In human T-lymphoblastoid cells, downstream signaling events of mammalian target of rapamycin (mTOR), including the activity of p70^{s6k} and phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), were dependent on amino acid concentration in the culture media, whereas other growth-related protein kinases were not. Amino acid-induced p70^{s6k} activation was completely inhibited by rapamycin but only partially inhibited by wortmannin. Moreover, amino acid concentration similarly affected the p70^{s6k} activity, which was dependent on a rapamycin-resistant mutant (S2035I) of mTOR. These data indicate that mTOR is required for amino acid-dependent activation of p70^{s6k}. The mechanism by which amino acids regulate p70^{s6k} activity was further explored: 1) amino acid analogs, which inhibit aminoacylation of tRNA by their competitive binding to tRNA synthetases, suppressed p70^{s6k} activity; 2) suppression of p70^{s6k} by amino acid depletion was blocked by cycloheximide or puromycin, which inhibit utilization of aminoacylated tRNA in cells; and 3) in cells having a temperature-sensitive mutant of histidyl tRNA synthetase, p70^{s6k} was suppressed by a transition of cells to a nonpermissible temperature, which was partially restored by addition of high concentrations of histidine. These results indicate that suppression of tRNA aminoacylation is able to inhibit p70^{s6k} activity. Deacylated tRNA may be a factor negatively regulating p70^{s6k}. 

The p70 S6 kinase (p70^{s6k}) is a serine/threonine kinase that is ubiquitously activated at the G_{1}/G_{2} transition of the cell cycle in mammalian cells (1–3). This protein kinase phosphorylates 40 S ribosomal S6 protein at five serine residues near the carboxyl terminus in vitro (4). Studies using targeted disruption of the p70^{s6k} gene (5) and using transfection of a dominant negative or a rapamycin-resistant mutant of p70^{s6k} (6, 7) have shown that p70^{s6k} mediates S6 phosphorylation in vivo. S6 phosphorylation has been proposed to be a factor regulating initiation of mRNA translation (see review in Ref. 8), and the studies described above (5, 6) concluded that the role of p70^{s6k} in cell proliferation resides in specific regulation of translation of mRNAs encoding ribosomal proteins. Thus, p70^{s6k} activity is considered to be a factor required for up-regulation of ribosomal biogenesis, which facilitates G_{1} progression during the cell cycle (9).

In regards to the activation of p70^{s6k}, it appears there are at least two upstream regulatory pathways; one is through phosphatidylinositol-3 kinase (PI3K) and the other is through mammalian target of rapamycin (mTOR; also termed FRAP and RAFT). Many growth factors, such as platelet-derived growth factor and epidermal growth factor, activate PI3K through their binding to specific receptors, and previous studies using chemical inhibitors of PI3K, mutant platelet-derived growth factor receptors, and mutant PI3Ks, all indicated that PI3K is involved in the activation of p70^{s6k} by growth factors (10–13). Indeed, it has been demonstrated recently that PDK1, a downstream kinase of PI3K, phosphorylates p70^{s6k} at Thr^{229} and activates the kinase (14, 15).

The involvement of mTOR in the regulation of p70^{s6k} has been initially demonstrated by studies using the immunosuppressive drug rapamycin (see reviews in Refs. 16 and 17). Rapamycin associates with a cellular protein FKBP12 in cells, and the rapamycin-FKBP12 complex then binds to mTOR. It has also been demonstrated that mTOR is required for the inhibitory action of rapamycin on p70^{s6k} (18). Moreover, mTOR has an intrinsic protein serine/threonine kinase activity and regulates the activity and phosphorylation of p70^{s6k} in vivo in a manner that is dependent on the kinase activity of mTOR (18). Although initial reports concluded that mTOR did not directly phosphorylate p70^{s6k}, it has been demonstrated that mTOR phosphorylates p70^{s6k} at Thr^{229} in vitro (19). In addition to p70^{s6k}, mTOR regulates another translation regulatory molecule, eIF-4E-binding protein 1 (4E-BP1) (20–23), independent of p70^{s6k} activity (5, 6). mTOR is demonstrated to phosphorylate the translation repressor protein 4E-BP1 at its serine and threonine residues (24). The hypophosphorylated species of 4E-BP1 binds tightly to eIF-4E (an N7-methylguanosine cap-binding subunit of eIF-4F complex) and prevents eIF-4E from associating with eIF-4G (a scaffolding protein in eIF-4F complex). The phosphorylation of 4E-BP1 by mTOR is thought to release eIF-4E and facilitate translational initiation of capped mRNA (21). Thus, mTOR regulates 1) ribosomal protein synthesis at the level of mRNA translation through p70^{s6k} activity, and 2) overall protein synthesis by controlling translational initiation of capped mRNA through 4E-BP1. In contrast to the PI3K pathway, which is activated by various growth factors, it was unclear what factor physiologically regulated mTOR. A study on proteolytic responses to amino acid deprivation showed that amino acid deprivation negatively regulates p70^{s6k} activity (25).
acid deprivation demonstrated that ribosomal S6 phosphorylation was induced by supplementation of amino acids (25), suggesting that amino acid concentration in culture media may be a regulatory factor for p70<sub>s6k</sub>. Indeed, Fox et al. (26) demonstrated that amino acids stimulate phosphorylation of p70<sub>s6k</sub> in rat adipocytes. More recently, Harra et al. (27) have reported that amino acid concentration regulates p70<sub>s6k</sub> activity and phosphorylation of 4E-BP1 in Chinese hamster ovary cells. The study demonstrated that a rapamycin-resistant mutant (p70ΔΔ—46/ACT104) of p70<sub>s6k</sub> was resistant to amino acid deprivation, indicating that amino acid sufficiency and mTOR both signal to p70<sub>s6k</sub> through a common effector, which could be mTOR itself or an mTOR-controlled downstream element. In this study, we further explored the mechanism by which amino acid concentration regulates p70<sub>s6k</sub>

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—Human T-lymphoblastoid Jurkat cells were obtained from ATCC. Human alveolar rhabdomyosarcoma Rh30 cells were obtained from the bone marrow of a patient with metastatic tumor (28). Rh30 cells constitutively expressing wild type mTOR (WT-mTOR) or a rapamycin-resistant mTOR (S2035I) were obtained by transfection of cells with pcDNA3-AU1mTORwt or pcDNA3-AU1mTORS2035I. BHK21 cells and their temperature-sensitive mutant temperature-sensitive (ts) BN250 were obtained from RIKEN cell bank (Tsukuba, Japan). Rapamycin and Wortmannin were obtained from Calbiochem (San Diego, CA), and stored in PBS at −80 °C. Methyl 2-aminoisobutyric acid (MeAIB) and 2-amino-2-norbornane-carboxylic acid were obtained from Sigma and stored in distilled water (5 M).

**Culture**—Jurkat cells and Rh30 cells were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (HyClone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). For amino acid deprivation, exponentially growing cells were washed twice with a medium containing 10% dialyzed FCS and incubated at 37 °C in a 5% CO<sub>2</sub> incubator. In some experiments, individual amino acids were deprived instead of total amino acid deprivation. For amino acid supplementation, cells (5 × 10<sup>6</sup> cells/ml) were incubated in the amino acid-free medium supplemented with 10% dialyzed FCS and incubated for the indicated times (amino acid supplementation).

**RESULTS**

**Amino Acid Deprivation Inactivated and Supplementation Activated p70<sub>s6k</sub> but Not Other Protein Serine/Threonine Kinases**—The human T-lymphoblastoid cell line, Jurkat cells, were transferred to an amino acid-free medium supplemented with 10% dialyzed FCS and incubated for the indicated times (amino acid deprivation). In addition, after culturing cells in the amino acid-free medium with 10% dialyzed FCS for 16 h, the cells were transferred to the regular RPMI 1640 medium containing amino acids with 10% dialyzed FCS and incubated at the indicated times (amino acid supplementation). Initially, the activity of p70<sub>s6k</sub> was measured in the system, as well as the activities of other growth-related serine/threonine kinases, Akt, p90<sub>rsk</sub>, and Cd2k (Fig. 1). p70<sub>s6k</sub> activity decreased within 15 min after deprivation of amino acids and became undetectable within 30 min; levels remained undetectable throughout the time course. Additionally, supplementation of amino acids increased p70<sub>s6k</sub> activity within 15 min, and the activity reached a plateau level within 60 min. In contrast, the activity of Akt, which is a downstream kinase of PI3K (19), was not inhibited by amino acid deprivation, nor did it increase following amino acid supplementation. p90<sub>rsk</sub> is a downstream kinase. 2-amino-2-norbornane-carboxylic acid and has the highest homology with p70<sub>s6k</sub> in the catalytic domains (30). In contrast to p70<sub>s6k</sub>, the activity of p90<sub>rsk</sub> transiently increased by transferring cells to an amino acid-free medium, and then decreased gradually. However, p90<sub>rsk</sub> remained partially active throughout the time course of the experiments. Additionally, amino acid supplementation transiently decreased p90<sub>rsk</sub> activity, but overall it did not dramatically alter kinase activity for 360 min. Cd2k is active especially at the late G<sub>1</sub> and S phases of the cell cycle. Cd2k activity was partially decreased by both amino acid deprivation and by supplementation within 30 min, but the kinase remained partially active throughout the time course.

**Amino Acid Supplementation Increased Phosphorylation of p70<sub>s6k</sub> and 4E-BP1**—The hyperphosphorylated species of p70<sub>s6k</sub>, which correspond to the active form of the kinase, is...
determined as a band(s) with a lower mobility in immunoblots (9). On the other hand, the hypophosphorylated p70s6k, which corresponds to the inactive form of the kinase, is determined as a band with the highest mobility. Cells treated with an amino acid-starved medium demonstrated only the hypophosphorylated species of p70s6k, and supplementation of amino acids increased the hyperphosphorylated species of p70s6k within 5–15 min (Fig. 2). In contrast to p70s6k, amino acid deprivation/supplementation essentially did not alter the phosphorylation status of p90rsk, similarly evaluated by a mobility shift in an immunoblot analysis (data not shown).

The phosphorylation status of 4E-BP1, another downstream event of mTOR, was also examined by a gel mobility shift in an immunoblot assay (20–23). Cells treated with an amino acid-starved medium demonstrated bands with higher mobilities, corresponding to the hypophosphorylated species of p70s6k, and supplementation of amino acids increased the hyperphosphorylated species of p70s6k within 5–15 min (Fig. 2). In contrast to p70s6k, amino acid deprivation/supplementation essentially did not alter the phosphorylation status of p90rsk, similarly evaluated by a mobility shift in an immunoblot analysis (data not shown).

The phosphorylation status of 4E-BP1, another downstream event of mTOR, was also examined by a gel mobility shift in an immunoblot assay (20–23). Cells treated with an amino acid-starved medium demonstrated bands with higher mobilities, corresponding to the hypophosphorylated species of 4E-BP1. Amino acid supplementation induced bands with lower mobilities, which correspond to hyperphosphorylated 4E-BP1, within 15 min. These hyperphosphorylated bands became more predominant within 1–3 h. Additionally, hyperphosphorylated species of p70s6k and 4E-BP1 were eliminated by amino acid deprivation within 5–15 min and 30 min, respectively (data not shown).

Rapamycin, but Not Wortmannin, Inhibited Amino Acid-induced p70s6k Activation—Because amino acid concentrations affected p70s6k without affecting Akt activity, it does not seem that amino acids regulate p70s6k through PI3K. Furthermore, similar effects of amino acids on p70s6k and 4E-BP1 suggested that mTOR, or an unidentified factor that mediates mTOR signals to both p70s6k and 4E-BP1, is involved in amino acid-induced activation of p70s6k. In order to further investigate these observations, the effects of wortmannin and rapamycin on amino acid-induced p70s6k activation were examined. Jurkat cells were treated in an amino acid-free medium for 16 h and then supplemented with total amino acids or none for 1 h in the presence or absence of specific inhibitors of PI3K and mTOR, wortmannin (100 nM) and rapamycin (10 ng/ml), respectively. As shown in Fig. 3, p70s6k activation induced by amino acid addition was inhibited by rapamycin. In contrast, wortmannin at the concentration that is known to inhibit PI3K activity over 95% inhibited p70s6k only by ~25%. In contrast, Akt, which was already active in the cell culture regardless of

FIG. 1. Effects of amino acid deprivation and supplementation on the activities of p70s6k, Akt, p90rsk, and Cdk2. A, amino acid deprivation. Exponentially growing Jurkat cells were washed twice with amino acid-free RPMI 1640 medium and resuspended at 5 × 10⁷ cells/ml in the same amino acid-free medium supplemented with 10% dialyzed FCS for the indicated times at 37 °C in a CO₂ incubator. B, amino acid supplementation. Jurkat cells (5 × 10⁶ cells/ml) were incubated in the amino acid-free medium with 10% dialyzed FCS for 16 h. The same volume of RPMI 1640 medium containing 2× concentrations of amino acids + 10% dialyzed FCS were added into the culture, and cells were incubated for the indicated times. Cells (2 × 10⁶) were harvested, and the specific activity of immunoprecipitated p70s6k, Akt1, p90rsk, or Cdk2 was measured by incorporation of ³²P into specific substrate peptides as described under “Experimental Procedures.” Error bars here and in figures below represent S.D. of the data. Results show one representative experiment out of three.

FIG. 2. Effects of amino acid supplementation on the phosphorylation of p70s6k and 4E-BP1. Jurkat cells (5 × 10⁵ cells/ml) were incubated in the amino acid-free medium with 10% dialyzed FCS for 16 h. The same volume of RPMI 1640 medium containing 2× concentrations of amino acids + 10% dialyzed FCS were added into the culture, and cells were incubated for the indicated times. The protein extracts from cells were separated in 7.5% (for p70s6k) or 15% (for 4E-BP1)-SDS-polyacrylamide gel and transferred to nitrocellulose filters. The specific proteins were detected by immunoblotting using the ECL method as described under “Experimental Procedures.” Results show one representative experiment out of three.
Regulation of p70<sup>60k</sup> by tRNA Aminoacylation

Fig. 3. Rapamycin but not wortmannin inhibits amino acid-induced p70<sup>60k</sup> activation. Jurkat cells (5 × 10<sup>5</sup> cells/ml) were incubated in amino acid-free medium with 10% dialyzed FCS for 16 h. Then, the same volume of either a medium containing 2× concentrations of amino acids + 10% dialyzed FCS (AA<sup>(+)</sup>) or amino acid-free medium + 10% dialyzed FCS (AA<sup>(−)</sup>) were added into the culture for 1 h. Rapamycin (RAP) (10 ng/ml) or wortmannin (WOR) (100 nM) was added to the culture 15 min prior to the addition of AA<sup>(+)</sup> medium or AA<sup>(−)</sup> medium. Cells were harvested, and the activities of p70 s6k and Akt were measured as described above. Results show one representative experiment out of two.

Amino acid concentrations, was inhibited by wortmannin by about 60%. Similar to the p70<sup>60k</sup> activity, 4E-BP1 phosphorylation induced by amino acid addition was inhibited by rapamycin but not by wortmannin (data not shown).

Rapid activation of p70<sup>60k</sup> (within 15 min) after re-addition of total amino acids suggested that this may be independent of newly synthesized proteins. In order to confirm this, cycloheximide was added to the culture before addition of total amino acids. Cycloheximide (100 μM), which inhibits protein synthesis over 95%, did not inhibit amino acid-induced p70<sup>60k</sup> activation (data not shown). These data indicate that amino acids activate p70<sup>60k</sup> independent of newly synthesized proteins.

Amino Acid Supplementation Induced p70<sup>60k</sup> Activation in the Presence of Rapamycin in Cells Expressing a Rapamycin-resistant Mutant of mTOR—In order to further investigate mechanisms by which amino acids regulate the mTOR pathway, we utilized cells constitutively expressing a rapamycin-resistant mTOR. Human rhabdomyosarcoma Rh30 cells constitutively expressing either WT-mTOR or a rapamycin-resistant mutant (S2035I-mTOR) were obtained by cotransfection of the mTOR expression vectors and a neomycin-resistant gene expression vector, followed by G418 selection as described previously (31). Parental Rh30 cells, WT-mTOR Rh30 cells, or S2035I-mTOR Rh30 cells were first incubated for 3 h in an amino acid-deprived medium. Rapamycin (10 ng/ml) was then added to the culture. Thirty min after addition of rapamycin, total amino acids were added, and cells were incubated for the indicated times. As shown in Fig. 4, amino acid supplementation was not able to induce p70<sup>60k</sup> in the presence of rapamycin in parental Rh30 cells or WT-mTOR Rh30 cells, as shown in Jurkat cells above. In contrast, amino acids did induce p70<sup>60k</sup> in the presence of rapamycin in S2035I-mTOR Rh30 cells.

Effects of Selective Inhibitors for Amino Acid Transport Systems on Amino Acid-induced p70<sup>60k</sup> Activation—The mechanism by which amino acids regulate p70<sup>60k</sup> activity was further explored. Fox et al. (26) reported that supplementation of a set of neutral amino acids, which were preferentially transported by system L transporter, was able to induce p70<sup>60k</sup> phosphorylation. In contrast, another set of neutral amino acids preferentially transported by system A or ASC, or a set of charged amino acids, was less effective. A potential explanation for these results is that a specific amino acid transporter, such as system L, is linked to activation of p70<sup>60k</sup>. In order to investigate this possibility, we examined the effects of specific inhibitors of various amino acid transporter systems on amino acid-induced p70<sup>60k</sup> activation. There are three major amino acid transporter systems known for uptake of neutral amino acids; system A, system L, and system ASC. It is reported that over 90% of neutral amino acids are transported through these three systems in mammalian cells (32). Each of the three systems has a model substrate that has a high affinity to the system. MeAIB has a high affinity to system A and competitively inhibits transport of other amino acids through the system. In contrast, MeAIB does not significantly affect transport of amino acids through other transporters, including system L and system ASC. Similarly, 2-amino-2-norbornane-carboxylic acid and cysteine can competitively inhibit amino acids-transport through system L and system ASC, respectively. Jurkat cells were initially incubated with amino acid-deprived medium for 16 h, and then 1/5 concentrations of total amino acids contained in regular RPMI 1640 medium were added to the culture in the presence or absence of these competitive model substrates (10 mM). This concentration of total amino acids was able to induce approximately one-half the activity of p70<sup>60k</sup> when compared with addition of 1× total amino acids of RPMI 1640 medium. As shown in Fig. 5, all of the model substrates partially inhibit p70<sup>60k</sup> activation induced by amino acid supplementation. This is inconsistent with the idea that a specific neutral transporter is linked to p70<sup>60k</sup> activation. If amino acid transporters are signaling mediators that sense extracellular
FIG. 5. Effects of selective inhibitors for neutral amino acid transport systems on amino acid-induced p70\(^{\text{S6K}}\) activation. Jurkat cells (5 \times 10^5 cells/ml) were incubated in amino acid-free medium with 10% dialyzed FCS for 16 h. \(1/16\) concentrations of total amino acids contained in RPMI 1640 were added to the culture in the presence of a vehicle (distilled water (control)) or 10 \(\mu\)M of 2-amino-2-norbornane-carboxylic acid, MeAIB, or Cys for 1 h. Cells were harvested, and the activity of p70\(^{\text{S6K}}\) was measured as described above. Results show one representative experiment out of three.

Amino Acid Alcohols Inhibit p70\(^{\text{S6K}}\) Activity—The mechanism by which the cell recognizes the lack of an amino acid seems reasonably well understood in bacteria and yeast, and basically it is through increased concentration of intracellular deacylated tRNA or, in some cases, through reduced availability of aminoacylated tRNA (see under “Discussion”). In order to investigate the involvement of the tRNA aminoacylation in the regulation of p70\(^{\text{S6K}}\) in mammalian cells, we initially utilized amino acid alcohols. The alcohol derivatives of amino acids inhibit their corresponding tRNA synthetases and thus prevent aminoacyl-tRNA formation (33, 34). For example, L-histidinol inhibits L-His binding to tRNA\(^{\text{His}}\) synthetase and thereby increases deacylated tRNA\(^{\text{His}}\). Various amino acid alcohols were added to exponentially growing Jurkat cells. As shown in Fig. 6A, addition of 2 \(\mu\)M of either leucinol, phenylalaninol, alaninol, histidinol, tyrosinol, or methioninol (all L-type) inhibited p70\(^{\text{S6K}}\) activity to various extents in 30 min. Addition of D-leucinol, in contrast, did not inhibit p70\(^{\text{S6K}}\) activity. It should also be noted that addition of \(\alpha\)-amino acids, including D-Leu and D-Gln, did not increase p70\(^{\text{S6K}}\) activity in total amino acid-starved cells, whereas addition of L-Leu or L-Gln did increase p70\(^{\text{S6K}}\) activity partially (data not shown). The dose response, time effect, and specificity of amino acid alcohols on p70\(^{\text{S6K}}\) was examined using histidinol, one of the most effective amino acid alcohols in the inhibition of p70\(^{\text{S6K}}\). Fig. 6B demonstrates the dose response of histidinol in the inhibition of p70\(^{\text{S6K}}\). Histidinol (10 \(\mu\)M) inhibited p70\(^{\text{S6K}}\) activity within 30 min and activity remained low for 24 h (Fig. 6C). The time course was compatible with that of p70\(^{\text{S6K}}\) activity upon total amino acid deprivation (Fig. 1A). For comparison, the time course of His deprivation on p70\(^{\text{S6K}}\) is shown in Fig. 6C as well. His deprivation decreased p70\(^{\text{S6K}}\) activity to an extent similar to histidinol within 30 min, but there was a rebound of the kinase activity within 1–6 h after deprivation of the amino acid. This transient rebound was also observed when lower concentrations of histidinol (0.5 or 2 \(\mu\)M) were added to the culture. The effects of histidinol on p70\(^{\text{S6K}}\) activity was considered to be specific and not due to its toxic effects on cells, because it did not affect p90\(^{\text{S6K}}\), Akt, or ck2 within 6 h (Fig. 6D). Additionally, we confirmed that the effect of histidinol was not mediated through inhibition of His transport (Fig. 6E). Uptake of histidine within 60 min after addition of radiolabeled His was not affected by the presence of 10 \(\mu\)M histidinol. These data support the idea that blockade of tRNA aminoacylation may inhibit p70\(^{\text{S6K}}\).

Effects of Inhibitors of Peptide Elongation—Cycloheximide is an inhibitor of peptidyl transferase, and puromycin interferes peptide elongation by accepting the nascent peptide chain from the peptidyl tRNA molecule on the ribosome (35). Because these drugs block the utilization of aminoacylated tRNA by interfering with peptide elongation, their action is expected to cause a shift in the aminoacylation equilibrium (36). The ratio of deacylated tRNAs decreases by addition of cycloheximide or puromycin in regular cell culture conditions (37). Additionally, the increase in deacylated tRNAs is thought to be minimized by the drugs when amino acid supply is limited (38). Exponentially growing Jurkat cells were transferred to amino acid-depleted medium in the presence or absence of cycloheximide (1 \(\mu\)M) or puromycin (100 \(\mu\)g/ml) (Fig. 7). A decrease in amino acid concentration to \(1/4\) did not change p70\(^{\text{S6K}}\) activity significantly, and a decrease to \(1/16\) reduced p70\(^{\text{S6K}}\) activity by about one-half within 1 h. Both cycloheximide and puromycin enhanced p70\(^{\text{S6K}}\) activity when cells were transferred to the medium containing \(1/4\) and \(1/16\) concentrations of amino acids. Inhibition of p70\(^{\text{S6K}}\) activity by decrease in amino acid concentration to \(1/16\) was completely blocked by the drugs. These data support the idea that increase in deacylated tRNA or decrease in aminoacylated tRNA is a factor suppressing p70\(^{\text{S6K}}\).

Study Using a Temperature-sensitive Mutant of tRNA Synthetase—There have been several reports describing temperature-sensitive mutants of mammalian cells that contain mutations in the tRNA synthetases. Here, we utilized tsBN250 cells that were derived from a golden hamster kidney BHK21 cell line (39). tsBN250 has a point mutation at the histidyl-tRNA synthetase gene, which likely alters normal protein folding (39). The activity of the mutated tRNA synthetase is very low or undetectable and cannot support cell growth unless excess amounts of His are supplemented at nonpermissive temperature (39 °C) (39). Using these lines, we examined the correlation of tRNA synthetase activity in the cells with p70\(^{\text{S6K}}\) activity. BHK21 and tsBN250 cells were maintained at permissible 33 °C. Cells were transferred to 39 °C incubator and cell numbers and p70\(^{\text{S6K}}\) activity were monitored. As shown in Fig. 8A, growth of tsBN250 cells was arrested in nonpermissive temperature, and it was partially recovered by addition of high concentrations of His, but not of Lys, to the culture medium. Transfer to nonpermissive temperature decreased p70\(^{\text{S6K}}\) in tsBN250 cells within 2–6 h after transferring cells to 39 °C as seen in a His deprivation experiment (Fig. 6C). The inhibition of p70\(^{\text{S6K}}\) in tsBN250 cells was partially recovered by addition of high concentrations of His, the corresponding amino acid for the mutated tRNA synthetase, but not by Lys (Fig. 8C). In contrast to p70\(^{\text{S6K}}\), activity of p90\(^{\text{S6K}}\) in tsBN250 cells was not altered by the changing temperature or by addition of extra amino acids. These data suggest that inhibition of tRNA\(^{\text{His}}\) aminoacylation led to the suppression of p70\(^{\text{S6K}}\) activity.

DISCUSSION

Amino acid deprivation inactivated and amino acid supplementation activated p70\(^{\text{S6K}}\), indicating that the kinase activity is dependent on amino acid concentration in cell culture media. The activities of other serine/threonine kinases were less af-
fected by amino acid concentration, and moreover, none of these kinases demonstrated the pattern for amino acid dependence that was seen in p70s6k. Amino acid concentration affected phosphorylation of not only p70s6k but also 4E-BP1, which is another translation regulatory molecule controlled by mTOR. Additionally, p70s6k activation and 4E-BP1 phosphorylation induced by amino acid addition was inhibited by rapamycin, a specific inhibitor of mTOR. In contrast, a concentration of wortmannin (100 nM) that almost completely inhibits PI3K activity had only a minor effect on the amino acid-induced p70s6k. mTOR activity is sensitive to only higher concentrations of wortmannin (IC50, 200 nM), and a partial inhibition of p70s6k by wortmannin (100 nM) may be explained by a partial inhibition of mTOR by the drug. Moreover, amino acid supplementation induced p70s6k in the presence of rapamycin in cells constitutively expressing a rapamycin-resistant mutant of mTOR (S2035I-mTOR). These data indicate that amino acid-dependent regulation of p70s6k requires mTOR but not PI3K. Our data here support the conclusion by Hara et al. (27) that amino acids regulate p70s6k through mTOR itself or an mTOR-controlled downstream element, such as a protein phosphatase.
stream element is affected by amino acids, amino acids should regulate this molecule independent of the mTOR-regulatory site because amino acid deprivation decreased S2035I-mTOR-dependent p70^65k activity.

The next question we attempted to answer is how amino acids affect the mTOR/p70^65k pathway. The results obtained using amino acid alcohols, inhibitors of mRNA translation elongation, and temperature-sensitive mutants of tRNA synthetase indicated involvement of tRNA aminoacylation in amino acid-dependent control of p70^65k. All of the inhibitors of neutral amino acid transporters partially suppressed amino acid-induced p70^65k activity, which does not conflict with the idea that intracellular amino acid concentration regulates the pathway through tRNA aminoacylation. Indeed, mechanisms by which unicellular organisms recognize and respond to amino acid starvation is all through the aminoacylation status of tRNA. One aspect of the response to amino acid starvation of *Escherichia* *coli* is the accumulation of a regulatory nucleotide, guanosine 3'-diphosphate, 5'-diphosphate (ppGpp), which inhibits several reactions, including transcription of rRNA (“stringent” control). The synthesis of ppGpp is induced by deacylated tRNA (42). Additionally, “attenuation” in amino acid biosynthetic operons is controlled by availability of aminoacyl tRNA (43). The best characterized mechanism by which eukaryotes cope with amino acid starvation is the amino acid-dependent control of yeast GCN2 in *S. cerevisiae* (44, 45). This serine/threonine kinase has a 530-amino acid sequence related to histidyl-tRNA synthetase at its carboxy terminal (46). Studies using an RNA binding assay have shown that deacylated tRNA can bind to this domain of GCN2 (47). Upon amino acid starvation, surplus deacylated tRNAs are thought to bind to GCN2 and activate GCN2 protein kinase activity (48). Although the carboxy terminal of GCN2 has homology to histidyl-tRNA synthetase, it seems that deacylated forms of other tRNAs in addition to histidyl-tRNA can activate GCN2 as well. It seems that there has been sufficient genetic drift in the histidyl-tRNA synthetase domain of GCN2 that it now lacks the ability to discriminate between the binding of different tRNAs (45). mTOR or p70^65k does not have any sequence homology with tRNA synthetases, implying that tRNA may not interact with mTOR or p70^65k directly. Indeed, addition of a yeast tRNA mixture to immunoprecipitated mTOR and p70^65k does not suppress activity of the kinases to phosphorylate 4E-BP1 and S6, respectively, in vitro (data not shown). If amino acid-dependent control of p70^65k is through mTOR, tRNA may interact with an unidentified upstream regulator of mTOR. Withdrawal of most individual amino acids inhibited p70^65k in Chinese hamster ovary cells, although with differing potency (27). In our system, a variety of L-amino acid alcohols was effective to inhibit p70^65k. These data suggest that most deacylated tRNAs would be sufficient to suppress p70^65k with differing potency, as they were demonstrated to activate GCN2 in yeast. One candidate for an upstream regulator of mTOR would be a mammalian homologue of GCN2, although it has not been cloned or identified yet to date. Histidinol was among the most effective amino acid alcohols in the suppression of p70^65k, which may support the idea that the upstream regulatory molecule of mTOR contains a domain homologous to histidyl-tRNA synthetase, as does yeast GCN2.

A rebound of p70^65k activity was detected during deprivation of His or by addition of lower doses of histidinol. Similar results were obtained by deprivation of other individual amino acids or by addition of other amino acid alcohols, although to various extents (data not shown). Additionally, a rebound of p70^65k activity was also observed when tsBN250 cells were transferred to nonpermissive temperature. These effects might be
explained by proteolytic responses induced by amino acid deprivation. Because TOR was demonstrated to be involved in autophagic responses in yeast (41), inhibition of mTOR by amino acid deprivation might induce a proteolytic response in mammalian cells, which in turn increases intracellular amino acid concentrations and subsequently reactivates the mTOR/p70<sub>66</sub>k pathway.

In contrast to intensive studies on signaling mechanisms regulated by growth factors, our understanding of intracellular signal transduction controlled by nutrients such as amino acids is very limited, especially in mammalian cells. p70<sub>66</sub>k (and potentially its upstream kinase mTOR) has been shown to be a protein kinase that is tightly controlled by amino acid concentration. Moreover, the study reported here demonstrates for the first time the involvement of tRNA aminoacylation for the regulation. Our data give us a basis for study directions to reveal an entire signaling mechanism for amino acid-dependence control of mRNA translation (and potentially protein degradation as well) in mammalian cells.

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REFERENCES

1. Reinhard, C., Thomas, G., and Kozma, S. C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4052–4057
2. Susa, M., Olivier, A. R., Fabbro, D., and Thomas, G. (1989) Cell 57, 817–824
3. Terada, N., Franklin, R. A., Lucas, J. J., Blenis, J., and Gelfand, E. W. (1993) J. Biol. Chem. 268, 12062–12068
4. Bandi, H. R., Ferrari, S., Krieg, J., Meyer, H. E., and Thomas, G. (1993) J. Biol. Chem. 268, 4530–4533
5. Kawasome, H., Papst, P., Webb, S., Keller, G. M., Johnson, G. L., Gelfand, E. W., and Terada, N. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5033–5038
6. Jefferies, H. B. J., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B., and Thomas, G. (1997) EMBO J. 16, 3703–3704
7. von Manteuffel, S. R., Dennis, P. B., Pullen, N., Gingras, A.-C., Sonenben, N., and Thomas, G. (1997) Mol. Cell. Biol. 17, 5426–5436
8. Preed, C. G. (1992) Curr. Top. Cell Regul. 32, 243–369
9. Terada, N., Lucas, J. J., Szepesi, A., Franklin, R. A., Takase, K., and Gelfand, E. W. (1992) 186, 1315–1321
10. Cheatham, B., Vlahos, C. L., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1984) 14, 4902–4911
11. Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994) Nature 370, 71–75
12. Weng, Q.-P., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1993) Science 279, 707–710
13. Abraham, R. T., and Wiederrecht, G. J. (1996) Annu. Rev. Immunol. 14, 483–510
14. Brown, R. J., and Schreiber, S. L. (1996) Cell 86, 517–520
15. Brown, R. J., Beal, F. A., Keith, C. T., Chen, J., Shiu, T. B., and Schreiber, S. L. (1995) Nature 377, 441–445
16. Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H., and Sabatini, D. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1432–1437
17. Lin, T.-A., Kong, X., Salsiui, A. R., Blackshear, P. J., and Lawrence, J. C., Jr. (1995) J. Biol. Chem. 270, 18531–18538
18. Beretta, L., Gingras, A.-C., Svitkin, Y. V., Hall, M. N., and Sonenberg, N. (1995) EMBO J. 15, 658–664
19. von Manteuffel, S. R., Gingras, A.-C., Ming, X.-F., Sonenben, N., and Thomas, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4976–4980
20. Hara, K., Yonezawa, K., Kozlowski, M. T., Sugimoto, T., Andjelkovic, K., Weng, Q.-P., Kasuga, M., Nishimoto, I., and Avruch, J. (1997) J. Biol. Chem. 272, 26457–26463
21. Brunn, G. J., Hudson, C. C., Sekulic, A., Williams, J. M., Hosoi, H., Houghton, P. J., Lawrence, J. C., Jr., and Abraham, R. T. (1997) Science 277, 99–101
22. Blommaart, E. F. C., Luiken, J. J. F. P., Blommaart, P. J. E., van Woerkom, G. M., and Meijer, A. J. (1995) J. Biol. Chem. 270, 2320–2326
23. Fox, H. L., Kimball, S. R., Jefferson, L. S., and Lynne, C. J. (1998) Am. J. Physiol. 274, C206–213
24. Hara, K., Yonezawa, K., Weng, Q.-P., Kozlowski, M. T., Belham, C., and Avruch, J. (1998) J. Biol. Chem. 273, 14484–14494
25. Douglas, E. C., Valentine, M., Etubanana, E., Parham, B. L., Weber, P. J., Houghton, P. J., Houghton, J. A., and Green, A. A. (1987) Cytogenet. Cell. Genet. 45, 145–155
26. Kumakura, T., Takase, T., Terada, N., and Gelfand, E. W. (1995) Life Sci. 57, 75–81
27. Banerjee, P., Ahmad, M. F., Grove, J. R., Kozlosky, C., Price, D. J., and Avruch, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8550–8554
28. Sugiyama, H., Papst, F., Fujita, M., Gelfand, E. W., and Terada, N. (1997) Oncogene 15, 443–452
29. Christensen, H. N. (1990) Physiol. Rev. 70, 43–77
30. Hansen, B. S., Vaughan, M. H., and Wang, L. (1972) J. Biol. Chem. 247, 3854–3857
31. Kilberg, M. S., Hutson, R. G., and Laine, R. O. (1994) FASEB J. 8, 13–19
32. Petstka, S. (1971) Annu. Rev. Microbiol. 25, 487–562
33. Thompson, L. H., Lofgren, D. J., and Adair, G. M. (1978) Somat. Cell Genet. 4, 423–435
34. Hamilton, T. A., and Litt, M. (1976) Biochim. Biophys. Acta 435, 362–375
35. Fukushima, K., Motomura, S., Kuraoka, A., Nakano, H., and Nishimoto, T. (1996) Genes Cells. 1, 1087–1099
36. Motomura, S., Fukushima, K., Nishibani, H., Nawata, H., and Nishimoto, T. (1996) Genes Cells. 1, 1101–1112
37. Barbet, N. C., Jeffrey, U., Helliwell, S. B., Stansfield, I., Tuite, M. F., and Barbet, A. (1996) Mol. Biol. Cell 7, 25–42
38. Noda, T., and Ohsumi, Y. (1998) J. Biol. Chem. 273, 3963–3966
39. Haseltine, W. A., and Block, R. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1564–1568
40. Kolter, R., and Yanofsky, C. (1982) Annu. Rev. Genet. 16, 113–134
41. Hinnebusch, A. G. (1988) Microbiol. Rev. 52, 248–273
42. Wek, R. C. (1994) Trends Biochem. Sci. 19, 491–496
43. Wek, R. C., Jackson, B. M., and Hinnebusch, A. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4579–4583
44. Wek, S. A., Zhu, S., and Wek, R. C. (1995) Mol. Cell. Biol. 15, 4497–4506
45. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992) Cell 68, 585–596