Temporal activation of p70 S6 kinase and Akt1 by insulin: PI 3-kinase-dependent and -independent mechanisms

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Somwar, Romel, Satoru Sumitani, Celia Taha, Gary Sweeney, and Amira Klip. Temporal activation of p70 S6 kinase and Akt1 by insulin: PI 3-kinase-dependent and -independent mechanisms. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E618–E625, 1998.—Several studies have suggested that activation of p70 ribosomal S6 kinase (p70 S6 kinase) by insulin may be mediated by the phosphatidylinositol 3-kinase (PI 3-kinase)-Akt pathway. However, by temporal analysis of the expression of each kinase in L6 muscle cells, we report that the activation of the two serine/threonine kinases (Akt and p70 S6 kinase) can be dissociated. Inhibition stimulated p70 S6 kinase in intact cells in two phases. The first phase (5 min) of stimulation was fully inhibited by wortmannin (IC_{50} = 20 nM) and LY-294002 (full inhibition at 5 µM). After this early inhibition, p70 S6 kinase was gradually stimulated by insulin in the presence of 100 nM wortmannin. After 30 min, the stimulation was 65% of the maximum attained in the absence of wortmannin. The IC_{50} of wortmannin for inhibition of this second phase was ∼150 nM. In contrast, activation of Akt1 by insulin was completely inhibited by 100 nM wortmannin at all time points investigated. Inhibition of mitogen-activated protein kinase/extracellular signal-regulated protein kinase kinase with PD-098059 (10 µM) or treatment with the protein kinase C inhibitor bisindolylmaleimide (10 µM) had no effect on the late phase of insulin stimulation of p70 S6 kinase. We have previously shown that GLUT-1 protein synthesis in these cells is stimulated by insulin via the mTOR-p70 S6 kinase pathway, based on its sensitivity to rapamycin. We therefore investigated whether the signals leading to GLUT-1 synthesis correlated with the early or late phase of stimulation of p70 S6 kinase. GLUT-1 synthesis was not inhibited by wortmannin (100 nM). In summary, insulin activates p70 ribosomal S6 kinase in L6 muscle cells by two mechanisms, one dependent on and one independent of the activation of PI 3-kinase. In addition, activation of Akt1 is fully inhibited by wortmannin, suggesting that Akt1 does not participate in the late activation of p70 S6 kinase. Wortmannin-sensitive PI 3-kinases and Akt1 are not required for insulin stimulation of GLUT1 protein biosynthesis.

Temporal activation of p70 S6 kinase by insulin.

The ribosomal p70 S6 kinase is a serine/threonine kinase that is activated in response to many growth factors by hierarchical phosphorylation of multiple serine and threonine residues in discrete functional domains (34). In vivo, the kinase controls protein synthesis by increasing the rate of translation of a family of mRNAs that encode essential components of the protein synthetic machinery (32). In addition, we have recently reported that the macrolide immunosuppressant rapamycin, which prevents the activation of p70 S6 kinase, blocks the insulin-induced upregulation of glucose transporter 1 (GLUT-1) expression (39, 40). The molecular mechanisms underlying the activation of p70 S6 kinase by insulin and other growth factors are poorly understood, and some of the intermediate participants that link growth factor receptors to the phosphorylation and activation of this serine/threonine kinase remain elusive.

Phosphatidylinositol 3-kinase (PI 3-kinase) is a lipid kinase that phosphorylates inositol lipids at the D-3 position (7), and several lines of evidence support the view that it is one of the primary signaling molecules linking growth factor receptors to the activation of p70 S6 kinase (8, 10). For example, platelet-derived growth factor (PDGF) failed to stimulate p70 S6 kinase in Chinese hamster ovary (CHO) cells overexpressing a mutant PDGF receptor lacking the binding sites for the p85 regulatory subunit of PI 3-kinase (Y740 and Y751) (10). Moreover, two structurally different inhibitors of PI 3-kinase, wortmannin and LY-294002, blocked the activation of p70 S6 kinase by insulin in 3T3-L1 adipocytes (8) and by PDGF in CHO cells overexpressing wild-type PDGF receptors (10). On the other hand, mutation of Y740 in the PDGF receptor completely abolished activation of PI 3-kinase by PDGF but did not prevent the activation of p70 S6 kinase (27). In addition, insulin can activate p70 S6 kinase in CHO cells and Rat1 fibroblasts, both overexpressing a dominant negative p85α subunit of PI 3-kinase, which lacks the binding site for the p110 catalytic subunit (17). Surprisingly, wortmannin (50 nM) completely inhibited insulin-induced activation of p70 S6 kinase in both cell types expressing dominant negative p85α (17). These results suggest that a PI 3-kinase-independent but wortmannin-sensitive pathway exists for the activation of p70 S6 kinase by insulin.

The molecular mechanism underlying the activation of p70 S6 kinase is complex, but one current model suggests that a PI 3-kinase-dependent kinase phosphorylates Thr^{380}, one of the most critical residues for p70 S6 kinase activation (12). Akt is a serine/threonine kinase that has been demonstrated to be a downstream target of PI 3-kinase in cells stimulated with insulin (23), PDGF, epidermal growth factor (EGF), or basic fibroblast growth factor (4, 15), since activation of Akt by these growth factors is blocked by wortmannin and LY-294002. Activation of Akt was also lost in cells overexpressing a dominant negative p85α subunit of PI...
3-kinase (4). In addition, Akt could not be activated by PDGF in cells overexpressing the PDGF-receptor mutants described above (4, 15). On the basis of the characteristics of the activation of Akt and the observation that overexpression of a gag fusion of Akt constitutively activates p70 S6 kinase (4), it has been speculated that Akt could be the protein kinase that mediates the PI 3-kinase-dependent phosphorylation of Thr389 of p70 S6 kinase. On the other hand, Akt cannot phosphorylate p70 S6 kinase in vitro (33), and a kinase-deficient mutant of Akt believed to have dominant negative action was unable to block p70 S6 kinase activation in vivo (22).

Given the controversy mentioned above, we have investigated the time course of p70 S6 kinase activation vis-à-vis its sensitivity to inhibition of PI 3-kinase. Surprisingly, two mechanisms of activation were uncovered, one dependent on and one independent of PI 3-kinase, based on sensitivity to wortmannin and LY-294002. We further demonstrate that, in contrast, activation of Akt1 is fully dependent on PI 3-kinase and can therefore be dissociated from the activation of p70 S6 kinase at longer times.

**MATERIALS AND METHODS**

Materials. α-MEM, FBS, and other tissue culture reagents were purchased from GIBCO BRL (Burlington, ON, Canada). Human insulin (Humulin) was obtained from Eli Lilly Canada (Toronto, ON, Canada). Protein A-Sepharose was from Pharmacia (Uppsala, Sweden). Polyclonal anti-GLUT-1 glucose transporter antisera was from East Aces Laboratories (South Bridge, MA). The monoclonal antibody 6H to the α-subunit of the Na+/K+-ATPase was a kind gift from Dr. M. Caplan (Dept. of Cellular and Molecular Physiology, Yale University). Polyclonal anti-p70 S6 kinase, anti-Akt1, and anti-IRS-1 antibodies, p70 S6 kinase substrate, and protein kinases A (PKA) and C (PKC) inhibitor peptides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Akt1 substrate peptide, Crossid, was purchased from Upstate Biotechnology (Lake Placid, NY). γ-32P-ATP (6,000 Ci/mmol) and enhanced chemiluminescence reagents were purchased from Amersham (Oakville, ON, Canada). Purified L-α-phosphatidylinositol (PI) was purchased from Avanti Polar Lipids (Alabaster, AL). Oxalate-treated TCA sonic gel H plates (250 µm) were from Analtech (Newark, DE). Wortmannin was from Sigma (St. Louis, MO). PD-098059 was a kind gift from Dr. Alan Saltiel (Parke-Davis, Ann Arbor, MI). LY-294002 and okadaic acid were from Biomol (Plymouth Meeting, PA). Rapamycin and bisindolylmaleimide (BIM) were from Calbiochem (La Jolla, CA). SB-203580 was a generous gift from Dr. John Lee (SmithKline Beecham Pharmaceuticals, King of Prussia, PA). All electrophoresis and immunoblotting reagents were purchased from Bio-Rad (Missisaga, ON, Canada). All other reagents were of the highest analytical grade.

Cell culture and incubations. A clonal cell line of L6 rat skeletal muscle cells selected for high fusion potential was grown in α-MEM containing 2% FBS; these cells were allowed to fuse and differentiate as previously described (29). Cultures were studied at >90% maximal fusion into fully differentiated myotubes. For total membrane preparation, L6 myotubes were incubated in the presence or absence of insulin (100 nM) for a total of 18 h. Incubation media were replaced every 5 h. Where indicated, the cells were incubated in the presence of 100 nM wortmannin or 20 ng/ml rapamycin for the entire period that insulin was present. For the kinetic analysis of the activation of p70 S6 kinase, the cells were serum deprived in 0.1% FBS-α-MEM for 12 h and stimulated with 100 nM insulin for the indicated time. For PI 3-kinase assay, the serum-deprived cells were stimulated with insulin as indicated in figure legends. All inhibitors were added 30 min before insulin stimulation and were present throughout the incubations.

Isolation of total membranes. Total membranes of L6 myotubes were prepared as previously described (28). Briefly, cells were rinsed twice with cold homogenization buffer (250 mM sucrose, 20 mM HEPES (pH 7.4), 5 mM NaN3, and 2 mM EGTA). Cell monolayers were scraped into cold homogenization buffer containing 1 µM leupeptin, 1 µM pepstatin, 10 µM E-64, and 200 µM phenylmethylsulfonyl fluoride (PMSF), homogenized with 20 strokes (Dounce type A homogenizer), and then centrifuged at 700 g for 5 min at 4°C. The supernatant from this low-speed spin was then centrifuged for 1 h at 190,000 g to obtain total membranes. Immunoblotting was carried out as previously described (28).

Preparation of cell extracts for p70 S6 kinase or PI 3-kinase assays. For p70 S6 kinase activity, L6 myotubes grown in 10-cm dishes were washed twice with ice-cold PBS and lysed in 1 ml of buffer A [50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM β-glycerophosphate, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM Na3VO4, and 1% (vol/vol) NP-40] containing a mixture of protease inhibitors (1 µM leupeptin, 1 µM pepstatin A, 10 µM E-64, and 200 µM PMSF). After 15 min of slow agitation and centrifugation (15,000 g for 15 min), the supernatant was subjected to immunoprecipitation. For PI 3-kinase activity, cell extracts were prepared in the same way except for using buffer D (20 mM Tris (pH 7.5), 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 100 mM Na3VO4, 1% (vol/vol) NP-40, and 10% (vol/vol) glycerol) and the same mixture of protease inhibitors.

Immunoprecipitation and assay of p70 S6 kinase activity. p70 S6 kinase kinase activity was assayed essentially as described (29) in a final volume of 50 µl of buffer C containing 1 µM PKA and PKC inhibitor peptides, 0.2 µM S6 peptide, and 0.25 mM Mg[γ-32P]ATP at 30°C for 10 min. Aliquots (30 µl) were transferred onto Whatman p81 filter papers and washed three times for 15 min with 175 mM phosphoric acid (pH 7.2), 25 mM β-glycerophosphate, 5 mM EDTA, 20 mM MgCl2, 2 mM Na3VO4, and 1 mM dithiothreitol (DTT). p70 S6 kinase activity was assayed essentially as described (24) in a final volume of 50 µl of buffer C containing 1 µM PKA and PKC inhibitor peptides, 0.2 µM S6 peptide, and 0.25 mM Mg[γ-32P]ATP at 30°C for 10 min. All aliquots (30 µl) were transferred onto Whatman p81 filter papers and washed three times for 15 min with 175 mM phosphoric acid (pH 7.2). 32P incorporated into the S6 peptide was measured by liquid scintillation counting. One unit of protein kinase activity corresponds to 1 µl of 32P incorporated into the substrate peptide under the assay conditions.

Immunoprecipitation and assay of PI 3-kinase activity. PI 3-kinase activity was measured on IRS-1 immunoprecipitates as described (41). Immunoprecipitation was performed as described above but using anti-IRS-1-antibody instead of anti-p70 S6 kinase antibody. The immunoprecipitates were washed three times with wash buffer 1 [PBS containing 1% (vol/vol) NP-40 and 100 µM Na3VO4], three times with wash buffer 2 [100 mM Tris (pH 7.5) containing 500 mM LiCl and 100 µM Na3VO4], and twice with wash buffer 3 [10 mM Tris (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, and 100 µM...
Na<sub>3</sub>VO<sub>4</sub>]. The pellets were resuspended in 50 µl of 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, 100 µM Na<sub>3</sub>VO<sub>4</sub>, 10 µl of 100 mM MgCl<sub>2</sub>, and 10 µl of PI (2 mg/ml) in 10 mM Tris (pH 7.5) and 1 mM EGTA. The reaction was initiated by the addition of 5 µl of 440 µM ATP containing 10 µCi of [γ<sup>32</sup>P]ATP. After 10 min at 30°C, the reaction was terminated by the addition of 20 µl of 8 M HCl and 180 µl of CHCl<sub>3</sub>-methanol (1:1 vol/vol). The samples were centrifuged for 5 min at maximal speed in a microcentrifuge, and 50 µl of the lower organic phase were removed and applied to a potassium oxalate (1%)-pretreated silica gel 60 TLC plate that had been prebaked for at least 1 h. Lipids were separated by TLC using CHCl<sub>3</sub>-CH<sub>3</sub>OH-H<sub>2</sub>O-NH<sub>4</sub>OH (60:47:11:3:2) as the running solvent. The detection and quantitation of [32P]PI3P on the TLC plates were done using a Molecular Dynamics Phosphorimager System (Sunnyvale, CA).

Immunoprecipitation and assay of Akt1 protein kinase activity. Immunoprecipitation of Akt1 and kinase assay were performed as described (23) with modifications. Anti-Akt1 antibody was precoupled to a mixture of protein A- and protein G-Sepharose beads by incubating 2 µg of antibody per well with 40 µl of protein A-Sepharose (100 mg/ml) and 20 µl of protein G-Sepharose (100 mg/ml) for 20 min. These anti-Akt1-bead complexes were washed twice with ice-cold PBS and once with ice-cold lysis buffer. Akt1 was immunoprecipitated by incubating 200 µg of total cellular protein with the anti-Akt1-bead complex for 2–3 h under constant rotation (4°C). Akt1 immunocomplex was isolated and washed four times with 1 ml of wash buffer (25 mM HEPES (pH 7.8), 10% glycerol (vol/vol), 1% Triton X-100 (vol/vol), 0.1% BSA (vol/vol), 1 M NaCl, 1 mM DTT, 1 mM PMSF, 1 µM microcystin, and 100 µM okadaic acid) and twice with 1 ml kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, and 1 mM DTT). This was then incubated under constant agitation for 10 min at 30°C with 30 µl of reaction mixture (kinase buffer containing 5 µM ATP, 2 µCi of [γ<sup>32</sup>P]ATP, and 100 µM Cysteide). After the reaction, 30 µl of the supernatant were transferred onto Whatman p81 filter paper and treated as described above for p70 S6 kinase assay.

Statistical analysis. Autoradiograms of X-ray films exposed to produce bands within the linear range for quantitation were scanned and quantitated by use of the computer software NIH Image. Statistical analysis was performed using the ANOVA test (Fisher, multiple comparisons). For analysis of PI 3-kinase, Akt1, and p70 S6 kinase activities, the Student's paired t-test was applied.

RESULTS

Time course of p70 S6 kinase activation by insulin and effect of inhibition of PI 3-kinase. To understand the relationship between PI 3-kinase and the activation of p70 S6 kinase, we examined the effect of wortmannin on insulin-induced activation of the latter. Previous studies advocating the positioning of PI 3-kinase upstream of p70 S6 kinase measured the activity of p70 S6 kinase after a 5-min incubation of cells with growth factors (8). To obtain a more detailed description of the regulation of p70 S6 kinase by insulin, we used an in vitro kinase assay to analyze the effect of wortmannin treatment on p70 S6 kinase catalytic activity in L6 skeletal muscle cells. L6 myotubes were pretreated for 30 min with 100 nM wortmannin before the addition of 100 nM insulin for 5–30 min. p70 S6 kinase was immunoprecipitated, and the kinase activity associated with the immunocomplex was determined by using an exogenous S6 peptide as substrate (a 9-amino acid peptide derived from ribosomal S6 protein). Preliminary experiments confirmed the linearity of the assay within the time frame used (data not shown). As shown in Fig. 1A, insulin increased p70 S6 kinase activity rapidly, reaching a peak in 10 min and remaining elevated for at least 30 min. Although wortmannin (100 nM) completely abrogated insulin-stimulated p70 S6 kinase activity, it had no effect on basal activity. Furthermore, insulin increased p70 S6 kinase activity within 5 min. These results are consistent with the idea that PI 3-kinase is involved in the regulation of p70 S6 kinase activity.

Statistical analysis of the data was performed using the ANOVA test (Fisher, multiple comparisons). For analysis of PI 3-kinase, Akt1, and p70 S6 kinase activities, the Student's paired t-test was applied.

Fig. 1. Effect of phosphatidylinositol (PI) 3-kinase inhibition on insulin-stimulated p70 S6 kinase activation. L6 myotubes were stimulated with 100 nM insulin for indicated time periods in absence or presence of 100 nM wortmannin (30-min pretreatment) or 5 µM LY294002 (30-min pretreatment). Cells were lysed, p70 S6 kinase was immunoprecipitated, and an in vitro kinase assay was performed with immunocomplex as described in MATERIALS AND METHODS. Results are representative of 2–3 independent experiments. Insulin-stimulated p70 S6 kinase activity at each time point in absence of inhibitors was considered as 100%. *P < 0.05, compared with 5-min insulin-treated cells. **P < 0.05, compared with 30-min insulin-treated cells. #P < 0.05, compared with corresponding treatment at 5 min.
lated p70 S6 kinase activity at 5 min, insulin treatment gradually stimulated p70 S6 kinase activity after this time in the continued presence of wortmannin. In two independent experiments, insulin was not able to fully restore the magnitude of p70 S6 kinase activity that was observed in the absence of wortmannin for up to 60 min (data not shown).

We also tested the ability of 5 µM LY-294002, another inhibitor of PI 3-kinase, to inhibit the first (5 min) and second (30 min) phases of p70 S6 kinase activation by insulin. In cells treated with insulin for 5 min in the presence of 5 µM LY-294002 or 100 nM wortmannin for comparison, p70 S6 kinase activity was completely inhibited by both agents (Fig. 1B), confirming the involvement of PI 3-kinase. However, in cells treated with insulin for 30 min in the continued presence of 5 µM LY-294002 or 100 nM wortmannin, 34 ± 13 or 65 ± 8%, respectively, of insulin-stimulated p70 S6 kinase activity was still measurable.

To determine whether PI 3-kinase was inhibited by wortmannin under these conditions, cells pretreated for 30 min with or without 100 nM wortmannin were stimulated with insulin for 5 or 30 min. IRS-1-associated PI 3-kinase activity was then determined. Insulin (5 min) stimulated PI 3-kinase activity by 34 ± 6.2-fold above control, and in the presence of wortmannin, insulin-stimulated PI 3-kinase activity was 3.1 ± 1.4-fold above control (P < 0.05, n = 5). Wortmannin also inhibited PI 3-kinase activity stimulated by 30 min of insulin treatment (insulin: 11.2-fold above control; wortmannin + insulin: 0.9-fold above control; n = 1).

Dose-dependent effect of wortmannin on p70 S6 kinase activation. To confirm the existence of a PI 3-kinase-dependent pathway and a PI 3-kinase-independent pathway leading to the activation of p70 S6 kinase, we examined the dose-dependent inhibition of p70 S6 kinase activity by wortmannin on the first (5 min) and second (30 min) phases of activation by insulin. L6 myotubes were treated with 100 nM insulin for 5 or 30 min in the presence of various concentrations of wortmannin, and p70 S6 kinase activity was then assayed. In cells that were treated with insulin for 5 min, wortmannin inhibited insulin-stimulated p70 S6 kinase activity, with an IC50 of ~20 nM (Table 1). In contrast, the IC50 of wortmannin for the inhibition of the second phase (30 min) of activation of p70 S6 kinase stimulated was ~150 nM (Table 1). This concentration of wortmannin is 5- to 15-fold higher than that for inhibition of class IA PI 3-kinases (11, 43). These results suggest that insulin stimulated the activation of p70 S6 kinase, with two phases having distinct wortmannin sensitivities.

Time course of Akt1 activation by insulin and inhibition by wortmannin. Overexpression of Akt was shown to activate p70 S6 kinase independent of any stimulus (4). To determine whether Akt could mediate the second phase (30 min) of insulin-stimulated p70 S6 kinase activation that has a reduced sensitivity to wortmannin, we utilized an in vitro kinase assay to investigate the time course of activation of Akt1 by insulin along with the effect of wortmannin. L6 myotubes were treated for 5–30 min with 100 nM insulin in the absence or presence of indicated concentrations of wortmannin. Cells in presence of wortmannin were treated with appropriate concentration for 30 min before addition of insulin. p70 S6 kinase activity was then determined as described under MATERIALS AND METHODS. Value of insulin-stimulated p70 S6 kinase activity above basal, in absence of wortmannin, was considered as 100%.

![Fig. 2. Effect of wortmannin on insulin-stimulated Akt1 activation. L6 myotubes were stimulated with 100 nM insulin for indicated time periods in absence (○) or presence (●) of 100 nM wortmannin (30-min pretreatment). Akt1 was immunoprecipitated from total cell lysates and an in vitro kinase assay performed as described in MATERIALS AND METHODS. Results are mean ± SE of 3–6 independent experiments. *Significantly different from corresponding time point in presence of wortmannin, P < 0.05.](http://ajpendo.physiology.org/)
is consistent with Akt1 being a downstream target of PI 3-kinase. Because activation of Akt1 by insulin was prevented by wortmannin, we conclude that Akt1 does not play a role in the second phase (30 min) of activation of p70 S6 kinase.

Analysis of second phase of p70 S6 kinase activation by insulin. The observation that insulin can activate p70 S6 kinase by a PI 3-kinase-independent pathway prompted us to examine other signaling pathway(s) that may mediate this activation of p70 S6 kinase. L6 myotubes were treated for 30 min with 100 nM insulin in the absence or presence of inhibitors of two insulin-stimulated kinases that have been implicated in the regulation of p70 S6 kinase: mitogen-activated protein kinase (MAPK) (14) and PKC (38). The results are shown in Table 2. As expected, rapamycin completely abrogated insulin-stimulated p70 S6 kinase activity (1 ± 0.1% of control, n = 3). In concert with the results shown in Fig. 1A, wortmannin only partially inhibited the stimulation of p70 S6 kinase by insulin (allowing 65 ± 8% of maximal stimulation, n = 3). BIM (10 μM), an inhibitor of all PKC isofoms at this concentration (26), had no effect on insulin-stimulated p70 S6 kinase activity (Table 2; 106 ± 17% of control, n = 3). This suggests that insulin does not utilize the PKC pathway to stimulate p70 S6 kinase. Similarly, the MAPK/extracellular signal-regulated protein kinase kinase (MEK) inhibitor PD-098059 (10 μM) had no effect on p70 S6 kinase activity (91 ± 5% of control). p38 MAPK, a stress-activated MAPK, has been shown to be activated by insulin in 3T3-L1 adipocytes and fibroblasts (6), and its phosphorylation is stimulated by insulin in L6 cells (40, 42). The specific inhibitor of p38 MAPK, SB-203580 (25), had no inhibitory effect on insulin-stimulated p70 S6 kinase activity (133 ± 44% of control, n = 3), indicating that p38 MAPK is not necessary for the regulation of p70 S6 kinase by insulin.

Wortmannin does not inhibit insulin-stimulated biosynthesis of GLUT-1. We have previously demonstrated that insulin-induced synthesis of GLUT-1 protein is dependent on the mTOR-p70 S6 kinase signaling pathway in L6 myotubes (39, 40). We therefore investigated which of the two phases of activation of p70 S6 kinase by insulin participates in this end-point response. L6 myotubes were treated with or without 100 nM insulin in the absence or presence of 100 nM wortmannin or 20 ng/ml rapamycin for a total of 18 h. To ensure the stability and bioavailability of wortmannin during the 18-h incubation period, we replaced the incubation medium with medium containing freshly added wortmannin every 5 h. Total membranes were then prepared as described under MATERIALS AND METHODS. As shown in Fig. 3A and quantified in Fig. 3B, insulin elicited a significant increase in GLUT-1 protein immunoreactivity above control (1.8 ± 0.6-fold, n = 3, P < 0.05). Consistent with our previous report (39), this increase was completely abolished by 20 ng/ml rapamycin (data not shown). Here we show that, in the absence of wortmannin, insulin still elicited a significant increase in GLUT-1 protein content above control (1.7 ± 0.2-fold, n = 3, P < 0.05). In the absence of insulin, the levels of GLUT-1 were not altered by either wortmannin or rapamycin treatment. After a 5-h pre-treatment of L6 myotubes with 100 nM wortmannin, a 5-min insulin challenge was unable to stimulate PI 3-kinase activity associated with IRS-1 immunoprecipitates (insulin: 6.5-fold above control; insulin + wortmannin: 0.9-fold above control). This underscores that wortmannin was still capable of completely inhibiting PI 3-kinase activity 5 h after its addition to cells. Taken

Table 2. Effect of inhibition of several kinases on stimulation of p70 S6 kinase activity by 30-min insulin stimulation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Kinase Inhibited</th>
<th>Insulin-Stimulated p70 S6 Kinase Activity, %</th>
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<tbody>
<tr>
<td>No inhibitor</td>
<td></td>
<td>100</td>
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<tr>
<td>Rapamycin</td>
<td>mTOR</td>
<td>0.6 ± 0.05</td>
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<tr>
<td>Wortmannin</td>
<td>PI 3-kinase</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>BIM</td>
<td>PKC</td>
<td>106 ± 17</td>
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<tr>
<td>PD-098059</td>
<td>MEK</td>
<td>106 ± 24</td>
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<tr>
<td>SB-203580</td>
<td>p38 MAPK</td>
<td>133 ± 44</td>
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Values are means ± SE of 3 independent experiments. L6 myotubes were pretreated with appropriate inhibitor for 30 min and then stimulated with 100 nM insulin for 30 min in absence or presence of each inhibitor. Insulin-stimulated p70 S6 kinase activity was subsequently measured as described in MATERIALS AND METHODS. Activity is expressed as percentage of the insulin-stimulated activity above basal in absence of inhibitors. BIM, bisindolylmaleimide PI 3-kinase; phosphatidylinositol 3-kinase; MEK, mitogen-activated protein kinase (MAPK)-1 extracellular signal-regulated protein kinase.
Together, these results suggest that wortmannin-sensitive PI 3-kinases are not essential for insulin stimulation of GLUT-1 protein synthesis in L6 skeletal muscle cells.

**DISCUSSION**

Two pathways leading to p70 S6 kinase activation. Here we show that activation of p70 S6 kinase by insulin in muscle cells occurs in two phases, distinguishable by their different sensitivity to inhibitors of PI 3-kinase. The IC50 of inhibition of the first phase by wortmannin (20 nM) is consistent with that for inhibition of class I A PI 3-kinases (11, 44). In vitro, the IC50 of wortmannin for the inhibition of p85/p110 PI 3-kinases is 2 nM (36, 43) and in intact cells is 10–30 nM (43). On the other hand, the higher IC50 value of wortmannin for inhibition of the second phase (150 nM) suggests that class I A PI 3-kinases are not involved and that wortmannin targets another upstream activator, possibly mTOR. This notion is supported by two recent reports that demonstrate that wortmannin irreversibly inhibits the serine-specific autokinase activity (3) and protein kinase activity (2) of mTOR with IC50 of ~200 nM. The higher sensitivity of the second phase of p70 S6 kinase activation to LY-294002 than to wortmannin also supports this hypothesis, since Brunn et al. (3) demonstrated that the IC50 of LY-294002 for inhibition of both mTOR and PI 3-kinase is 5 µM. The yeast and mammalian TOR proteins are now recognized as members of a growing family of high-molecular-weight kinases whose catalytic domains resemble those of PI 3-kinases (19) and are necessary for the activation of p70 S6 kinase by all known stimuli, including insulin (9, 32, 34). Although mTOR protein is upregulated as 3T3-L1 fibroblasts differentiate into the more insulin-responsive adipocytes (46), it is unknown how insulin regulates the kinase activity of this protein.

The protein kinase activity of mTOR is stimulated by serum in vivo, and this precedes the activation of p70 S6 kinase (5). Full activation of p70 S6 kinase by growth factors is achieved with phosphorylation of three critical sites (Thr229, Thr389, and Ser404) (16, 31), and phosphorylation of these sites is prevented by both wortmannin and rapamycin. Late phase is probably sufficient to mediate insulin-induced synthesis of GLUT-1, since this process is inhibited by rapamycin. Late phase is probably sufficient to mediate insulin-induced synthesis of GLUT-1, since this process is inhibited by rapamycin.

**Fig. 4.** Schematic model of regulation of p70 S6 kinase and GLUT-1 protein synthesis by insulin. Insulin stimulates p70 S6 kinase via 2 pathways, one sensitive to inhibitors of PI 3-kinase (early phase, indicated by solid arrows) and the other less sensitive to these inhibitors (late phase, indicated by dashed arrows) at concentrations at which they inhibit class IA PI 3-kinase. Both phases of activation are inhibited by rapamycin. Late phase is probably sufficient to mediate insulin-induced synthesis of GLUT-1, since this process is inhibited by rapamycin but not wortmannin.
its involvement in insulin-stimulated p70 S6 kinase activity. The specific p38 MAPK inhibitor, SB-203580 (25), had no effect on insulin-induced p70 S6 kinase activity (30-min stimulation).

Role of Akt1 in activation of p70 S6 kinase. Akt has recently been identified as a direct downstream substrate of PI 3-kinase in many growth factor signaling pathways (18). However, Akt could be activated by basic fibroblast growth factor without prior activation of PI 3-kinase (21), whereas overexpression of Akt in cells suggested that it had a role in the activation of p70 S6 kinase (4). To determine whether the kinetics of activation of Akt matched those of the activation of p70 S6 kinase, we first looked at the effect of wortmannin on the activation of Akt1 by insulin. Activation of Akt1 was fully inhibited by 100 nM wortmannin under conditions in which only 35% of insulin-stimulated p70 S6 kinase was inhibited. Thus Akt1 activation by insulin could not support the latent activation of p70 S6 kinase in the face of complete inhibition of PI 3-kinase. Together with the results presented here, this suggests that if Akt1 is involved in the regulation of p70 S6 kinase, it may mediate only the first phase of p70 S6 kinase activation.

Akt2 and Akt3 are two additional isoforms of Akt that have been suggested to be downstream of PI 3-kinase (13). No detailed time course showing activation of these two isoforms has been reported. Hence, we do not know whether the kinetics of Akt2 and Akt3 activation match those for activation of p70 S6 kinase. More detailed studies of Akt2 and Akt3 are required before we can assign a role for either one in the regulation of p70 S6 kinase. However, we can speculate that these two isoforms may participate in the first phase of activation of p70 S6 kinase because their activation is PI 3-kinase dependent.

GLUT-1 protein synthesis occurs via second phase of p70 S6 kinase activation. Finally, we demonstrate that wortmannin was unable to block insulin-stimulated GLUT-1 protein synthesis in L6 muscle cells, whereas rapamycin did. It is unlikely that degradation of wortmannin during the incubation caused reactivation of PI 3-kinase, since repetitive addition of wortmannin every 5 h was still effective in preventing the rapid stimulation of PI 3-kinase by insulin. A similar protocol for prolonged treatment with wortmannin was shown to inhibit differentiation of L6E9 cells (20). Wortmannin covalently modifies Lys802 in the lipid kinase domain of the p110 catalytic subunit, resulting in the irreversible inhibition of PI 3-kinase activity (47). Thus PI 3-kinase is not required for insulin to upregulate GLUT-1 protein, and it is likely that the second phase of activation of p70 S6 kinase is sufficient to upregulate GLUT-1 protein content.

In summary, the activation of p70 S6 kinase by insulin in L6 skeletal muscle cells is composed of two separable components: an early phase dependent on class IA PI 3-kinase and possibly Akt1, and a second phase independent of class IA PI 3-kinases (Fig. 4). The latter phase does not involve PKC, MEK, or p38 MAPK. In addition, insulin upregulates GLUT-1 protein despite complete inhibition of PI 3-kinase and Akt1 activation with wortmannin. Because the second phase of activation of p70 S6 kinase remains sensitive to higher levels of wortmannin, it is possible that PI 3-kinase-related mTOR or another PI 3-kinase enzyme is involved in the maintenance of insulin-stimulated p70 S6 kinase activity and GLUT-1 protein biosynthesis.

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