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Regulation of Translational Effectors by Amino Acid and Mammalian Target of Rapamycin Signaling Pathways

POSSIBLE INVOLVEMENT OF AUTOPHAGY IN CULTURED HEPATOMA CELLS*

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Amino acid deprivation of Chinese hamster ovary cells overexpressing human insulin receptors results in deactivation of p70 S6 kinase (p70) and dephosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which become unresponsive to insulin; readdition of amino acids restores these responses in a rapamycin-sensitive manner, suggesting that amino acids and mammalian target of rapamycin signal through common effectors. Contrarily, withdrawal of medium amino acids from the hepatoma cell line H4IIE does not abolish the ability of insulin to stimulate p70 and 4E-BP1. The addition of 3-methyladenine (3MA) to H4IIE cells deprived of amino acids inhibited the increment in protein degradation caused by amino acid withdrawal nearly completely at 10 mM and also strongly inhibited the ability of insulin to stimulate p70 and 4E-BP1 at 10 mM. Treatment of H4IIE cells with 3MA did not alter the ability of insulin to activate tyrosine phosphorylation, phosphoinositide 3-kinase, or mitogen-activated protein kinase. In conclusion, the ability of H4IIE cells to maintain the insulin responsiveness of the mammalian target of rapamycin-dependent signaling pathways impinging on p70 and 4E-BP1 without exogenous amino acids reflects the generation of amino acids endogenously through a 3MA-sensitive process, presumably autophagy, a major mechanism of facultative protein degradation in liver.

Considerable progress has been achieved recently in the understanding of the signal transduction pathways involved in translational control. In addition to the many eukaryotic initiation factors (eIFs)† that are regulated by phosphorylation, two translational regulatory proteins, i.e. p70 S6 kinase (p70) and PHAS1/eIF-4E-binding protein 1 (4E-BP1), have been extensively studied as targets of growth factor-regulated signaling pathways (for review, see Refs. 1 and 2). p70 is activated in response to insulin and mitogens through a multisite phosphorylation and catalyzes the multiple phosphorylation of 40 S ribosomal protein S6 in vivo; this kinase plays a critical regulatory role in the translation of a class of transcripts that contain a 5′ oligopyrimidine tract at their transcriptional start site structure (3). 4E-BP1 exhibits rapid and multiple phosphorylation in vivo in response to insulin and mitogens (2). The initiation factor eIF-4E binds to the 5′ cap structure of mRNA (m7GpppN, where N is any nucleotide) and through its binding to the scaffold protein eIF-4G recruits the mRNA to a complex, known as the eIF-4F (4). 4E-BP1 in the phosphorylated form binds to eIF-4E (5) in a manner competitive with eIF-4G, thereby inhibiting translation of a subset of mRNAs (4). The insulin- and mitogen-stimulated phosphorylation of 4E-BP1 inhibits 4E-BP1 binding to eIF-4E, making eIF-4E available for incorporation into the eIF-4F complex via eIF-4G (6) and restoring translation.

At least two distinct signaling pathways underlying activation of p70 and phosphorylation of 4E-BP1 have been identified. One input controlling p70 and 4E-BP1 is provided by phosphoinositide 3-kinase (PI3-k), which is recruited to the activated receptor tyrosine kinases in response to insulin and mitogens. Thus, treatment of cells with PI3-k inhibitors such as wortmannin inhibits activation of p70 and phosphorylation of 4E-BP1 in response to insulin and mitogens (7, 8). Most importantly, 3-phosphoinositide-dependent protein kinase-1, phosphorylates p70 in vitro selectively at Thr-252, a PI3-k-stimulated and wortmannin-sensitive phosphorylation site in vivo (9), with a resultant increase in p70 activity (10, 11). 3-phosphoinositide-dependent protein kinase-1 was first identified as an activator of protein kinase B (also called Akt and RacPK), another downstream effector of PI3-k; protein kinase B activation in vivo activates both p70 and 4E-BP1 indirectly, through an unknown mechanism (12, 13). Another pathway contributing to activation of p70 and phosphorylation of 4E-BP1 is regulated by mammalian target of rapamycin proteins (named FRAP, RAPT-1, RAPT-1, or mTOR; for review, see Refs. 14 and 15). The macrolide immunosuppressant rapamycin is known to cause a dephosphorylation and deactivation of p70 (for review, see Refs. 14 and 15), and dephosphorylation of 4E-BP1 (16, 17). Rapamycin, in complex with the cytosolic 12-kDa FK506-binding protein (FKBP12), binds to mTOR and inhibits its protein kinase activity in vitro (18), and recent studies have established that mTOR is the rapamycin-sensitive upstream regulator of both p70 (18) and 4E-BP1 (19, 20). These two targets,
however, are regulated by separate mTOR-controlled pathways that bifurcate at or downstream of mTOR (19, 21).

The phosphorylation of ribosomal S6, p70, and 4E-BP1 in vivo is governed by the availability of amino acids (22–24). Rat hepatocytes incubated in the absence of amino acids exhibit a decrease in the phosphorylation of S6 that is rapidly reversed by addition of amino acids but blocked by rapamycin (22). Withdrawal of amino acids from the nutrient medium of CHO-IR or HEK293 cells results in a rapid deactivation of endogenous or recombinant p70 and dephosphorylation of 4E-BP1, which become unresponsive to various agonists, including insulin and recombinant PI3-K (24). Amino acid readdition restores p70 activity and the phosphorylation of 4E-BP1. The effect of amino acid repletion on p70 and 4E-BP1 is blocked by rapamycin; moreover, the p70 mutant Δ2–46/ΔCT104, which is resistant to inhibition by rapamycin, is also resistant to inhibition by amino acids withdrawal, suggesting that the amino acids and mTOR signal to p70 through a common effector mechanism (24). Based on these findings, we proposed that amino acid insufficiency and loss of mTOR activity both act as override switches, which inhibit p70 activation and 4E-BP1 phosphorylation irrespective of the receptor tyrosine kinase and PI3-K signals.

Cells regulate amino acid pools in part by the degradation of endogenous proteins; this becomes the only source of amino acids when exogenous amino acids are unavailable. A major mechanism for degrading intracellular proteins in response to amino acid deprivation is autophagy or macroautophagy (for

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Effects of wortmannin, rapamycin, and PD98059 on the ability of insulin and amino acids to stimulate p70 activity after amino acid withdrawal. *A*, after serum starvation for 24 h, H4IIE cells were incubated with DMEM lacking amino acids for 2 h. Five plates (lanes 1–5) were incubated for another 10 min with vehicle (lane 1) or 100 nM insulin (lanes 2–5). Before insulin stimulation, the cells were pretreated with vehicle (lane 2), 100 nM wortmannin (lane 3), or 100 ng/ml rapamycin (lane 4) for 30 min or with 40 μM PD98059 (lane 5) for 1 h. After incubation with DMEM lacking amino acids for 2 h, another four plates (lanes 6–9) were incubated in complete DMEM containing vehicle (lane 6), 100 nM wortmannin (lane 7), or 100 ng/ml rapamycin (lane 8) for 30 min or 40 μM PD98059 (lane 9) for 1 h. p70 activity was determined as described under “Experimental Procedures.” Numbers at the top of each lane represent %P incorporated into S6 expressed as a percentage of that catalyzed by p70 immunoprecipitated from cell extracts treated with 100 nM insulin after amino acid withdrawal (lane 2). *B* and *C*, after serum starvation for 24 h, H4IIE cells were incubated with DMEM lacking amino acids for 2 h. The cells were then incubated for another 10 min with 100 nM insulin (open circles). Before insulin stimulation, cells were pretreated with the indicated concentrations of wortmannin (*B*) or rapamycin (*C*) for 30 min. After amino acid deprivation, cells were incubated in complete DMEM (closed circles) containing the indicated concentrations of wortmannin (*B*) or rapamycin (*C*) for 30 min. p70 activity was determined as described under “Experimental Procedures.” p70 activity stimulated with either 100 nM insulin or complete DMEM in the absence of inhibitors was regarded as maximal stimulation. Data are expressed as a percentage of maximal stimulation of p70 activity by either insulin or amino acids. Data are the means ± S.D. of triplicates.
review, see Refs. 25 and 26). Autophagy is a major source of endogenous amino acids for gluconeogenesis and other critical pathways early in starvation, and extracellular amino acids have been shown to be the primary regulators of autophagy in hepatocytes of perfused liver (27, 28) and isolated hepatocytes (29). Autophagic proteolysis is enhanced in the absence of amino acids, and it is suppressed by amino acids at concentrations equivalent to the upper physiological limit (27, 30). The molecular mechanisms underlying the control of autophagy by amino acids are largely unknown. In yeast, autophagy is activated in response to nutrient insufficiency and appears to be regulated by TOR, the yeast homolog of mTOR (31).

In view of the prominent role of autophagy in hepatic proteolysis, and the evidence for TOR regulation of autophagy in yeast, we reasoned that cultured hepatocytes might provide an attractive system in which to study the role of amino acids and mTOR signaling pathways in the regulation of translation and autophagy. H4IIE cells, a cultured hepatoma cell line, have been used to study the molecular mechanism of p70 activation by various agonists, including insulin (32). In H4IIE cells, in contrast to our previous finding in CHO-IR cells or HEK293 cells, withdrawal of amino acids from the nutrient medium did not eradicate the ability of insulin to stimulate p70 activity and 4E-BP1 phosphorylation.

DEVELOPMENT PROTOCOLS

Materials— Dulbecco’s modified Eagle’s medium (DMEM), minimal essential medium α and fetal calf serum were purchased from Life Technologies, Inc. Protein G-Sepharose 4FF and mGTP-Phosphorase were from Amersham Pharmacia Biotech. Radiolabeled proteins were obtained from Amersham Pharmacia Biotech. Human insulin was supplied from Boehringer Mannheim. 3-Methylenedioxime and wortmannin were purchased from Sigma. PD98059 and rapamycin were obtained from New England Biolabs and Calbiochem, respectively.

Antibodies—The polyclonal antiserum against the C-terminal 104 amino acids of p70 used for immunoprecipitation of endogenous p70 and the monoclonal antibody against 4E-BP1 were described previously (9, 19). The anti-eIF-4E antibody and the anti-phosphoryrosynine antibody (PY20) were purchased from Transduction Laboratories. The phosphospecific (Thr-202/Tyr-204) and the non-phospho-specific antibodies against p44/42 mitogen-activated protein kinase (MAPK) were from New England Biolabs.

Cell culture and treatments— Rat hepatoma H4IIE cells were grown in minimal essential medium α with 10% fetal calf serum on 6-cm culture dishes. Cells were first incubated in minimal essential medium α without fetal calf serum for 24 h, washed once with DMEM lacking amino acids, and incubated in the same medium for the times indicated for up to 2 h. Reedition of amino acids restored the concentration of amino acids to the level found in complete DMEM. Treatments of cells were terminated by removal of the medium followed by freezing down with liquid nitrogen; then cells were stored at −80 °C until lysis. Cells were extracted into ice-cold buffer A (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 20 mM β-glycerophosphate, 0.5 mM dithiothreitol, 50 μM p-amidinophenylmethanesulfonyl fluoride hydrochloride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and the extracts were centrifuged at 10,000 × g for 20 min at 4 °C before analysis.

Immunoprecipitation and p70 S6 Kinase Assays—p70 activity was determined by immunocomplex assay using 40 S ribosomal subunits as substrates. Immunoprecipitation of p70 and the kinase assay conditions were as described (9, 19). Assay samples containing [35S]-S6 were separated by SDS-PAGE on a 12% acrylamide gel, and radioactivity was quantified with a BAS-2000 Bioimaging analyzer (Fuji).

Immunoblot Analysis and 4E-BP1 Assay—Aliquots of the supernatants of cell extracts were heated for 7 min at 90 °C, cooled on ice, and recentrifuged. The heat-soluble proteins were separated by SDS-PAGE on a 15% polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, immunoblotted with the polyclonal antibody against 4E-BP1 (1:500 dilution), and visualized using the ABC kit according to the manufacturer’s protocol (Vectastain).

4E-BP1 Binding Assay to eIF-4E—Cell extracts were incubated with mGTP-Phosphorase, and bound proteins were eluted in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM dithiothreitol, 2 mM EDTA, mixed with SDS-sample buffer, and heated for 7 min at 90 °C. Samples were separated by SDS-PAGE on 15% polyacrylamide gels, transferred onto a polyvinylidene difluoride membrane, immunoblotted with the polyclonal antibody against 4E-BP1 (1:500 dilution), and visualized using the ABC kit according to the manufacturer’s protocol (Vectastain).

Autophagy Assay—Autophagic activity was determined by measurement of the degradation of long-lived proteins as described previously (35) with a slight modification. H4IIE cells were incubated for 6 h at 37 °C with serum-free minimal essential medium α and labeled in the same medium containing 0.2 μCi/ml [3H]valine for 18 h at 37 °C. After three rinses with phosphate-buffered saline, pH 7.4, to remove unincorporated radioisotope, the cells were incubated in DMEM lacking amino acids, complete DMEM, or DMEM lacking amino acids with 10 mM 3MA, containing 0.1% of bovine serum albumin and 10 μg unlabeled valine for 1 h, at which time short-lived proteins were being degraded. Then incubation to measure the degradation of long-lived proteins was initiated by replacing the medium with the same fresh medium and continued for up to 2 h. At the indicated times, the cells and media were collected separately. The cells were then frozen and scraped into 0.5 ml of phosphate-buffered saline. Radiolabeled proteins...
in the cells and media were precipitated in 10% trichloroacetic acid (TCA) at 4 °C. The precipitated proteins were separated from the soluble radioactivity by centrifugation at 600 x g for 10 min and then dissolved in 1 ml of Soluene 350 (Packard). The radioactivities in the fractions soluble and insoluble in TCA recovered from the cells and media were measured by scintillation counting. The rate of protein degradation attributable to autophagic activity was calculated as a percentage of the radioactivities in the fractions soluble in TCA recovered from the cells and media to total radioactivity recovered from the cells and media.

PI3-kinase Assay—Aliquots of cell extracts lysed in ice-cold buffer A containing 1 mM vanadate were subjected to immunoprecipitation with the anti-phosphotyrosine antibody. PI3-kinase activity in the immunoprecipitates was determined as described previously (36).

RESULTS AND DISCUSSION

The Ability of Insulin to Stimulate p70 Activity and 4E-BP1 Phosphorylation in H4IIE Cells Is Maintained Despite Amino Acid Withdrawal—As reported previously using CHO-IR and HEK293 cells (24), we observed in H4IIE cells that brief (2-h) withdrawal of amino acids from serum-deprived culture medium diminished substantially the activity of endogenous p70, and readdition of amino acids activated p70 activity (Fig. 1A, lanes 1 and 6). In the previous study in CHO-IR and HEK293 cells, withdrawal of amino acids from the culture medium inhibited almost completely the ability of insulin to stimulate p70 activity (24). By contrast, insulin activation of p70 in H4IIE cells was robust despite the withdrawal of exogenous amino acids (Fig. 1A, lane 2). The ability of insulin to stimulate p70 activity in amino acid-deprived H4IIE cells was still maintained when the duration of amino acid withdrawal was increased from 2 to 6 h (data not shown). This pattern is similar to that exhibited when amino acid-deprived CHO-IR cells are treated with very low levels of exogenous amino acids. Readdition of amino acids to CHO-IR cells at 10–12% the level usually found in DMEM does not increase basal p70 activity but largely restores the responsiveness of p70 to insulin (24).

Previous work showed that amino acid deprivation in CHO-IR and HEK293 cells abolished the insulin-stimulated phosphorylation of 4E-BP1 (24). The effect of amino acid deprivation on 4E-BP1 and its association with eIF-4E in H4IIE cells was therefore examined. 4E-BP1 binds to eIF-4E; previous work has established that the insulin- and mitogen-stimulated phosphorylation of 4E-BP1 inhibits its binding to eIF-4E (5), whereas rapamycin-induced dephosphorylation of 4E-BP1 enhances its binding to eIF-4E. Phosphorylation states of 4E-BP1 in H4IIE cell extracts were monitored indirectly by shifts in the migration of 4E-BP1 polypeptides on SDS-PAGE and by estimation of the amount of 4E-BP1 complexed with eIF-4E, the latter isolated using m7GTP-Sepharose. H4IIE cells deprived of amino acids for 2 h exhibited a predominance of rapidly migrating bands (Fig. 2A, lane 1) and considerable binding of 4E-BP1 to eIF-4E (Fig. 2B, lane 1). Readdition of amino acids diminished the abundance of the more rapidly migrating bands, increased the abundance of the slowest migrating, hyperphosphorylated bands (Fig. 2A, compare lane 5 with lane 1), and decreased the amount of 4E-BP1 recovered in association with eIF-4E (Fig. 2B, compare lane 5 with lane 1). Addition of insulin in the absence of amino acids also caused an upshift in the mobility of 4E-BP1 (Fig. 2A, lane 2) and the decrease of 4E-BP1 complexed with eIF-4E (Fig. 2B, lane 2). Thus, in H4IIE cells, withdrawal of exogenous amino acids promotes 4E-BP1 dephosphorylation but does not abolish insulin stimulation of 4E-BP1 phosphorylation.

Effect of Wortmannin and Rapamycin on Insulin- and Amino
Acid-induced p70 Activation and 4E-BP1 Phosphorylation in H4IIE Cells—The ability of insulin to maintain p70 activity in amino acid-deprived H4IIE cells enabled us to examine whether the upstream inputs required for the insulin-induced increase in p70 activity are also necessary for the increase in p70 activity caused by amino acid repletion. H4IIE cells were subjected to amino acid withdrawal for 2 h, and they were pretreated with wortmannin, rapamycin (each for 30 min), or PD98059 for 1 h before stimulation with insulin (Fig. 1A, lanes 3–5) or during readdition of amino acids (Fig. 1A, lanes 7–9). As expected, insulin activation of p70 was strongly inhibited by wortmannin and rapamycin but minimally altered by the mitogen-activated protein kinase kinase-1 inhibitor PD98059. The increase in p70 activity induced by amino acid readdition exhibited a sensitivity to inhibition by rapamycin indistinguishable from that shown by p70 activated in response to insulin (Fig. 1A, compare lane 8 with lane 4, and C); however, the activation of p70 by amino acids was considerably more resistant to inhibition by wortmannin than was the activation induced by insulin (Fig. 1A, compare lane 7 with lane 3, and B). Inhibition of the insulin-activated p70 by wortmannin was complete and maximal at 30 nM (Fig. 1B), whereas much higher concentrations of wortmannin failed to inhibit amino acid-induced p70 activation to the extent achieved by much lower concentrations of wortmannin acting on insulin-stimulated cells (Fig. 1B).

With regard to 4E-BP1, pretreatment of H4IIE cells with 100 ng/ml rapamycin inhibited the ability of both insulin and amino acids to cause the upshift of 4E-BP1 (Fig. 2A, lanes 4 and 7) and the decrease of 4E-BP1 binding to eIF-4E (Fig. 2B, lanes 4 and 7). Pretreatment with 100 nM wortmannin before insulin stimulation resulted in a maximal downshift of 4E-BP1 mobility and increment in eIF-4E binding (Fig. 2, A, lane 3, and B, lane 3). However, as with p70, the ability of amino acids to promote the phosphorylation of 4E-BP1 was much less sensitive to inhibition by wortmannin than was the insulin-stimulated 4E-BP1 phosphorylation (Fig. 2, A, lane 6, and B, lane 6).

Taken together, these data show that in H4IIE cells after amino acid withdrawal, as in amino acid-replete cells, both rapamycin- and wortmannin-sensitive signaling pathways are required for the insulin stimulation of p70 activity and 4E-BP1 phosphorylation. Because mTOR is the rapamycin-sensitive molecule in both circumstances, a conclusion supported by the observation is that inhibition of mTOR in vivo by rapamycin abolishes the response to amino acids and insulin with a parallel sensitivity and to a comparable extent. By contrast, the much lower potency of wortmannin in inhibiting the response of p70 and 4E-BP1 to amino acids compared with insulin could reflect the requirement for a lesser input from PI3-kinase (28–30) in the response to amino acids compared with insulin. Alternatively, the ability of wortmannin to inhibit the response to amino acids may not reflect wortmannin inhibition of PI3-kinase as much as the ability of the higher concentrations of wortmannin to inhibit mTOR (8).

Amino Acid Release by Autophagy—The sustained responsiveness of p70 and 4E-BP1 to insulin in H4IIE cells after amino acid withdrawal, mimicking the behavior of CHO-IR cells incubated in the presence of the low concentrations of exogenous amino acids (24), suggested that H4IIE cells might be better able than are CHO-IR cells to generate amino acids from endogenous sources during the amino acid deprivation.

Autophagy is a ubiquitous mechanism for the degradation of endogenous proteins and is especially active in liver and isolated hepatocytes (28–30). Amino acid deprivation is known to
enhance and probably initiate this process. We assessed the effects of amino acid sufficiency on the degradation of endogenous H4IIE proteins by labeling cells with [14C]valine for 16 h. After washing in phosphate-buffered saline to remove the bulk of unincorporated [14C]valine, the cells were transferred to the medium containing or lacking amino acids, and the degradation of long-lived proteins was determined by the release of TCA-soluble [14C]valine. Approximately 3–4% of total [14C]valine was released per hour during 2 h of incubation in the presence of exogenous amino acids; [14C]valine release was ~50% higher when H4IIE cells were incubated in medium lacking amino acids (Fig. 3, A and B, compare lane 1 with lane 2). Moreover, 3MA, a widely used inhibitor of autophagy (33, 34) inhibited [14C]valine release from amino acid-deprived H4IIE cells to an extent similar to that achieved by amino acid readdition (Fig. 3B, compare lane 3 with lane 2). Addition of 3MA in the presence of exogenous amino acids did not decrease the rate of [14C]valine release beyond the lower rate induced by the readdition of amino acids per se (data not shown). These findings are consistent with earlier reports and support the conclusion that autophagy is the major mechanism for the facultative increase in endogenous protein degradation in H4IIE cells in response to amino acid withdrawal and can be inhibited by amino acid readdition or 3MA.

Effects of 3MA on the Ability of Insulin to Stimulate p70 Activity and 4E-BP1 Phosphorylation in H4IIE Cells after Amino Acid Withdrawal—Reasoning that any effects of 3MA that are abrogated by amino acid repletion can be attributed to the ability of 3MA to inhibit autophagy, we next examined the effect of 3MA on insulin activation of p70 and 4E-BP1 phosphorylation in both the presence and absence of exogenous amino acids. H4IIE cells were serum-deprived for 24 h, followed by incubation for an additional 2 h in DMEM or DMEM lacking amino acids, in the absence or presence of 10 mM 3MA. 100 nM insulin was then added to some incubations, and the cells were harvested 10 min thereafter. In H4IIE cells incubated in the absence of exogenous amino acids, the ability of insulin to activate p70 was inhibited ~90% by 3MA (Fig. 4A, compare lane 3 with lane 4). By contrast, the insulin activation of p70 in amino acid-replete cells was inhibited only modestly (~30% inhibition) by the same concentration of 3MA (Fig. 4A, compare lane 5 with lane 6). We also examined dose-dependent effects of 3MA on insulin- and amino acid-induced activation of p70 during incubation in the culture medium lacking exogenous amino acids. The inhibition of insulin activation of p70 by 3MA was observed in a dose-dependent manner (Fig. 4B). By contrast, 3MA barely inhibited activation of p70 by amino acids (Fig. 4B), suggesting that the inhibitory effect of 3MA on insulin activation of p70 in amino acid-deprived cells is not attributable to the direct inhibition of p70 activity by 3MA in situ.

With regard to 4E-BP1 phosphorylation, the ability of insulin to upshift the mobility of 4E-BP1 (Fig. 5A, lane 3) and decrease the amount of 4E-BP1 complexed with eIF-4E (Fig. 5B, lane 3) in amino acid-deprived cells was completely inhibited by 10 mM 3MA (Fig. 5, A, lane 4, and B, lane 4), whereas the inhibitory effect of 3MA on 4E-BP1 phosphorylation in amino acid-replete H4IIE cells was greatly attenuated; in amino acid-replete cells, considerable 4E-BP1 persisted in the most slowly migrating band, and the amount of 4E-BP1 complexed with eIF-4E greatly diminished despite the presence of 10 mM 3MA (Fig. 5, A and B, compare lanes 4 and 6). Thus, 3MA inhibits insulin-induced p70 activation and 4E-BP1 phosphorylation to a much greater extent in amino acid-deprived cells than in amino acid-replete cells, consistent with the conclusion that in H4IIE cells incubated without exogenous amino acids, amino acids derived from autophagic proteolysis are critical to maintain the responsiveness of p70 and 4E-BP1 to insulin.

3MA Does Not Inhibit Insulin Activation of Insulin Receptor Kinase, PI3-k, or MAPK—We examined effects of 3MA on several steps of insulin signaling upstream of p70 and 4E-BP1. Treatment of H4IIE cells with 3MA had no significant inhibitory effects on insulin-stimulated receptor tyrosine phosphorylation and tyrosine phosphorylation of endogenous insulin receptor substrate proteins (Fig. 6A, lanes 3, 4, 7, and 8) or on the insulin-stimulated increase in PI3-k immunoprecipitated by anti-phosphotyrosine antibodies (Fig. 6B, lanes 3, 4, 7, and 8), either in the presence or absence of exogenous amino acids. The insulin-stimulated phosphorylation at activating sites (TEY) on the endogenous p44/42 MAPK, monitored by immunoblotting with the phosphopeptide antibody against p44/42 MAPK, was not significantly altered by 3MA (Fig. 6C, lanes 3, 4, 7, and 8). Thus, the inhibitory effects of 3MA on insulin stimulation of p70 and 4E-BP1 in amino acid-deprived H4IIE cells reflect the inhibition of a signal transduction response clearly distinguished from those controlled directly by insulin. Nevertheless, the provision of endogenous amino acids through autophagy is indispensable to the ability of insulin to effectively increase the phosphorylation of p70 and 4E-BP1 in amino acid-deprived cells. When amino acids are supplied exogenously, however, autophagy is largely dispensable for insulin signaling to p70 and 4E-BP1.

Conclusion—Previous work in several cell lines had shown that removal of amino acids from the nutrient medium blocked selectively the ability of insulin to regulate the phosphorylation...
of p70 and 4E-BP1 (24, 37). The present study was undertaken to explore the mechanism that enabled H4IIE hepatoma cells deprived of amino acids to maintain the responsiveness of p70 and 4E-BP1 to insulin. The resistance of hepatoma cells to amino acid withdrawal was also observed by Patti et al. (38), who reported that insulin was able to activate p70 activity and 4E-BP1 phosphorylation in FAO hepatoma cells after the complete omission of amino acids from the nutrient medium. The similarity in the responses to insulin shown by H4IIE cells deprived completely of exogenous amino acids and CHO-IR cells replete with low concentrations of amino acids (<0.25 of concentrations usually present in DMEM) led us to inquire as to whether H4IIE cells had a means to generate amino acids from endogenous sources.

Inasmuch as autophagy offered an attractive candidate mechanism for the provision of endogenous amino acids, we examined the effect of 3MA, a relatively potent, widely used inhibitor of autophagy (33, 34), on the ability of insulin to regulate p70 and 4E-BP1 in the presence and absence of exogenous amino acids in H4IIE cells. Omission of amino acids from the medium increased the rate of endogenous proteolysis ([14C]valine release) presumably by autophagy, resulting in the release of amino acids into the nutrient medium (Fig. 3A). This increment in proteolysis was inhibited by 3-MA (Fig. 3B). The inhibition of autophagy in amino acid-deprived H4IIE cells by 3MA was accompanied by a dephosphorylation of 4E-BP1 (Fig. 5, A and B), a strong inhibition of the ability of insulin to promote 4E-BP1 phosphorylation (Fig. 5, A and B) and the activation of p70 (Fig. 4, A and B). The inhibitory effects of 3MA on basal and insulin-stimulated 4E-BP1 phosphorylation and p70 activation were greatly attenuated by reintroduction of amino acids into the medium (Figs. 4, A and B, and 5, A, and B). Moreover, 3MA did not inhibit signal transduction pathways activated by insulin (Fig. 6). These data support the conclusion that the inhibition of autophagy by 3MA resulted in the reduction of amino acid supply and a shutoff of amino acid-activated, rapamycin-sensitive signal that is necessary to maintain the responsiveness of these translational regulators to insulin. Further studies are necessary to clarify the signaling components that regulate translational control and autophagy via amino acid and mTOR signaling pathways.

**FIG. 6. Effects of 3MA on insulin signaling.** After serum starvation for 24 h, H4IIE cells were incubated with DMEM lacking amino acids (lanes 1–4) or complete DMEM (lanes 5–8) for 2 h, and 10 mM 3MA was added for the last 30 min to lanes 2, 4, 6, and 8. Cells were then stimulated with vehicle (lanes 1, 2, 5, and 6) or 100 nM insulin (lanes 3, 4, 7, and 8) for another 10 min. Supernatants of cell extracts were prepared as described under "Experimental Procedures." A and B, tyrosine-phosphorylated proteins were immunoprecipitated with the anti-phosphotyrosine antibody (PY20). In A, tyrosine phosphorylation of β-subunits of insulin receptors (IR) and insulin receptor substrates (IRS) were determined by immunoblotting with PY20 as the first antibody. Positions of IRS and IR are shown by arrows. In B, PI3-k activity in the immunoprecipitates was determined as described under "Experimental Procedures." Positions of phosphoinositide 3-phosphate (PIP) and thin layer chromatography origin (Origin) are shown by arrows. C, the supernatants were separated by SDS-PAGE and immunoblotted with the non-phospho-specific antibody against p44/42 MAPK (upper panel) or the phospho-specific (Thr-202/Tyr-204) antibody against p44/42 MAPK (lower panel) as the first antibody. Positions of p44 and p42 MAPK are shown by arrows.

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