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Assessment of Cell-Signaling Pathways in the Regulation of Mammalian Target of Rapamycin (mTOR) by Amino Acids in Rat Adipocytes

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Abstract Enhanced phosphorylation of the ribosomal protein s6 kinase, $p70^{s6k}$, and the translational repressor, 4E-BP1, are associated with either insulin-induced or amino acid-induced protein synthesis. Hyperphosphorylation of $p70^{s6k}$ and 4E-BP1 in response to insulin or amino acids is mediated through the mammalian target of rapamycin (mTOR). In several cell lines, mTOR or its downstream targets can be regulated by phosphatidylinositol (PI) 3-kinase; protein kinases A, B, and C; heterotrimeric G-proteins; a PD98059-sensitive kinase or calcium; as well as by amino acids. Regulation by amino acids appears to involve detection of levels of charged t-RNA or t-RNA synthetase activity and is sensitive to inhibition by amino acid alcohols. In the present article, however, we show that the rapamycin-sensitive regulation of 4E-BP1 and $p70^{s6k}$ in freshly isolated rat adipocytes is not inhibited by either L-leucinol or L-histidinol. This finding is in agreement with other recent studies from our laboratory suggesting that the mechanism by which amino acids regulate mTOR in freshly isolated adipocytes may be different than the mechanism found in a number of cell lines. Therefore we investigated the possible role of growth factor-regulated and G-protein–regulated signaling pathways in the rapamycin-sensitive, amino acid alcohol-insensitive actions of amino acids on 4E-BP1 phosphorylation. We found, in contrast to previously published results using 3T3-L1 adipocytes or other cell lines, that the increase in 4E-BP1 phosphorylation promoted by amino acids was insensitive to agents that regulate protein kinase A, mobilize calcium, or inhibit protein kinase C. Furthermore, amino acid-induced 4E-BP1 phosphorylation was not blocked by pertussis toxin nor was it mimicked by the G-protein agonists fluoroaluminate or MAS-7. However, amino acids failed to activate either PI 3-kinase, protein kinase B, or mitogen-activated protein kinase and failed to promote tyrosine phosphorylation of cellular proteins, similar to observations made using cell lines. In summary, amino acids appear to use an amino acid alcohol-insensitive mechanism to regulate mTOR in freshly isolated adipocytes. This mechanism is independent of cell-signaling pathways implicated in the regulation of mTOR or its downstream targets in other cells. Overall, our study emphasizes the need for caution when extending results obtained using established cell lines to the differentiated nondividing cells found in most tissues. J. Cell. Biochem. 79:427–441, 2000.

Key words: phosphatidylinositol 3-kinase; rapamycin; leucinol; histidinol; protein kinases

Amino acids, insulin, and growth factors stimulate the activity of the serine/threonine protein kinase mTOR (mammalian target of rapamycin, also FRAP and RAFT) in many mammalian cells and cell-lines [Fox et al., 1998a; Fox et al., 1998b; Hara et al., 1998; Iiboshi et al., 1999; Kimball et al., 1999; Lawrence et al., 1997; Patti et al., 1998; Shigemitsu et al., 1999; Thomas and Hall, 1997; von Manteuffel et al., 1997; Wang et al., 1998; Xu et al., 1998a]. Activation of mTOR is important in dividing cells for G_0/G_1 transition of the cell cycle [Brooks, 1977; for review see Thomas and Hall, 1997] and, in nondividing cells, may facilitate hypertrophic cell growth and regulation of protein synthesis. In adipocytes, mTOR

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activity has been additionally implicated in two tissue-specific events: the output of leptin, a hormone that regulates appetite and energy expenditure [Bradley and Cheatham, 1999] and the formation of multicellular clusters of adipocytes cultured within Matrigel [Fox et al., 1998a], an in vitro behavior that may reflect steps in adipose tissue morphogenesis [Brown et al., 1997].

Activation of mTOR modulates protein synthesis in part through effects on the initiation phase of mRNA translation [Dumont and Su, 1996; Jackson and Wickens, 1997; Kleijn et al., 1998; Lawrence and Abraham, 1997; Lawrence et al., 1997; Thomas and Hall, 1997]. This translational control is accomplished through at least two mechanisms. The first is associated with multisite phosphorylation of the translational repressor eIF4E binding protein-1 (4E-BP1, also called PHAS-I) [Brunn et al., 1997a; Lin et al., 1995]. Phosphorylation of 4E-BP1 leads to release of bound eIF4E, allowing eIF4E to complete the formation of the eIF4F multiprotein complex [Brunn et al., 1997b; Pause, 1994]. This mechanism is believed to be important for enhancing translation of mRNAs containing extensive secondary structure in the 5′-capped untranslated region. The second known mechanism through which mTOR regulates translation initiation involves activation of the ribosomal protein kinase, $p70^{s6k}$ [for review see Thomas and Hall, 1997]. Activated $p70^{s6k}$, in turn, phosphorylates ribosomal protein S6, an event that is required for rapamycin-sensitive increases in translation of mRNAs containing a tract of polypyrimidine residues in their 5′-untranslated region [for review see Thomas and Hall, 1997].

An area of interest in the field of translational control has been the delineation of the mechanism involved in the activation of mTOR by amino acids and how this mechanism of action compares to that of insulin. Insulin stimulation of mTOR appears to be dependent on the activation of phosphatidylinositol (PI) 3-kinase and the serine, threonine protein kinase, protein kinase B (PKB, also AKT) [Gingras et al., 1998; Scott et al., 1998b; Takata et al., 1999]. This observation has led to speculation that mTOR may be regulated through phosphorylation by PKB or another PKB-regulated kinase. Indeed, increased phosphorylation of mTOR on Ser-2448 is associated with insulin stimulation of the kinase [Scott et al., 1998a; Nave, 1999 #262]. However, the direct role of PKB in this phosphorylation is controversial. Nave et al. [1999] reported that PKB could phosphorylate mTOR in vitro; however, others were unable to detect direct phosphorylation of mTOR [Scott et al., 1998a]. A complicating factor is that mTOR is autophosphorylated on at least two sites, and autokinase activity towards at least one of these sites (Ser 2481) is rapamycin-insensitive [Peterson et al., 2000].

In contrast to the action of insulin, which requires PI 3-kinase and PKB for activation of mTOR, amino acids did not activate PKB or PI 3-kinase in several cell lines where this was tested [e.g., Hara et al., 1998; Iiboshi et al., 1999; Kimball et al., 1999; Patti et al., 1998; Wang et al., 1998]. In lymphoblastoid cell lines, amino acid-sensitive mTOR signaling is sensitive to inhibition by L- but not D-amino acid alcohols of the regulatory amino acids [Iiboshi et al., 1999]. Furthermore, cells expressing a temperature-sensitive mutant of histidyl t-RNA synthetase had decreased $p70^{s6k}$ activity at the nonpermissive temperature, which was partially relieved by adding excess histidine [Iiboshi et al., 1999]. These observations imply that these cell lines possess a mechanism that can recognize the t-RNA charging state, reminiscent of the yeast GCN2 kinase that is activated by deacylated t-RNAs [Hinnebusch, 1997]. Thus, detection of t-RNA charging state has been posited to represent a mechanism whereby amino acids regulate mTOR signaling.

All of the above-mentioned studies on the mechanistic aspect of amino acid regulation of mTOR have been performed in immortal cell lines. Recently we have noted several differences in the regulation of mTOR by amino acids between such cell lines and freshly isolated adipocytes [Lynch et al., 2000]. One of differences cited was that stimulation of rapamycin-sensitive 4E-BP1 phosphorylation by amino acids was not inhibited by the amino acid alcohol, L-leucinol, in freshly isolated adipocytes [Lynch et al., 2000] as was the case for activation of $p70^{s6k}$ in lymphoblastoid cell lines [Iiboshi et al., 1999]. In addition, mTOR signaling was activated in rat adipocytes by norleucine [Lynch et al., 2000], an amino acid that is not incorporated into mammalian proteins [ul Hassan and Greenberg, 1952]. These observations have led us to postulate that freshly isolated

adipocytes may use a different mechanism to regulate mTOR that does not involve recognition of the t-RNA charging state or t-RNA synthetase activity, as may be the case in the above-mentioned cell lines. This as yet unelucidated mechanism may be of general interest because amino acid regulation of protein synthesis also appears to be independent of the t-RNA charging state in skeletal muscle, cardiac muscle, and liver [Ojamaa et al., 1993; Tischler et al., 1982].

Recent studies have suggested that amino acid regulation of mTOR in hepatocytes might involve heterotrimeric G-protein signaling pathways [Blommaart et al., 1995; Blommaart et al., 1997]. Calcium, β-adrenergic receptors, cAMP, a PD98059-sensitive protein kinase, and protein kinase Cδ have also been implicated in the regulation of mTOR or its downstream targets in various cell lines [Conus et al., 1998; Kumar et al., 2000; Moule et al., 1997; Scott et al., 1996; Scott and Lawrence, 1997; Scott and Lawrence, 1998; Wilson et al., 1996]. If the regulation of mTOR by amino acids in freshly isolated adipocytes is different from cell lines in terms of amino acid alcohol sensitivity, it seems likely that other mechanistic aspects of this regulation might also be different. Therefore, in the study reported herein, we have systematically evaluated potential signaling mechanisms whereby amino acids may be regulating mTOR in freshly isolated rat adipocytes, beginning with the effects of L-leucinol and L-histidinol, and including insulin and G-protein signaling pathways.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). BioMag goat-anti mouse IgG magnetic beads were obtained from PerSeptive Biosystems (Framingham, MA) and the magnetic sample rack from Promega (Madison, WI). Amino acids were purchased from Sigma (St. Louis, MO) and United States Biochemical Corporation (Cleveland, OH). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), disodium ethylenediaminetetraacetic acid (EDTA), and benzamidine were all purchased from Sigma. Sodium vanadate and Triton X-100 were from Calbiochem (San Diego, CA). PY-20 anti-phosphotyrosine antibody was obtained from Transduction Laboratories (Lexington, KY). Anti-rat p85 (PI 3-kinase regulatory subunit) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Polyvinylidene difluoride (PVDF) membrane was from BioRad (Hercules, CA) and NitroBind nitrocellulose membrane from MSI, Inc. (Westborough, MA). Protein kinase B anti-peptide antibody, "anti-active" phospho-specific AKT (Ser 473) antibody and "anti-active" phospho-specific p44/42 mitogen-activated protein (MAP) kinase (Thr202/Tyr204) E10 monoclonal antibody were from New England BioLabs (Beverly, MA). Horseradish peroxidase-linked sheep-anti mouse Ig secondary antibody, horseradish peroxidase-linked goat-anti rabbit Ig secondary antibody, and the enhanced chemiluminescence (ECL) Western blotting detection kit were all obtained from Amersham (Arlington Heights, IL).

Isolation of Adipocytes

Adipocytes were isolated from epididymal fat pads of 7–8-week-old male Sprague-Dawley rats by collagenase digestion as previously described [Fox et al., 1998a; Fox et al., 1998b]. The cells were washed three times in Krebs-Ringer HEPES (KRH; 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM HEPES, 1 mM EDTA, 2 mM glucose, and 200 nM adenosine) buffer containing 2% bovine serum albumin fraction V (BSA) and then incubated at 37°C in albumin-free KRH buffer for 20 min prior to the start of an experiment. After this preincubation period, the underlying buffer was removed from beneath the cells with a syringe, resulting in a cell suspension with a 60–80% cytocrit and allowing the cells to be more readily aliquoted into other tubes. Aliquots of cells (150 μl, 60–80% cytocrit) were generally added to 500 μl of either BSA-free KRH, BSA-free KRH with other additives as indicated (e.g., insulin), or buffer A containing additional additives as indicated in the figure legends (e.g., leucine or leucine and insulin) and allowed to incubate at 37°C for the indicated time. Buffer A consisted of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM HEPES, 1 mM EDTA, 2 mM glucose, 200 nM adenosine, 2395 μM Ala, 312 μM Asn, 176 μM Asp, 651 μM Arg, 389 μM Cys, 2,278 μM Gln, 1,950 μM Gly, 385 μM His, 649 μM Ile, 2,335 μM Lys, 259 μM Met, 326 μM Phe, 651 μM Pro,

1,302 μM Ser, 1,170 μM Thr, 454 μM Trp, 371 μM Tyr, 1,170 μM Val (these concentrations of amino acids become diluted when 500 μl are added to 150 μl of cells suspended in KRH). In certain experiments, immediately prior to the preincubation period, cells were exposed in KRH to potential inhibitors for an indicated period to allow time for the inhibitors to act on their targets.

Phosphorylation of 4E-BP1, $p70^{s6k}$, PKB, and MAP Kinase

Phosphorylation of 4E-BP1 and $p70^{s6k}$ was assessed by examining changes in electrophoretic mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [Fox et al., 1998a; Fox et al., 1998b; Lynch et al., 2000]. The following procedure was used to measure total PKB and PKB phosphorylated at Ser 473 (phospho-PKB) and p42 and p44 MAP kinase doubly phosphorylated on Thr 202 and Tyr 204. Aliquots of cells (150 μl, 60–80% cytocrit) were added to BSA-free KRH buffer (500 μl) containing amino acids or insulin as indicated. After a 10-min incubation at 37°C, the buffer was withdrawn from beneath the cells with a syringe and the cells were frozen in liquid nitrogen. Homogenization buffer (450 μl of 20 mM HEPES, pH 7.4; 2 mM EGTA; 50 mM NaF; 100 mM KCl; 0.2 mM EDTA; 50 mM β-glycerophosphate; 1 mM dithiothreitol (DTT); 0.1 mM PMSF; 1 mM benzamidine; 0.5 mM sodium vanadate) was added to 150-μl aliquots of frozen cells. The mixture was sonicated over ice and the samples were centrifuged at 10,000*g* for 10 min at 4°C. Aliquots (50 μl) of the resulting fat-free infranatant were solubilized in SDS-PAGE sample buffer and heated to 100°C for 5 min. Proteins in the samples were separated by SDS-PAGE on a 7.5% (PKB) or 10 % (MAP kinase) acrylamide gel and transferred to PVDF. The blots were probed with either AKT antibody (1:1000), "anti-active" phospho-specific AKT (Ser 473) antibody (1:1000) or "anti-active" phospho-specific p44/42 MAP kinase (Thr 202/Tyr 204) E10 monoclonal antibody (1:2000).

Immunoprecipitation and Assay of PI 3-Kinase

After experimental treatments, adipocytes were homogenized in a ground-glass homogenizer with homogenization buffer containing the following: 250 mM sucrose, 20 mM Tris HCl (pH 7.4) 5 mM EDTA, 10 mM NaF, 2 mM PMSF, 2 mM diisopropylfluorophosphate (DFP), 2 mM orthovanadate, 2 mM ammonium molybdate, and 1 μg/ml each of aprotinin and leupeptin. The resulting mixture was centrifuged at 600*g* for 10 min at 4°C. The supernatant (called the cytosolic fraction by Lavan and Lienhard [1993]) was then centrifuged at 360,000*g* for 1 h at 4°C. NP-40 was added to the harvested infranatant (called the cytosolic fraction by Lavan and Lienhard [1993]) to a final concentration of 1% and the pellet was solubilized in a Dounce homogenizer with the above homogenization buffer containing 1% NP-40. Phosphotyrosine-containing associated proteins in the solubilized membranes were immunoprecipitated with PY-20 antibodies as previously described [Kelly and Ruderman, 1993]. The resulting immunoprecipitates were assayed for PI 3-kinase activity using phosphatidylinositol as a substrate and thin layer chromatography to resolve the products as previously described [Kelly and Ruderman, 1993]. In other experiments, the amounts of p85 in the 360,000*g* pellet and supernatant fractions were determined by Western blotting according to the manufacturer's protocol.

Protein Tyrosine Phosphorylation

Isolated adipocytes (660 μl of cell suspension containing 60–80% cells) were incubated in KRH (control) or KRH containing either 100 nM insulin or buffer A with a final concentration of 0.8 mM L-leucine. Soluble membrane and cytoskeletal fractions were then prepared immediately by a modification of the method of Lavan and Lienhard [1993]. Briefly, cells were homogenized with a motorized Teflon pestle homogenizer in 3.75 ml of buffer A. The homogenates were centrifuged at 116,000*g* for 1 h at 4°C. The soluble infranatant was retained and the fat cake was discarded. The pellet was resuspended in 3.75 ml of buffer A containing 1% Triton X-100 and briefly sonicated. The mixture was then recentrifuged as above. The resulting supernatant (called membrane fraction by Lavan and Lienhard [1993]) was removed and reserved. The precipitate (called cytoskeletal fraction by Lavan and Lienhard [1993]) was dissolved by sonication and heating (5 min at 100°C) in 1× SDS-PAGE sample buffer modified according to Lavan and Lienhard [1993]. The other fractions were similarly treated with

a 1:1 dilution of 2× sample buffer. Proteins were separated by SDS-PAGE electrophoresis on a 10% acrylamide gel and transferred by Western blotting to an MSI NitroBind membrane. Phosphotyrosine-containing proteins were detected by ECL using PY-20 primary antibody at a dilution of 1:1,000 and anti-mouse horseradish peroxidase-linked secondary antibody diluted 1:10,000 in Tris buffered saline and Tween 20 solution (TBST) 0.3% BSA. The blocking step was performed using 5% BSA in TBST.

Pertussis Toxin Treatment and ^{32}P-ADP-Ribosylation of G_i

For pertussis toxin treatment, rats were injected intraperitoneally with 25 μg of pertussis toxin per 100 g body weight with phosphate-buffered saline (PBS) as the vehicle. Adipocytes were isolated from sham and injected rats either 24 or 72 h after the pertussis toxin injection. Some of the cells were used for 4E-BP1 studies and the rest were reserved for determining the proportion of total G_i modified. The latter was accomplished by comparing pertussis toxin-catalyzed incorporation of ^{32}P-ADP into the α-subunits of G_i in membranes (42,000*g* for 30 min) prepared from the same number of control and pertussis-toxin treated adipocytes as previously described [Lynch et al., 1989]. Radioactivity in the gel was quantitated using a Molecular Dynamics phosphoimager.

RESULTS

Studies by Iiboshi et al. [1999] have shown that several amino acid alcohols, including L-histidinol or L-leucinol, can inhibit the rapamycin-sensitive effects of amino acids on p70^{s6k} in Jurkat cells and related cell lines. In contrast, in a more recent study, we reported that L-leucinol did not inhibit the rapamycin-sensitive phosphorylation of 4E-BP1 in freshly isolated adipocytes incubated with amino acids. We wondered whether the differences between the results of these two studies might simply be due to differences in either the experimental design (timing of inhibitor additions and incubation length) or the target studied (p70^{s6k} versus 4E-BP1). To address these differences in experimental design, a study was performed using rat adipocytes subjected to conditions that closely mimicked those described by Iiboshi et al. [1999]. The effects of a second amino acid alcohol, L-histidinol, that was fully efficacious in the studies of Iiboshi et al. [1999] at modulating p70^{s6k} was examined. With the modified protocol, adipocytes were stimulated with amino acids for 5 min to allow full activation of mTOR and 4E-BP1 phosphorylation. Then, either L-leucinol, L-histidinol, or rapamycin was added and the incubation was continued for 30 min. Phosphorylation of 4E-BP1 and p70^{s6k} was then examined. As shown in Fig. 1, rapamycin strongly inhibited the amino acid-induced stimulation of both 4E-BP1 and p70^{s6k} phosphorylation, as expected for an event mediated by mTOR. In contrast,

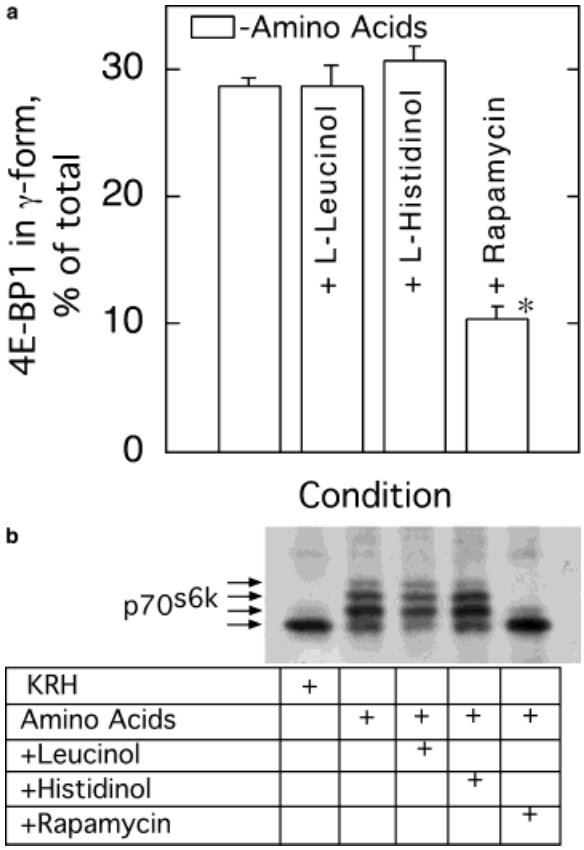

Fig. 1. Effects of L-leucinol and L-histidinol on rapamycin-sensitive amino acid stimulation of 4E-BP1 and p70^{s6k} phosphorylation in adipocytes. Adipocytes were incubated with Krebs-Ringer HEPES (KRH) buffer or Buffer A with a final concentration of 0.8 mM L-leucine. After 5 min, putative inhibitors were added (either 5 mM L-leucinol, 5 mM L-histidinol, or 100 ng/ml rapamycin) and the incubation was continued for an additional 30 min. Cells were then frozen and 4E-BP1 (**A**) and p70^{s6k} phosphorylation (**B**) were assessed as described in Materials and Methods. The results are the mean ± standard error of three experiments.

neither L-histidinol (the most efficacious amino acid alcohol inhibitor of $p70^{s6k}$ in Jurkat cells [Iiboshi et al., 1999] nor L-leucinol had any significant effect on amino acid-induced stimulation of 4E-BP1 or $p70^{s6k}$ phosphorylation. These new findings, together with our previous results [Lynch et al., 2000], indicate that the mechanism through which amino acids act to activate mTOR in freshly isolated rat adipocytes is insensitive to amino acid alcohols, in contrast to the situation in lymphoblastoid cell lines.

Potential Roles of PI 3-Kinase, Tyrosine Phosphorylation, and AKT

Previous studies using cell lines have indicated that the effects of amino acids on mTOR are not associated with changes in growth factor signaling components such as PI 3-kinase or AKT [Hara et al., 1998; Kimball, 1999; Iiboshi et al., 1999; Patti et al., 1998; Shigemitsu et al., 1999; Wang et al., 1998]. However, these studies were performed on cell lines that potentially use an amino acid alcohol-sensitive mechanism to regulate mTOR, as described by Iiboshi et al. [1999]. With this caveat in mind, we evaluated the potential role of the insulin-signaling pathway in the amino acid alcohol-insensitive regulation of mTOR by amino acids in freshly isolated rat adipocytes. The "classical" mechanism by which growth factors activate PI 3-kinase involves the interaction of one or both SH2 domains on the enzyme's p85 regulatory subunit with phosphotyrosyl residues in other proteins. Agents that stimulate PI 3-kinase by this mechanism thus increase PI 3-kinase activity in antiphosphotyrosine immunoprecipitates.

To examine PI 3-kinase involvement in amino acid signaling, adipocytes were incubated with amino acids as in Fig. 1, and PI 3-kinase activity was assayed in antiphosphotyrosine immunoprecipitates from the detergent-solubilized 360,000*g* particulate fractions [Kelly and Ruderman, 1993; Kelly et al., 1992]. On average, insulin stimulated PI 3-kinase activity approximately fivefold (Fig. 2). In contrast, amino acids caused a statistically significant (Student's *t*-test, $P < 0.05$) reduction in PY-20 immunoprecipitable PI 3-kinase activity (Fig 2). This is in agreement with previous findings of Patti et al. [1998]. Coincident with PI 3-kinase, activation by insulin is the redistribution of cytosolic PI 3-kinase to membranes [Heller-Harrison et al., 1996; Lavan and Lienhard, 1993]. To examine the effects of amino acids on PI 3-kinase translocation, soluble and 360,000*g* particulate fractions were prepared from freshly isolated adipocytes and p85 was quantitated by Western blotting. Approximately 20% of the total p85 immunoreactivity was associated with the particulate fractions prepared from control adipocytes (Fig. 3), in agreement with earlier estimates [Kelly and Ruderman, 1993; Kelly et al., 1992; Lavan and Lienhard, 1993]. Insulin treatment, for either 2 or 3 min, consistently increased p85 immunoreactivity in the 360,000*g* particulate fractions (Fig. 3), as previously reported [Heller-Harrison et al., 1996; Lavan and Lienhard, 1993]. However, this parameter in cells exposed to amino acids was not statistically different from that of controls (Fig. 3).

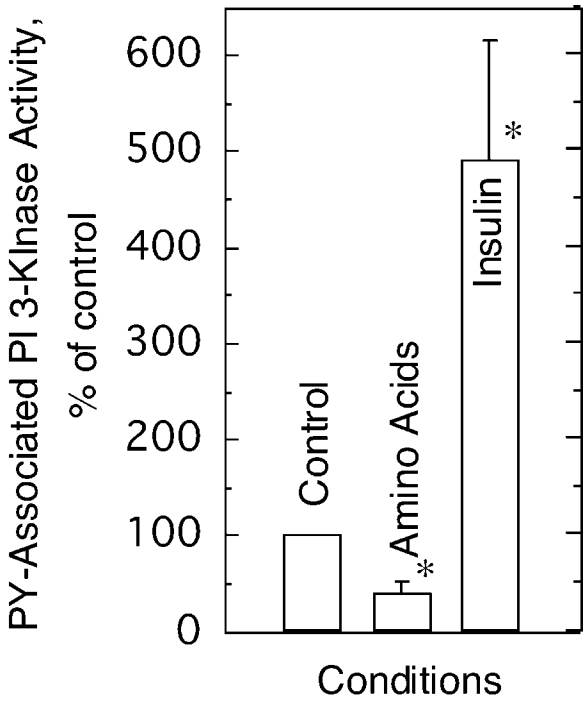

Fig. 2. Effects of amino acids and insulin on phosphatidylinositol (PI) 3-kinase activity. Adipocyte suspensions were added to bovine serum albumin-free Krebs-Ringer HEPES buffer in the absence (**Control**) or presence of either 100 nM insulin (**Insulin**), or buffer A plus a final concentration of 0.8 mM L-leucine (**Amino Acids**) as indicated. After a 3-min incubation at 37°C, 360,000*g* particulate fractions were prepared from the adipocytes. PI 3-kinase was assayed in anti-PY immunoprecipitates after solubilization with 1% NP-40. Results presented are the average from three separate experiments on different cell preparations.

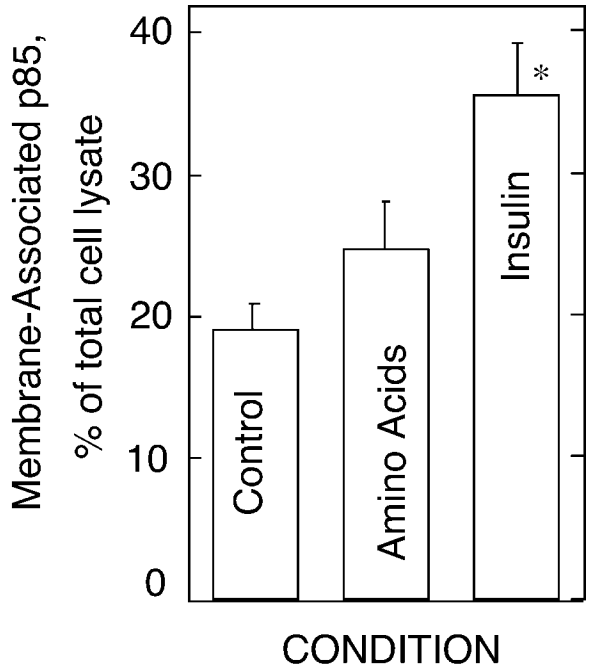

Fig. 3. Changes in membrane-associated p85 in fat cells incubated with either insulin or amino acids. Adipocyte suspensions were added to bovine serum albumin-free Krebs-Ringer HEPES buffer in the absence (**Control**) or presence of either 100 nM insulin (**Insulin**) or buffer A plus a final concentration of 0.8 mM L-leucine (**Amino Acids**) as indicated. After a 2–3-min incubation at 37°C, the cells were homogenized and a 360,000*g* particulate fraction was prepared from the post-nuclear supernatant. Phosphatidylinositol 3-kinase immunoreactivity was examined by Western blotting. Densitometric values for each condition were normalized for total p85 in both membrane and soluble fractions. Averages and standard errors from twelve separate experiments on different cell preparations are shown.

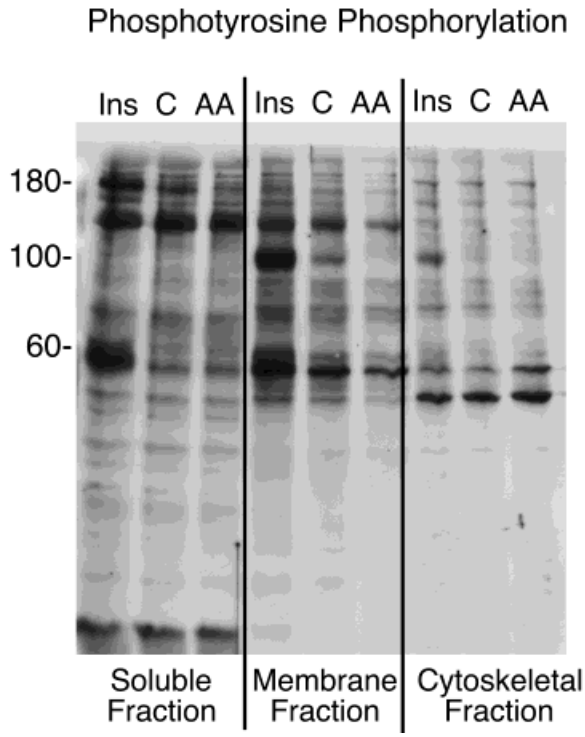

Fig. 4. Protein phosphotyrosine content in subcellular fractions prepared from adipocytes. Aliquots (660 μl) of adipocyte suspensions were added to or buffer A with 0.8 mM leucine (**AA**) or bovine serum albumin-free Krebs-Ringer HEPES buffer in the absence (**C**) or presence of either 100 nM insulin (**Ins**) as indicated. Subcellular fractions were analyzed for PY-20 immunoreactivity as described in Materials and Methods. The position and M_r of three immunoreactive proteins are indicated. A blot from a single experiment is shown that is representative of four such studies from different cell preparations.

The effect of amino acids on overall tyrosine phosphorylation was also examined. For these experiments, subcellular fractions were prepared as described by Lavan and Lienhard [1993]. Figure 4 shows that insulin increased tyrosine phosphorylation of bands at approximately 180 and 60 kDa in the soluble fractions, and 100 kDa in the membrane and cytoskeletal fractions as previously reported [Lavan and Lienhard, 1993]. These phosphoproteins have been identified as IRS-1, IRS-3, and the insulin receptor, respectively [see for example Lavan et al., 1997; Lavan and Lienhard, 1993]. Amino acids caused no change in the PY-20 immunoreactivity of most proteins in the soluble fraction or cytoskeletal fractions (Fig. 4). However, amino acids reproducibly decreased phosphotyrosine content in the membrane fraction and p180 phosphotyrosine content in the soluble fraction (Fig. 4). The inhibitory effect of amino acids on phosphotyrosine phosphorylation may explain the decrease in PI 3-kinase activity in antiphosphotyrosine immunoprecipitates.

Next we examined the potential role of the serine/threonine kinase, PKB in the response to amino acids. Protein kinase B is an integral component of the pathway leading to the phosphorylation of 4E-BP1 in response to insulin [Klippel et al., 1997; Mendez et al., 1996]. Activation of PKB requires phosphorylation in its activation loop at Ser 473 [for review see Downward, 1998]. Figure 5 (top panel) shows that an anti-PKB antibody recognized a peptide doublet of approximately 60 kDa in infranatants from control cells or cells incubated with amino acids. In contrast, only the upper band was observed in infranatants from cells incubated with insulin. Figure 5 (bottom panel) shows

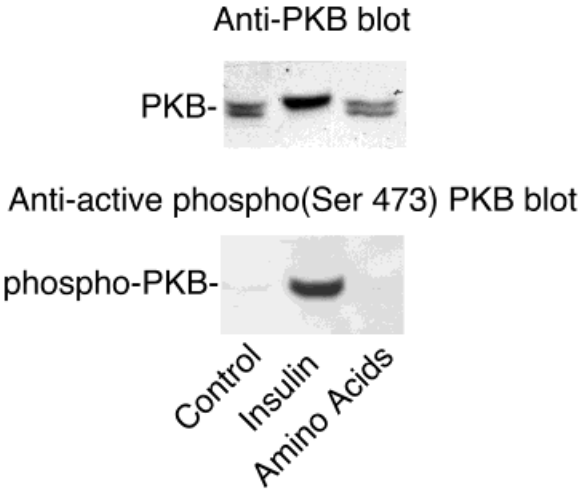

Fig. 5. Total protein kinase B (PKB) and "Active" (phospho-Ser 473) PKB immunoreactivity in adipocytes. Aliquots (150 μl) of cell suspension were placed in bovine serum albumin-free Krebs-Ringer HEPES buffer with either no addition (**Control**), 100 nM insulin (**Insulin**) or Buffer A plus 0.8 mM L-leucine (**Amino Acids**) as indicated. Infranatant proteins from frozen cells were separated on a 7.5% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Proteins in the gels were transferred to PVDF membrane and the membranes were probed with a 1:1000 dilution of either anti-PKB or phospho-Ser 473-PKB antibody per the manufacturer's instructions.

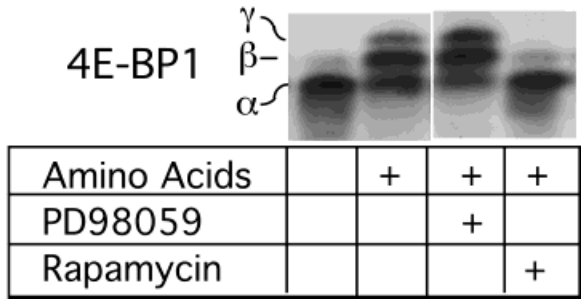

Fig. 6. Effects of rapamycin and PD98059 on 4E-BP1 phosphorylation. Adipocytes suspended in bovine serum albumin-free Krebs-Ringer HEPES (KRH) buffer were incubated for 15 min in the absence or presence of either 50 μM PD98059 or 98 nM rapamycin as indicated. The buffer was withdrawn from underneath the cells using a syringe and 150-μl aliquots of cells were added to 500 μl of KRH (**lane 1**), buffer A plus 0.8 mM L-leucine (**lane 2**), or buffer A plus L-leucine and one of the aforementioned drugs as indicated. Changes in 4E-BP1 phosphorylation were examined following immunoprecipitation as described in Materials and Methods. Representative of two such experiments.

that an anti-phospho-Ser 473–specific PKB antibody recognized only the upper band and only in the insulin-treated cells. These results indicate that amino acids, unlike insulin, are not capable of activating PKB and argue against a potential involvement of PKB in the mechanism by which amino acids stimulate 4E-BP1 phosphorylation.

Scott and Lawrence [1997] have suggested that a PD98059-sensitive kinase in Chinese hamster ovary (CHO) cells may be partially involved in the insulin-induced activation of mTOR. Since PD98059 is a MEK-1 inhibitor, this unknown kinase could be MAP kinase. We also observed that PD98059 could partially decrease the amount of 4E-BP1 in the γ form in cells incubated with insulin (data not shown). However 50 μM PD98059 did not affect the rapamycin-sensitive stimulation of 4E-BP1 phosphorylation by amino acids (Fig. 6). Subsequently, MAP kinase activity, in vivo, was assessed using a monoclonal antibody that recognizes only the doubly (Thr 202 and Tyr 204) phosphorylated form of MAP kinase that is found after activation by an upstream MAP kinase [Payne et al., 1991; Sturgill et al., 1988]. Insulin stimulated and PD98059 (50 μM) inhibited the phosphorylation of the p42 and p44 forms of MAP kinase at theses sites, indicating activation and inhibition, respectively (Fig. 7). In contrast, amino acids had no effect on the activation state of MAP kinase.

Potential Role of Heterotrimeric G-Protein-Linked Pathways

G-proteins and their downstream targets, including cAMP, calcium, and protein kinase C (PKC), have been implicated in the regulation of the mTOR signaling pathway [Diggle et al., 1996; Graves et al., 1995; Han et al., 1996; Lawrence et al., 1997, Kumar et al., 2000; Conus et al., 1998; Lin and Lawrence, 1996; Moule et al., 1997; Rybkin et al., 2000; Xu et al., 1998b]. For example, isoproterenol and BRL37344s stimulate $p70^{s6k}$ activity in adipocytes [Diggle et al., 1996; Moule et al., 1997], although it is unclear whether this effect is mediated by mTOR or another kinase. On the other hand, analogs of cAMP and agents that raise cAMP in cells have been reported to inhibit FRAP/TOR signaling in some, but not all, cells tested [Graves et al., 1995; Han et al., 1996; Lawrence et al., 1997; Lin and Lawrence, 1996; Xu et al., 1998b]. We first investigated the possibility that a recognition site for amino acids might couple to the G-protein that activates adenylate cyclase ($G\alpha_s$) or its down-

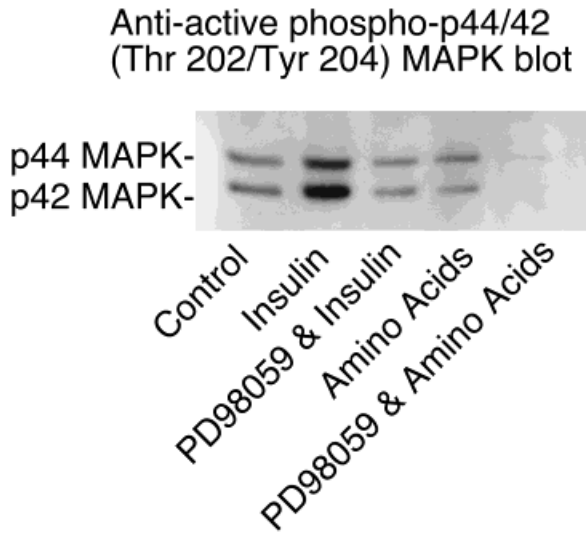

Fig. 7. "Active" (phospho Thr202/Tyr204) mitogen-activated protein (MAP) kinase (MAPK) immunoreactivity in adipocytes. Adipocytes suspended in bovine serum albumin-free Krebs-Ringer HEPES buffer were incubated for 15 min in the absence or presence of 50 μM PD98059 and then for 10 min with either no addition (**Control**), 100 nM insulin (**Insulin**), or buffer A plus 0.8 mM L-leucine (**Amino Acids**) as indicated. Infranatant proteins from frozen cells were subsequently separated on a 10% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to PVDF and probed with a 1:2000 dilution of phospho-p44/42 (Thr 202/Tyr 204) E10 monoclonal antibody. The blot is from a representative experiment of which two were performed.

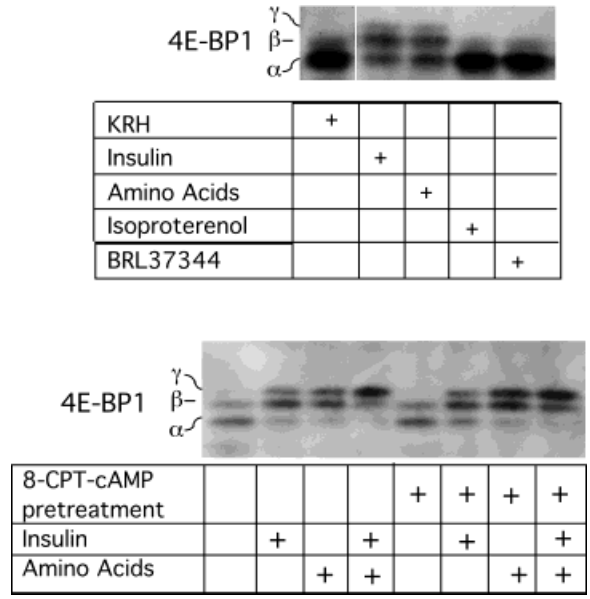

Fig. 8. Effects of insulin, amino acids, β-adrenergic agonists or 8-chlorophenylthio-cAMP on 4E-BP1 phosphorylation. **Top panel:** Aliquots (150 μl) of cell suspension were placed in Buffer A plus 0.8 mM L-leucine (Amino Acids), bovine serum albumin (BSA)-free Krebs-Ringer HEPES (KRH) buffer alone or BSA-free KRH buffer in the presence of either 100 nM insulin, 1 μM isoproterenol, or 10 nM BRL37344 as indicated for 10 min. **Bottom panel:** Effect of 8-chlorophenylthio-cAMP pretreatment (8-CPT-cAMP) on 4E-BP1 phosphorylation. Cell suspensions to be treated with 8-CPT-cAMP were incubated in bovine serum albumin-free Krebs-Ringer HEPES with 0.1 mM 8-CPT-cAMP for 15 min prior to and during the incubation with either 100 nM insulin, Buffer A plus 0.8 mM Leu, both or no addition as indicated. The figure shows the results of a single experiment that is representative of two such studies.

stream targets. In agreement with previous reports [Diggle et al., 1996; Moule et al., 1997], we found that both isoproterenol and BRL37344 could stimulate the phosphorylation of $p70^{s6k}$, but not to the same extent as insulin or amino acids (data not shown). In contrast, neither β_3-adrenergic agonist had a significant effect on 4E-BP1 phosphorylation (Fig. 8, top panel). Next, the possibility that amino acids might regulate mTOR activity through changes in protein kinase A activity in adipocytes was explored. Adipocytes were exposed to supramaximal concentrations of 8-chlorophenyl cAMP (8-CPT-cAMP, a cell-permeable, poorly hydrolyzable cAMP analog) before and during treatment with vehicle, insulin, or amino acids. However, neither basal, insulin-stimulated, nor amino acid-stimulated 4E-BP1 phosphorylation was affected by 8-CPT-cAMP (Fig. 8, bottom panel).

Pertussis toxin (also known as islet-activating protein) enter cells and selectively ADP-ribosylates and uncouples α-subunits in the $G\alpha_i$ family of heterotrimeric G-proteins (i.e., $G\alpha_{i\text{-}1}$, $G\alpha_{i\text{-}2}$, $G\alpha_{i\text{-}3}$, $G\alpha_t$, and $G\alpha_o$). In contrast, other G-proteins, like $G\alpha_q$ and $G\alpha_s$, are not substrates for the toxin's ADP ribotransferase activity, because they lack the target Cys residue found four amino acids in from the C-terminus in the $G\alpha_i$ family. Rat adipocytes have been reported to contain as many as four potential substrates for pertussis toxin: $\alpha_{i\text{-}1}$, $\alpha_{i\text{-}2}$, $\alpha_{i\text{-}3}$, and α_o [Denis-Henriot et al., 1996]. In the experiments reported here, the effectiveness of in vivo pertussis toxin treatment was first evaluated in terms of the percent of the $G\alpha_i$-family proteins that were ADP-ribosylated 24 and 72 h after toxin injection. The evaluation was performed by comparing pertussis toxin-stimulated incorporation of ^{32}P-ADP into $G\alpha_i$ proteins prepared from control and pertussis toxin-injected rats. Figure 9 shows that injection of pertussis toxin resulted in an ≈85% reduction in the amount of $G\alpha_i$ family proteins

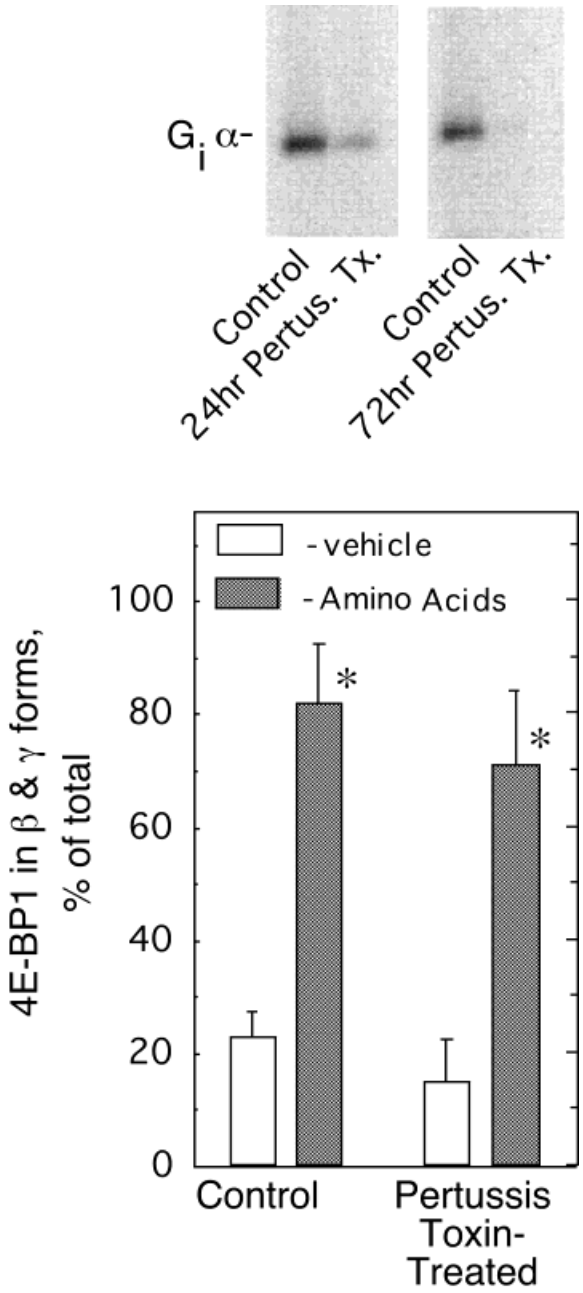

Fig. 9. Effect of pertussis toxin treatment on 4E-BP1 phosphorylation. **Top panel:** Efficacy of pertussis toxin treatment. The position of the 40–41 kDa α-subunits of G_i found in adipocytes is indicated. **Bottom panel:** Effect of pertussis toxin treatment on 4E-BP1 phosphorylation. The results represent the mean ± standard error of three experiments.

left unmodified one day after injection and a complete loss of available substrate after three days.

The effects of pertussis toxin on adipocyte 4E-BP1 phosphorylation were the same either one or three days after the injection (data not shown). Therefore, the results from 24-h and 72-h treatments were combined in Fig. 9. Pertussis toxin treatment had no significant effect on either basal or amino acid-stimulated 4E-BP1 phosphorylation (Fig. 9). These findings indicate that neither G_i nor $G\alpha_o$ proteins of adipocytes play a role in the mechanism leading to 4E-BP1 phosphorylation in response to amino acids.

Several agents are available that stimulate a broad spectrum of heterotrimeric G-proteins including G-proteins like $G\alpha_q$ that are involved in calcium mobilization and activation of protein kinase C. Two examples are MAS-7 and fluoroaluminate. MAS-7 is an analog of the cell-permeable Wasp venom mastoparan. Fluoroaluminate is the name given to AlF_4^- complexes that are formed in aqueous solutions when fluoride is present in excess of aluminum salt; these complexes stimulate heterotrimeric G-proteins [Sternweis and Gilman, 1982]. Mastoparan and fluoroaluminate have been shown to activate G_q, and several other heterotrimeric G-proteins in intact cell experiments [e.g., Blackmore et al., 1985; Miller et al., 1994]. Neither MAS-7 nor fluoroaluminate mimicked the effect of amino acids on 4E-BP1 phosphorylation (Fig. 10, top panel) indicating that these G-proteins were not involved in amino acid signaling.

Kumar et al. [2000] have recently shown a functional interaction between mTOR and protein kinase Cδ (PKCδ) in the regulation of serum-stimulated 4E-BP1 phosphorylation in the 293T cell line. PKCδ and other PKC isoforms are inhibited by either staurosporine, bisindolylmaleimide (BIM I), or both [for review see Deng et al., 1997; Geiges et al., 1997]. However, neither BIM-I nor staurosporine had any significant effect on the stimulation of 4E-BP1 phosphorylation by amino acids (Fig 10., bottom panel). Furthermore, the calcium ionophore, ionomycin, had no significant effect on 4E-BP1 phosphorylation at a concentration that mobilizes calcium in adipocytes [Draznin et al., 1987], confirming that the CAM kinase and Ca^{2+}-sensitive (α β δ) PKCs are not involved. These findings indicate that neither PKCδ nor other PKCs are required for amino acid-stimulated 4E-BP1 phosphorylation in freshly isolated adipocytes.

DISCUSSION

In this communication, we report the results of experiments designed to investigate the

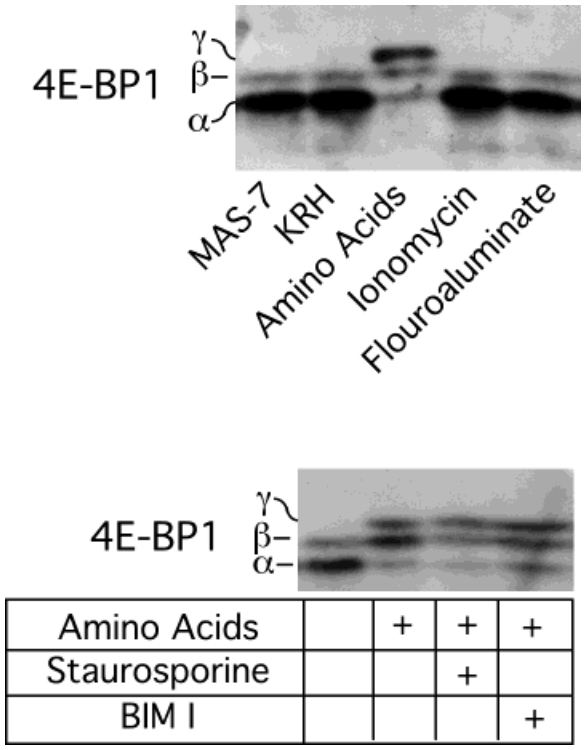

Fig. 10. Effects of G-protein agonists, ionomycin, or protein kinase C inhibitors on 4E-BP1 phosphorylation. **Top panel:** Aliquots (150 μl) of adipocytes were added to Buffer A plus 0.8 mM L-leucine (**Amino Acids**) or 500 μl of bovine serum albumin-free Krebs-Ringer HEPES buffer in the absence (**KRH**) or presence of one of the following: 100 μM aluminum chloride with 10 mM NaF (**Flouroaluminate**), 10 μM mastoparan 7 (**MAS-7**), or 1 μM ionomycin (**Ionomycin**). 4E-BP1 phosphorylation was measured after a 20-min incubation. **Bottom panel:** Effects of protein kinase C inhibitors (10 μM staurosporine or 10 μM bisindolylmaleimide (BIM I), as indicated) on 4E-BP1 phosphorylation. A blot from a representative experiment are shown of which a total of two were performed.

mechanism used by amino acids to bring about regulation of the mTOR signaling pathway in freshly isolated adipocytes. Recent studies by Iiboshi et al. [1999] indicated that in lymphoblastoid cells lines the rapamycin-sensitive (i.e., mTOR-mediated) regulation of $p70^{s6k}$ by amino acids was sensitive to amino acid alcohols (e.g., L-histidinol and L-leucinol) and therefore may involve a mechanism that detects t-RNA charging state or tRNA synthetase activity. We had previously found that the effects of amino acids on the regulation of mTOR signaling in adipocytes was not inhibited by L-leucinol, however those experiments were optimized to study 4E-BP1 phosphorylation [Lynch et al., 2000]. In the Iiboshi et al. [1999] protocol, amino acids and amino acid alcohols were added to the culture medium 30 min prior to measurement of $p70^{s6k}$ activity, whereas in our experiments only a 10-min treatment period was used (i.e., a time that is optimal for measuring 4E-BP1 phosphorylation state). Furthermore, in the experiments of Iiboshi et al. [1999], the amino acids were added before the amino acid alcohol inhibitors. In contrast, in our previous study the additions were in the opposite sequence (i.e., to give the potential inhibitors an opportunity to exert an effect). Here, we considered the possibility that the apparent differences between our previous results and those of Iiboshi et al. [1999] were not due to cell-specific differences, but rather differences in experimental design. To address this possibility, we used protocols that closely resembled those of Iiboshi et al. [1999]; examined both 4E-BP1 and $p70^{s6k}$ phosphorylation; and added one of the amino acid alcohols, L-histidinol, that was reported to be more efficacious in lymphoblastoid cell lines [Iiboshi et al., 1999]. However, neither L-histidinol nor L-leucinol inhibited amino acid stimulation of rapamycin-sensitive $p70^{s6k}$ or 4E-BP1 phosphorylation in freshly isolated rat adipocytes. These findings provide additional support, in conjunction with our previous findings [Lynch et al., 2000], for the hypothesis that amino acids use an amino acid alcohol-insensitive mechanism for regulating mTOR in rat adipocytes that may be different from lymphoblastoid and a number of other cell lines.

Role of Components of the Insulin-Signaling Pathway

In cell lines where this regulation is likely to be amino acid alcohol-sensitive, the mechanism used by amino acids to regulate mTOR does not appear to involve the upstream activation of PI 3-kinase or PKB [e.g. Hara et al., 1998; Iiboshi et al., 1999; Kimball et al., 1999; Patti et al., 1998]. We investigated the possible involvement of the insulin-signaling pathway in the regulation of mTOR, because in adipocytes amino acids appear to be activating mTOR by a different, amino acid alcohol-insensitive mechanism. Our data indicate that, despite the difference in the amino acid alcohol sensitivity between rat adipocytes and cell lines, stimulation of tyrosine phosphorylation, PI 3-kinase, and PKB is not involved in the regulation of mTOR by amino acids in freshly isolated rat adipocytes—as is the case in the

cell lines. Scott and Lawrence [1997] have reported that a PD 98059-sensitive kinase, possibly MAP kinase, was involved in the regulation of $p70^{s6k}$ and 4E-BP1 phosphorylation by insulin, an effect that was seen in our experiments. We wondered, therefore, if a PD98059-sensitive kinase might be involved in the regulation of mTOR by amino acids. However, PD98059 had no effect on amino acid-stimulated 4E-BP1 phosphorylation, even at the same concentration at which it inhibited phosphorylation of MAP kinase. Furthermore, in contrast to insulin, amino acids failed to stimulate phosphorylation of MAP kinase, as insulin did. Therefore a PD 98059-sensitive kinase does not seem to be involved in the activation of mTOR by amino acids in adipocytes.

Potential Role of cAMP-Dependent Protein Kinase Pathway

Graves et al. [1995] reported that incubating NIH-3T3 cells with forskolin to raise cAMP caused a marked dephosphorylation of 4E-BP1, and increased the amount of the protein bound to eIF4E. Similarly, in metabolically radiolabeled 3T3-L1 adipocytes, agents that increase cAMP or cAMP analogs caused a decrease in the incorporation of ^{32}P into 4E-BP1 [Scott and Lawrence, 1998]. Thus increasing cAMP reduces 4E-BP1 phosphorylation in a number of cell lines. In contrast, our findings indicate that neither β-adrenergic receptors, G_s, nor protein kinase A, the downstream target of β-adrenergic receptors and G_s, are involved in amino acid-stimulated phosphorylation of 4E-BP1 in freshly isolated rat adipocytes. Thus, it seems unlikely that regulation of these effectors could explain the effects of amino acids on mTOR signaling in rat adipocytes. The lack of effect that we observed of 8-CPT-cAMP and β-adrenergic agonists on 4E-BP1 phosphorylation in freshly isolated rat adipocytes, even in insulin-stimulated cells, represents a significant departure from the results observed in cell lines [Graves et al., 1995; Scott et al., 1996], particularly in 3T3-L1 adipocytes [Scott and Lawrence, 1998]. This difference further underscores the impression that conclusions drawn about the mechanisms of mTOR signaling in cell lines may not be generally applicable to the bona fide post-mitotic cells they are intended to represent.

Potential Role of Other Heterotrimeric G-protein Pathways

We also evaluated G-protein–coupled signaling pathways as potential mediators of the effects of amino acids on mTOR in rat adipocytes. Indeed it has been proposed previously that amino acids regulate rapamycin-sensitive S6 phosphorylation by stimulating a G-protein–linked receptor [Blommaart et al., 1995; Blommaart et al., 1997]. G-protein–coupled receptors have been previously shown to be capable of regulating cell-signaling components that can potentially regulate mTOR or its downstream targets [e.g., Kurosu et al., 1997; Moule et al., 1997; Moxham and Malbon, 1996], and at least one potential target, PKCδ, seems to be required for rapamycin-sensitive 4E-BP1 phosphorylation in the 293T cell line. Moreover, in rat-1 fibroblasts, calcium ionophores and agonists of α-adrenergic receptors stimulate rapamycin-sensitive 4E-BP1 phosphorylation [Rybkin et al., 2000]. Despite these reports, four results from the present studies are not compatible with the hypothesis that the rapamycin-sensitive regulation of 4E-BP1 by amino acids in adipocytes involves G-proteins. First, application of a calcium ionophore had no effect on 4E-BP1 phosphorylation. If amino acids worked by mobilizing calcium from intracellular stores or by stimulating calcium influx, the calcium ionophore should have mimicked the effects of amino acids on 4E-BP1 phosphorylation. Second, pertussis toxin treatment, which inactivates G_i-proteins, also had no effect on the response to amino acids. Third, two direct-acting G-protein agonists that activate a broad spectrum of G-proteins, fluoroaluminate and MAS-7, did not mimic the effect of amino acids on 4E-BP1 phosphorylation. Lastly, neither staurosporine nor BIM I attenuated the response to amino acids. Both of these are potent inhibitors of all PKCs including, PKCδ.

In conclusion, the regulation of 4E-BP1 by amino acids appears to involve a rapamycin- and LY294002-sensitive step, probably the protein kinase mTOR. Although the mechanism leading to activation of mTOR in response to amino acids requires further study, the results of the present study indicate that it does not involve a number of previously characterized cell-signaling mechanisms. Other possibilities include, but are not limited to the following: 1) Amino acids bind to a regulatory site on mTOR

and thereby stimulate mTOR directly; 2) amino acids act indirectly on mTOR by altering the binding of a positively or negatively acting adapter or regulatory protein (for example, FKBP12 is a small molecular weight protein that binds to and regulates mTOR activity); or 3) amino acids may stimulate a protein kinase (other than those investigated here) that regulates the phosphorylation state and activity of mTOR.

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