinase activity is required for IGF-I-induced cell proliferation but is distinct from the state of mTOR(Ser2448) phosphorylation and is not regulated by Akt. IGF-I-induced, PI3K-dependent activation of the atypical PKC isoform PKC-ζ also contributes to p70S6 kinase activation and growth.

The evidence supporting the sequential activation of PI3K, PDK-1, and PKC-ζ leading to IGF-I-induced p70S6 kinase activation and proliferation can be summarized as follows: 1) activation of PI3K was blocked by the PI3K inhibitor but not affected by the PDK-1, PKC-ζ, or p70S6 kinase antagonists; 2) both the phosphorylation and activation of PDK-1 were abolished by the PI3K inhibitor or the PDK-1 inhibitor and were inhibited by the PKC-ζ inhibitor but were not affected by the p70S6 kinase antagonist or expression of kinase-inactive Akt; 3) phosphorylation of PKC-ζ was blocked by the PI3K inhibitor and the PDK-1 inhibitor but was not affected by the p70S6 kinase antagonist or kinase-inactive Akt; 4) phosphorylation of p70S6 kinase(Thr421/Ser424) was blocked by the PI3K inhibitor and the p70S6 kinase inhibitor and was partially inhibited by the PKC-ζ inhibitor but was not affected by the PDK-1 inhibitor or kinase-inactive Akt; 5) phosphorylation of p70S6 kinase(Thr229) and p70S6 kinase-(Thr389), activation of p70S6 kinase, and cellular proliferation were blocked by the PI3K, PDK-1, and p70S6 kinase inhibitors and were partially inhibited by the PKC-ζ inhibitor but were not affected by kinase-inactive Akt.

PDK-1, a serine/threonine kinase, links the activation of PI3K and Akt. PDK-1 can also phosphorylate and activate other PI3K-dependent effectors: atypical PKC isoforms and p70S6 kinase. PDK-1 was initially thought to be constitutively active, associated with the plasma membrane, and not subject to growth factor (including IGF-I)-induced phosphorylation or activation (12). The findings of the current study corroborate and extend more recent information showing that insulin (10) and IGF-I (this study) can induce the phosphorylation of PDK-1 on Ser241. Mutants of PDK-1 with disrupted pleckstrin homology domains that do not

Fig. 6. Effects of kinase-inactive Akt1(K179M) on IGF-I-induced, PI3K-dependent signaling intermediates and phosphorylation and activation of p70S6 kinase. A: representative Western blots of expression of Myc-tagged Akt1(K179M) and of IGF-I-induced Akt1(Ser308) and Akt1(Ser473) phosphorylation. The Myc tag was identified in lysates of intestinal smooth muscle cells transiently transfected with Myc-tagged, kinase-inactive Akt1(K179M) but not empty vector. Expression of kinase-inactive Akt1(K179M) inhibited IGF-I-induced phosphorylation of Akt1(Ser308) but did not affect phosphorylation of Akt1(Ser473). B: densitometric analysis of the effects of kinase-inactive Akt1(K179M) on IGF-I-induced phosphorylation of Akt1(Ser308) and Akt1(Ser473). Expression of kinase-inactive Akt1(K179M) inhibited IGF-I-induced phosphorylation of Akt1(Ser473) but did not affect IGF-I-induced phosphorylation of Akt1(Ser308). C: expression of kinase-inactive Akt1(K179M) inhibited the ability of IGF-I to elicit phosphorylation of mTOR(Ser2448) but had no effect on the phosphorylation of PI3K, PDK-1, or PKC-ζ. D: expression of kinase-inactive Akt1(K179M) did not affect the ability of IGF-I to elicit phosphorylation of p70S6 kinase(Thr421/Ser424), p70S6 kinase(Thr229), or p70S6 kinase (Thr389) or to activate p70S6 kinase. Results are expressed as %increase in relative density above basal levels determined by Western blot analysis or the increase in kinase activity (pmol phosphate incorporated/min/mg protein) determined by in vitro kinase assay (see METHODS). Values are means ± SE of 3–6 separate experiments. *P < 0.05 vs. basal.
not localize to the plasma membrane, however, are not activated by growth factors (10). Binding of growth factor-stimulated PIP₃ to the pleckstrin homology domain of Akt causes translocation of Akt to the plasma membrane, relieves steric inhibition, and exposes the activation loop of Akt to PDK-1-mediated phosphorylation of Akt(Ser³⁰⁸) (2). Activation of Akt, however, requires the phosphorylation of both Ser³⁰⁸ and Ser⁴⁷³ in the carboxy terminus. The exact mechanism of Akt(Ser⁴⁷³) phosphorylation, termed the PDK-2 site, and the identity of PDK-2 have remained elusive. Recently, integrin-linked kinase (ILK) has been identified as a putative PDK-2 (3). Whether ILK regulates Akt(Ser⁴⁷³) phosphorylation in human intestinal smooth muscle cells is unknown. Alternative mechanisms for Akt(Ser⁴⁷³) phosphorylation have been proposed. Evidence suggesting that Akt(Ser⁴⁷³) is autophosphorylated by an intrinsic Akt kinase activity that is stimulated by PDK-1-dependent Akt(Ser³⁰⁸) phosphorylation was derived from studies in which kinase-inactive Akt1(K179M) was expressed in human embryonic kidney cells, (HEK-293E cells), Hep G2 cells (47), and Chinese hamster ovary cells (51). This mechanism of Akt activation is supported by the findings of the current paper: when the effects of PDK-1 are blocked by TPCK, the ability of IGF-I to elicit phosphorylation of Akt(Ser³⁰⁸) or Akt(Ser⁴⁷³) is abolished. In contrast, expression of kinase-inactive Akt1(K179M) in human intestinal smooth muscle does not affect IGF-I-induced Akt(Ser³⁰⁸) phosphorylation but does abolish Akt(Ser⁴⁷³) phosphorylation, implying that the autophosphorylation of Akt following IGF-I-induced Akt(Ser³⁰⁸) phosphorylation is required for Akt(Ser⁴⁷³) phosphorylation to occur. Other kinases have been shown to regulate Akt activity, e.g., p38 MAPK-dependent Akt(Ser⁴⁷³) phosphorylation occurs in human neutrophils (34). These mechanisms do not appear to operate in human intestinal smooth muscle cells, because the p38 MAPK and the MKK1/2 inhibitors do not affect the ability of IGF-I to phosphorylate Akt. Interestingly, activation of Akt does not appear to be a requirement for IGF-I-mediated p70S6 kinase activation or proliferation of human intestinal muscle cells. In cells expressing kinase-inactive Akt1(K179M), IGF-I retained its ability to activate p70S6 kinase and to stimulate proliferation. Activation of Akt by IGF-I in human intestinal muscle cells likely regulates other cellular processes, such as inhibition of apoptosis, a process that has also been observed in this and other cell types (23, 27).

The homologous atypical PKC isoforms PKC-ζ and PKC-λ are downstream targets in the PI3K pathway. PI3K-dependent activation of PKC-ζ occurs through three interrelated mechanisms: PDK-1-dependent phosphorylation of PKC-ζ on Thr⁴¹⁰ in the activation loop, autophosphorylation of Thr³⁹⁰, and phosphorylation-independent conformational relief of pseudosubstrate inhibition (perhaps via interaction with PIP₃) (44). Evidence that PKC-ζ represents a physiological target of PDK-1 comes from PDK-1-deficient embryonic stem cells that show markedly decreased levels of conventional (PKC-α, PKC-β₁, PKC-γ) and novel (PKC-δ, PKC-ε) PKC isoforms compared with PDK-1-positive cells (4). In contrast, levels of atypical PKC-ζ are unchanged, but PKC-ζ cannot be phosphorylated on Thr⁴¹⁰. IGF-I-mediated proliferation has been shown to involve the atypical PKC isoform PKC-ζ in rat adipocytes (45) and in the rat clonal β-cell line RIN 1046–38 (19). In the present study, IGF-I elicited phosphorylation of atypical PKC-ζ on Thr⁴¹⁰ of the activation loop and/or PKC-λ on the homologous residue Thr⁴⁰³. Activation of PKC-ζ/λ by IGF-I participated in p70S6 kinase activation and proliferation of muscle, and these effects were inhibited by the cell-permeant PKC-ζ pseudosubstrate inhibitor myr-PKC-ζ-PS (which also blocks the effects of PKC-λ). These two atypical PKC isoforms appear to act interchangeably in their ability to mediate PI3K-dependent cellular processes such as Glut4 translocation in response to insulin or serum-induced activation of p70S6 kinase (6). This is not to say that conventional and novel PKC isoforms are not regulated by PDK-1 (see above) or do not contribute to the complex mechanism of p70S6 kinase activation and regulation of proliferation.

mTOR is a constitutively active kinase that plays a permissive role in regulating p70S6 kinase activity and thus the ribosomal biogenesis necessary for cell division. mTOR senses the level of amino acids and ATP that are available to the cell (13, 38). The current study shows that basal levels of mTOR(Ser²⁴⁴⁸) phosphorylation can be augmented by IGF-I via an Akt-dependent mechanism as has been shown for insulin (37). Akt-dependent mTOR(Ser²⁴⁴⁸) phosphorylation does not appear to regulate p70S6 kinase activity, however. Other investigators have shown that mutation of mTOR(Ser²⁴⁴⁸) to the nonphosphorylated alanine, stimulation with mitogens, withdrawal of amino acids, or treatment with rapamycin all have little affect on mTOR kinase activity (17, 38). Deletion of the domain surrounding Ser²⁴⁴⁸ in mTOR (amino acids 2430–2450), in fact, increases p70S6 kinase activity, suggesting that a repressor function for mTOR resides within this region (41). The rapamycin-FKBP12 gain-of-function complex inhibits signaling downstream of mTOR by disinhibiting the effects of mTOR on protein phosphatase 2A (PP2A) (33), which is normally associated with p70S6 kinase and in an inactive form. Once activated, PP2A rapidly dephosphorylates numerous residues of p70S6 kinase with Thr³⁸⁹ dephosphorylation, most closely paralleling the loss of p70S6 kinase activity (32). Although addition of rapamycin to human intestinal smooth muscle cells did affect IGF-I-induced mTOR activity, this effect was a minor one. Similar effects of rapamycin have been observed in 3T3 L1 adipocytes treated with insulin (39). mTOR has also been shown to directly phosphorylate p70S6 kinase on Thr³⁸⁹ in HEK-293 cells (8). The present study supports the view that mTOR functions in a co-regulatory fashion with the PI3K-dependent signaling pathway involving PDK-1 and PKC-ζ activation. Activation of both pathways is required for p70S6 kinase activation and cell proliferation to occur.
In summary, the present study shows that, in human intestinal smooth muscle cells, IGF-I activates a sequence of events consisting of initial PI3K activation followed by PDK-1 and PKCζ phosphorylation and activation that leads to phosphorylation of specific residues within p70S6 kinase that result in full p70S6 kinase activation and proliferation. PI3K, perhaps via PKCζ, regulates phosphorylation of serine/threonine residues in the autoinhibitory domain of p70S6 kinase. PI3K-dependent activation of PDK-1 and PDK-1-dependent PKCζ activation, in turn, regulate phosphorylation of serine/threonine residues in the catalytic domain of p70S6 kinase, activation of p70S6 kinase, and muscle cell growth. IGF-I-induced Akt activation, although sufficient to increase mTOR(Ser2448) phosphorylation, does not significantly contribute to the regulation of mTOR kinase activity and is not required for either IGF-I-induced p70S6 kinase activation or cell proliferation to occur.

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REFERENCES


