Deficiency of dietary EAA preferentially inhibits mRNA translation of ribosomal proteins in liver of meal-fed rats

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Received 6 November 2000; accepted in final form 21 March 2001

Anthony, Tracy G., Ali K. Reiter, Joshua C. Anthony, Scot R. Kimball, and Leonard S. Jefferson. Deficiency of dietary EAA preferentially inhibits mRNA translation of ribosomal proteins in liver of meal-fed rats. Am J Physiol Endocrinol Metab 281: E430–E439, 2001.—The goal of these studies was to investigate the mechanisms by which amino acid supply regulates global rates of protein synthesis as well as the translation of ribosomal protein (rp) mRNAs in liver. In the experiments conducted, male weanling rats were trained over a 2-wk period to consume their daily food intake within 3 h. On day 14, rats were fed the control diet or an isocaloric, isonitrogenous diet lacking glycine, tryptophan, leucine, or the branched-chain amino acids (BCAA) for 1 h. Feeding Trp-, Leu-, or BCAA-deficient diets resulted in significant reductions in serum insulin, hepatic protein synthesis, eukaryotic initiation factor 2B (eIF2B) activity, and phosphorylation of eIF4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (S6K1). Phosphorylation of eIF2α was inversely related to eIF2B activity under all conditions. Alterations in the hepatic synthesis of rp were assessed by changes in the distribution of rp (S4, S8, L26) mRNAs across sucrose density gradients and compared with non-rp (β-actin, albumin) mRNAs. In all dietary treatments, non-rp mRNAs were mostly polysome associated. Conversely, the proportion of rp mRNAs residing in polysomes was two- to fivefold less in rats fed diets lacking tryptophan, leucine, or BCAA compared with rats fed the control diet. Total hepatic abundance of all mRNAs examined did not differ among treatment groups. For all parameters examined, there were no differences between rats fed the glycine-deficient diet and rats fed the control diet. The data suggest that essential amino acid (EAA) deficiency inhibits global rates of liver protein synthesis via a block in translation initiation. Additionally, the translation of rp mRNAs is preferentially repressed in association with decreased S6K1 phosphorylation.

amino acid deficiency; messenger ribonucleic acid translation; ribosomal protein S6 kinase; essential amino acids

ESSENTIAL AMINO ACID DEFICIENCY and/or imbalance depresses growth in animals. Among the processes involved in the growth response, protein synthesis is a key component. Feeding a diet lacking an essential amino acid results in disaggregation of liver polyribo-

somes and repression of liver protein synthesis in rats and pigs (9, 38, 48). These effects have been attributed to a decrease in caloric intake, a widely known phenomenon that accompanies the consumption of a diet lacking an essential amino acid (13). However, studies controlling for food intake by force-feeding the deficient diet demonstrate that increases in energy do not compensate for the inhibitory effects of amino acid deficiency on polysome profiles and protein synthesis (42, 47). Therefore, the mechanism(s) responsible for the inhibition of liver protein synthesis caused by consuming a diet deficient in an essential amino acid are still largely unstudied.

Previously, we showed that in both isolated hepatocytes and perfused liver, removal of total amino acids or single essential amino acids from culture medium or perfusate inhibits liver protein synthesis and polysome aggregation (14, 15, 23). These effects are accompanied by an inhibition of 43S initiation complex formation, implying a block in the initiation step of translation. The mechanism responsible for these effects is proposed to include an increase in phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2) (15). When phosphorylated, eIF2 bound to GDP possesses heightened affinity for the guanine nucleotide exchange factor eIF2B, sequestering it into an inactive eIF2-GDP-eIF2B complex. This causes stalling of guanine nucleotide exchange and, hence, decreased ternary initiation complex formation. Although this mechanism is reported in both cell culture and perfusion systems, it has yet to be demonstrated as a result of an amino acid deficiency in vivo.

Among the products of protein synthesis, the translational components themselves, most notably the ribosomal proteins (rp), are principal players in promoting growth and development (33). The rp are members of a special class of mRNAs that contain an oligopyrimidine tract (TOP) in the 5′-untranslated region of the transcript. This arrangement of pyrimidines adjacent to the cap site serves as the cis-regulatory element in selectively controlling their translational efficiency (2). Specific regulation via the 5′TOP serves the purpose of...
altering protein synthetic capacity (ribosome biogenesis) in accord or discord with global rates of protein synthesis, depending on the physiological needs of the cell.

One of the principal intracellular signaling molecules involved in the regulation of growth is the mammalian target of rapamycin (mTOR) protein kinase. Activation of mTOR by mitogens or growth factors results in enhanced phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and the 70-kDa rp S6 kinase (S6K1) in parallel (19, 36). Hyperphosphorylation of 4E-BP1 results in enhanced assembly of the mRNA cap-binding protein complex eIF4F, facilitating the translation of capped mRNAs. Concomitantly, hyperphosphorylation of S6K1 culminates in increased phosphorylation of the 40S rp S6. Phosphorylation of rp S6 by S6K1 is thought to enhance the affinity of the ribosome for binding TOP mRNAs, resulting in their selective translation (33). This effect is specific for TOP mRNAs, because treatment of cells with rapamycin, an inhibitor of S6K1 phosphorylation, selectively suppresses S6 phosphorylation and translation of TOP mRNAs (22).

Recently, we showed that oral administration of the branched-chain amino acid (BCAA) leucine selectively enhances the translation of rp mRNAs in association with increased activation of the S6K1 signaling pathway in the liver (4). However, it is unclear to what extent amino acid deficiency affects this signaling pathway and the subsequent translation of this special class of mRNAs in vivo. The purpose of this study was to investigate the mechanism through which essential amino acid deficiency results in the inhibition of liver protein synthesis in vivo. Particular emphasis was placed on the regulation of rp mRNA translation by amino acid supply and the role of S6K1 in mediating preferential translation of these TOP mRNAs in vivo.

EXPERIMENTAL PROCEDURES

Animals, experimental design, and sample collection. The animal facilities and protocol were reviewed and approved by the Institutional Animal Care and Use Committee of The Pennsylvania State College of Medicine. Weanling (day 21) male Sprague-Dawley rats were maintained on a reverse 12:12-h light-dark cycle, with water provided freely. The day after arrival, all rats were subjected to a feeding regimen that allowed them free access to rat chow (Harlan-Teklad Rodent Chow, Madison, WI) for 3 h each day at the beginning of the dark cycle. All animals were meal-trained over 10 days to consume their daily requirement within this time period. Food intake and body weight increased steadily throughout this period. On day 11, all animals were switched to a pelleted control diet (39). On day 13, animals were randomly assigned to either the control diet (+AA) or one of four isocaloric, isonitrogenous experimental diets: lacking glycine (-Gly), tryptophan (-Trp), leucine (-Leu), or the BCAA leucine, isoleucine, and valine (-BCAA) (Table 1). The nitrogen content of diets lacking one or more essential amino acids was compensated for by use of a mixture of alanine, aspartate, glutamate, and glycine. The nitrogen content of diets lacking glycine was compensated for by use of a mixture of alanine, aspartate, and glutamate. Food intake from each

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<th>Table 1. Composition of experimental diets</th>
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<td>l-Alanine</td>
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<td>Vitamin Mix AIN-93</td>
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<tr>
<td>Choline bitartrate</td>
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Diet composition is expressed in g/kg. AA, amino acids; BCAA, branched-chain AA.
Experimental diet on day 13 was recorded and used to determine the amount of food provided to all animals on the next day. On the day of the experiment (day 14), rats were provided their respective experimental diet for 1 h. The amount of food provided was equal to the diet consumed in the lowest amount the previous day (4 g per rat). All rats were allowed free access to water during the experimental meal.

Exactly 1 h after commencement of meal feeding, animals were killed by decapitation. Trunk blood was collected and centrifuged at 1,800 g for 10 min to obtain serum. The whole liver was excised, blotted, weighed, and divided into four parts. One portion of liver was weighed and homogenized in 3 volumes of buffer A, consisting of (in mM) 20 HEPES (pH 7.4), 100 KCl, and 5 magnesium chloride. The buffer A homogenate was centrifuged at 3,000 g for 15 min (4°C) for subsequent polysome profile analysis, as will be described. A second portion of liver was homogenized in 7 volumes of buffer B, consisting of (in mM) 20 HEPES (pH 7.4), 100 KCl, 0.2 EDTA, 2 EGTA, 50 NaF, 50 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 benzamidine, and 0.5 sodium vanadate. The supernatant was used for the measurement of liver protein synthesis, as will be described. The remaining buffer B homogenate was immediately centrifuged at 10,000 g for 10 min at 4°C. The resulting supernatant was used for measurement of Akt/protein kinase B, S6K1, and 4E-BP1 phosphorylation, as will be described. A third portion of liver was homogenized in 5 volumes of buffer C, consisting of (in mM) 45 HEPES (pH 7.4), 0.375 magnesium acetate, 0.075 EDTA, 95 potassium acetate, 2.03 digitonin, 10% glycerol, and 3 μM microcystin. Buffer C homogenates were centrifuged at 12,000 g for 10 min (4°C). Resulting supernatants were immediately assayed for the guanine nucleotide exchange activity of eIF2B, as will be described. The remaining portion of liver was stored in RNA later RNA Stabilization Solution (Ambion) for the subsequent isolation of total RNA. All serum and tissue samples were stored at -70°C.

Polysome profiles. One volume of detergent (10% Triton X-100, 10% sodium deoxycholate) was mixed with nine volumes of buffer A supernatant and layered over 10–70% linear sucrose gradients. The gradients were centrifuged at 26,000 rpm for 210 min at 4°C in a Beckman SW28 rotor. After centrifugation, the gradients were fractionated on an Lsco gradient fractionator. The ultraviolet (UV) absorption at 254 nm was continuously recorded. Five-milliliter fractions were collected for subsequent extraction of total RNA.

Serum insulin. Serum insulin concentrations were measured using a commercial RIA kit for rat insulin (Linco Research, St. Charles, MO).

Measurement of skeletal muscle protein synthesis. A metabolic tracer consisting of a flooding dose (1.0 ml/100 g body wt) of 1-[2,3,4,5,6-3H] phenylalanine (150 mM containing 3.70 GBq/mol) was injected via the tail vein 50 min after the commencement of meal feeding for the measurement of synthesis of total mixed proteins in liver (17). The elapsed time from injection of the metabolic tracer until homogenization of liver was recorded as the actual time for incorporation of the radiolabeled amino acid into protein. Fractional rates of liver protein synthesis were estimated from the rate of incorporation of radioactive phenylalanine into liver protein using the specific radioactivity of serum phenylalanine as representative of the precursor pool (29).

Measurement of eIF2B activity. The guanine nucleotide exchange factor (GEF) activity of eIF2B in liver homogenates was measured by the exchange of [3H]GDP bound to eIF2 for nonradioactively labeled GDP, as described previously (24). Briefly, 35 μl of preformed eIF2-[3H]GDP complex were combined with 20 μl of liver homogenate, 103 μl of water, and 140 μl of Assay Buffer (consisting of in mM: 52.1 MOPS, pH 7.4, 104.2 KCl, 1.04 dithiothreitol, 2.08 magnesium acetate, and 250 μg/ml bovine serum albumin) and incubated at 30°C. At four time points (0, 1, 2, and 3 min), a 60-μl aliquot was removed and added to 2.5 ml of ice-cold Wash Buffer (50 mM MOPS, pH 7.4, 100 mM KCl, and 1 mM dithiothreitol). The contents were filtered through a nitrocellulose filter disc under vacuum, and the filter was rinsed twice with 2.5 ml of ice-cold Wash Buffer. The amount of eIF2-[3H]GDP complex bound to the filters was determined by scintillation counting, and the resulting values over time were plotted to produce a slope measurement representing the rate of guanine nucleotide exchange.

Quantitation of eIF2α phosphorylation. Phosphorylation of eIF2α was assessed by protein immunoblot analysis by use of an antibody that recognizes eIF2α only when it is phosphorylated on Ser51 (26). Results were normalized for total eIF2α with an antibody that recognizes the protein irrespective of phosphorylation state.

Phosphorylation state of 4E-BP1. Phosphorylation of the 70-kDa rp S6K1 was assessed by protein immunoblot analysis, as previously described (18). Additionally, phosphorylation of S6K1 at Thr389, a site whose phosphorylation is associated with maximal activation of the kinase, was assessed by protein immunoblot analysis by use of an anti-phospho-S6K1 (Thr389) antibody (Cell Signaling Technology, Beverly, MA).

Examination of 4E-BP1 phosphorylation state. An aliquot of the 10,000 g supernatant (buffer B) was boiled for 10 min and then centrifuged at 10,000 g for 30 min at 4°C. The resulting supernatant was mixed with an equal volume of SDS sample buffer and then subjected to protein immunoblot analysis, as described previously (27). Total RNA extraction from whole liver and sucrose gradients. Total RNA from frozen liver samples was isolated by the method of Chomczynski (7) using Trizol (Molecular Research Center, Cincinnati, OH). Sucrose fractions were linearized with EcoRI to produce 250, 212, and 185 bp fragments.

Preparation of RNA probes. The full-length cDNAs for rp S4, S8, and L26 were kindly provided by Dr. Ira Wool (Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL). The plasmid pUC8 was digested with PstI to produce 250, 212, and 185 bp fragments for rpL26, rpS4, and rpS8, respectively. The cDNA for albumin, initially in the pBR322 vector (25), was digested with EcoRV, followed by AccI to produce a 280-bp albumin fragment. All four fragments were subsequently cloned into the pBluescript II SK+ vector (Stratagene) and transformed into bacteria K101 One-Shot Cells, Invitrogen). Colonies were isolated, and DNA was purified (Wizard Plus Mini and Maxi-preps DNA Purification System, Promega). Plasmid stocks of pBluescript-rlpL26, pBluescript-rpS4, pBluescript-rpS8, and pBluescript-Alb were then linearized with EcoRI, BamHI, HindIII, and AccI, respectively, to produce DNA template stocks of each probe for in vitro transcription. A linearized β-actin DNA template (126 bp) was purchased from Ambion. One microgram of each DNA template was mixed with [α-32P]UTP (800 Ci/mmol, 20 μCi/ml; Amersham) and limiting (0.1 mM) nonradioactively labeled UTP in a 20-μl transcription reaction according to the manufacturer’s instructions (MAXiScript In Vitro Transcription
Kits, Ambion) to produce single-stranded RNA probes. The resulting reactions were treated with DNase I, heat denatured, and gel purified by loading onto a 5% acrylamide/8 M urea mini-slab gel. After electrophoresis, the full-length probes were located by short-term (20-s) exposure of the gel to X-ray film. The full-length RNA probes were subsequently excised from the gel with a clean blade and incubated overnight in probe elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.2% SDS; RPA III, Ambion). The gel was briefly reexposed to X-ray film after excision to ensure that the correct bands were isolated.

ribonuclease protection assay. Five micrograms of total RNA isolated from either whole liver or sucrose density gradient fractions were coprecipitated with rpl26, rps4, rps8, and β-actin RNA probes in a single tube. Albumin mRNA expression was analyzed in a separate ribonuclease protection assay. Samples were heat denatured and incubated overnight in a 42°C water bath in Hybridization Buffer (RPA III, Ambion). Samples were digested with RNaseA/T1 on the following day, and the protected double-stranded mRNA fragments were ethanol precipitated, heat denatured, and loaded onto 5% acrylamide/8 M urea slab gels. After electrophoresis, gels were wrapped in plastic and exposed to X-ray film for up to 4 h at −80°C.

statistical analysis. All data were analyzed by the STATISTICA statistical software package for the Macintosh, volume II (StatSoft, Tulsa, OK). Data were analyzed using a one-way ANOVA to assess main effects, with treatment group (meal condition) as the independent variable. When a significant overall effect was detected, differences among group (meal condition) as the independent variable. When a significant overall effect was detected, differences among individual means were assessed with Duncan’s Multiple Range post hoc test. The level of significance was set at \( P < 0.05 \) for all statistical tests.

RESULTS

All rats were adapted to the meal training protocol used in this study for the purpose of encouraging similar food intake over a defined period of time. This approach is considered more physiological and less stressful to the animal than force-feeding by oral gavage (41, 48). All rats adjusted within a few days to the feeding schedule and gained weight steadily throughout the remainder of the meal-training period. It is well recognized that animals consuming diets lacking an essential amino acid voluntarily reduce their food intake (13). In an effort to control for this, all animals on the day of the experiment were provided an amount of food that was equal to the lowest intake of the previous day (4 g). Nevertheless, animals consuming diets lacking one or more essential amino acids consumed on average ~75% as much as rats consuming the control or glycine-deficient diets. However, all rats consumed 30–50% of their daily intake of the control diet over the 1-h period, and consumption of 25% of daily intake is adequate to produce a significant stimulation in liver protein synthesis 1 h postprandially (50).

Serum insulin in rats fed the glycine-deficient diet was similar to that of controls (Fig. 1A). Conversely, serum insulin concentrations in rats fed the −Trp, −Leu, and −BCAA diets were 11–22% of the values observed in rats fed the control diet (Fig. 1A). Liver protein synthesis rates in rats fed the glycine-deficient diet were also equivalent to those of controls (Fig. 1B).

In contrast, protein synthesis rates in rats fed the −Trp, −Leu, and −BCAA diets were significantly lower and 50–60% of those observed in rats fed the control diet.

The GEF activity of eIF2B in liver homogenates from rats consuming diets lacking one or more essential amino acids was reduced to 75% of controls (Fig. 2A). On the other hand, feeding a meal lacking glycine did not alter eIF2B activity compared with controls. The phosphorylation state of eIF2α was inversely related to eIF2B activity in all treatment groups. In the liver of rats fed the control and glycine-deficient diets, no phosphorylation of eIF2α at Ser51 was detected (Fig. 2B). In contrast, rats fed diets lacking one or more essential amino acids demonstrated increased phosphorylation of eIF2α at Ser51 compared with controls.

Phosphorylation of 4E-BP1 was estimated by comparing the electrophoretic migration patterns of the protein among treatment groups. 4E-BP1 resolves into three bands on SDS gels, with the slowest migrating species (γ-form) representing the most highly phosphorylated form. In rats fed the control and glycine-
deficient diets, a large proportion (~60%) of the protein was observed in the γ-form (Fig. 3, A and B). Conversely, only a small percentage (~10–15%) of the protein was observed in the γ-form in rats fed diets lacking one or more essential amino acids. Only a very small proportion of the protein existed in the α-form in all treatment groups.

To investigate the relationship between S6K1 signaling and the specific translation of rp mRNAs after amino acid deprivation, we first examined the electrophoretic migration pattern of S6K1 on SDS polyacrylamide gels. Similar to 4E-BP1, S6K1 was found mostly in hyperphosphorylated forms in rats fed the control or glycine-deficient diets (Fig. 4A). In contrast, in rats fed a diet lacking one or more essential amino acids, S6K1 was hypophosphorylated. To confirm that the phosphorylation pattern seen corresponded to a potential change in activity, we examined the phosphorylation of S6K1 at Thr389, a site that, when phosphorylated, is associated with maximal activation of the protein. Phosphorylation at this site was significant in the liver of rats fed either the control diet or the diet lacking glycine (Fig. 4B). In contrast, rats fed diets lacking tryptophan, leucine, or all three BCAAs demonstrated little or no Thr389 phosphorylation (Fig. 4B). These results suggest that essential amino acid deficiency downregulates S6K1 signaling in the liver.

To examine relative changes in the translation of specific mRNAs, we isolated total RNA from liver extracts fractionated on 10–70% linear sucrose density gradients.
The absorbance profiles obtained during gradient fractionation were used to designate the ribosomal contents of the fractions collected. On the basis of polysome profiles obtained (Fig. 5), the first two fractions collected were designated “subpolysomal.” These fractions contained protein, RNA, free ribosomal subunits, and monosomes. The remaining two fractions contained disomes, trisomes, and polysomes of increasing number bound to (and therefore, actively translating) mRNA, and so were designated “polysomal.” The distribution of three TOP mRNAs (rp L26, rpS4, and rpS8) and two non-TOP messages (albumin and β-actin) among the fractions was examined. This established approach provides a relative measure of how actively each message is being translated under the experimental conditions (1, 6, 45). The relative abundance of all transcripts in whole liver was also examined. There were no differences in either TOP or non-TOP mRNA expression among treatment groups in whole liver (Fig. 6).

In all treatment groups, the β-actin gene was present only in the polysome fractions (Fig. 5 and Table 2). The albumin gene was also mostly polysome associated (62–74%) in all treatment groups (Fig. 5 and Table 2). Feeding a meal lacking one or more essential amino acids resulted in a slight shift of the albumin gene to lighter polysomes, but redistribution to subpolysome fractions was not significant (P < 0.09). In contrast, rp mRNAs demonstrated significant movement between the subpolysome and polysome fractions, depending on nutritional state. After consumption of the control or glycine-deficient diet, the majority of rp mRNAs resided in the polysome fractions (Fig. 5 and Table 2). Feeding diets lacking one or more essential amino acids caused significant redistribution of rp mRNAs to the subpolysome fractions (Fig. 5 and Table 2). The results suggest that the translation of mRNAs that encode the ribosomal proteins are more profoundly affected in liver by amino acid supply than messages that do not contain a TOP sequence.
The liver is acutely responsive to amino acid supply, rapidly altering its protein content in response to protein consumption and periods of protein or food deprivation. Studies performed over 30 years ago established the profound influence that dietary essential amino acid deprivation and/or imbalance has on plasma and tissue concentrations of the limiting amino acid. These studies revealed reduced intracellular levels of the amino acid deficient in the diet (8, 21). This led to the hypothesis that lack of substrate causes stalling of mRNA translation via a decrease in tRNA-charging levels. In vitro studies performed to examine this idea determined that a 35% decline in the tRNA-charging level of an essential amino acid would produce only a 10% inhibition of protein synthesis (41). In comparison, meal-feeding an isoleucine-imbalanced diet resulted in only a 25% decline in the tRNA-charging level in liver extracts (41). On the basis of this report, diminished tRNA charging alone appears to be insufficient to account for the reduction in hepatic protein synthesis observed in the current study. However, it is possible that the concentration of the limiting amino acid incorporated into tRNA after the feeding of an essential amino acid-deficient diet may be lower than what was estimated in the study by Shenoy and Rogers (41). This is reasonable to consider, on the basis of findings by Stoll et al. (44). In that study, the utilization of amino acids from portal blood, arterial blood, and via proteolysis for hepatic protein synthesis was determined in piglets fed a high-protein diet containing U-13C-labeled algal protein. It was determined that essential amino acids from the diet are preferentially channeled for use as substrate in the stimulation of hepatic protein synthesis (44). When these results are extended to the current study, it is tempting to suggest that, in the fed state, hepatic tRNAs may be preferentially charged with amino acids of portal origin. However, there is no direct evidence to support a connection between metabolic compartmentation of amino acids and a decline in tRNA charging in vivo, and so this remains speculation.

Studies in eukaryotic cells report changes in tRNA charging to be a viable mechanism in the regulation of protein synthesis. In yeast, deacylated tRNA is shown to activate the eIF2α protein kinase GCN2 (12). Activation of this kinase in response to amino acid starvation results in increased phosphorylation of eIF2α on Ser51 and a shutdown of protein synthesis (10). In the current study, protein synthesis rates were inversely related to the phosphorylation of eIF2α, similar in fashion to previous studies in hepatocytes and perfused liver after amino acid deprivation (14, 15, 24). Recently, a murine homologue of GCN2 (mGCN2) was characterized by two independent laboratories (5, 43). The identification of this mammalian homologue raises the possibility that increased eIF2α phosphorylation in the current study may relate to the activity of mGCN2, because eIF2α is refractory to phosphorylation by amino acid deprivation in GCN2−/− cells in culture (20). However, increased phosphorylation of eIF2α after perfusion of liver with histidinol (a competitive inhibitor of histidinyl-tRNA synthetase) was associated with a decrease in eIF2α phosphatase activity, rather than an increase in eIF2α kinase activity (24). Therefore, a mechanism involving increased eIF2α phosphorylation via increased mGCN2 kinase activity remains controversial.

An amino acid receptor-mediated mechanism directing the changes in liver protein synthesis by amino acid imbalance/deprivation is an alternate possibility. A study from Miotto et al. (34) suggests the presence of a leucine recognition site at the cell surface that serves to regulate hepatic macroautophagy and proteolysis. In further support of this idea, diminished tRNA charging alone appears to be insufficient to account for the reduction in hepatic protein synthesis observed in the current study. However, it is possible that the concentration of the limiting amino acid incorporated into tRNA after the feeding of an essential amino acid-deficient diet may be lower than what was estimated in the study by Shenoy and Rogers (41). This is reasonable to consider, on the basis of findings by Stoll et al. (44). In that study, the utilization of amino acids from portal blood, arterial blood, and via proteolysis for hepatic protein synthesis was determined in piglets fed a high-protein diet containing U-13C-labeled algal protein. It was determined that essential amino acids from the diet are preferentially channeled for use as substrate in the stimulation of hepatic protein synthesis (44). When these results are extended to the current study, it is tempting to suggest that, in the fed state, hepatic tRNAs may be preferentially charged with amino acids of portal origin. However, there is no direct evidence to support a connection between metabolic compartmentation of amino acids and a decline in tRNA charging in vivo, and so this remains speculation.

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S. cerevisae, there is a plasma membrane protein complex called Ssy1p-Ptr3p-Ssy5p that functions to sense the presence of amino acids in the extracellular environment (16). Although this complex exhibits no amino acid transport activity, it is required for transcriptional induction of several genes that encode amino acid permeases and peptide transporters (11). Ssy1p signals through a component of an ubiquitin-protein ligase complex and is required for degradation of some G1 cyclins (30). These findings implicate Ssy1p-Ptr3p-Ssy5p in the regulation of protein degradation and possibly couple increases in leucine availability to gene expression and cell cycle regulation. However, a role for Ssy1p in regulating protein synthesis remains to be determined. Furthermore, it is unknown whether a mammalian homologue of Ssy1p exists. Hence, current evidence does not support a role for a cell surface amino acid-binding protein in the regulation of hepatic protein synthesis.

A surprising finding in the current study was the reduction in plasma insulin concentration after feeding a diet deficient in one or more essential amino acids. To the authors' knowledge, the effect on plasma insulin of feeding a diet deficient in an essential amino acid has not been previously reported in rats. However, feeding pigs a diet limiting in tryptophan has been shown to reduce postprandial plasma insulin at 1 h compared with feeding a tryptophan-adequate diet (9). The mechanism for this effect is unclear, but tryptophan in the diet of pigs is suggested to play an indirect role in insulin secretion either via stimulation of an insulinotropic gastrointestinal peptide or by accelerating the rate of gastric emptying (40). The reduction in plasma insulin after consumption of diets lacking leucine (−Leu and −BCAA) in the present study may be related to leucine functioning as a recognized insulin secretagogue (32). Alternatively, the reduction in food intake in rats consuming diets lacking one or more essential amino acids may have shifted the insulin peak to earlier than 1 h or resulted in a smaller increase in plasma insulin. Whatever the reason, the current results would appear to support a role for insulin deficiency in the observed changes in protein synthesis and rp mRNA translation after dietary essential amino acid restriction. However, whereas it initially appears that changes in insulin could alone account for the changes observed in protein synthesis and translation initiation, several lines of evidence argue against this notion. Our laboratory (3, 18, 50) and others (49) have shown that feeding 18-h food-deprived rats isocaloric meals containing or lacking amino acids increases plasma insulin similarly over time. However, only meals containing amino acids stimulated protein synthesis, enhanced eIF4F assembly, and increased the hyperphosphorylation of 4E-BP1 and S6K1. Studies in hepatocytes report that amino acids alone activate intermediates important in the initiation of protein synthesis (37). Furthermore, removal of singular or all essential amino acids from culture medium attenuates insulin-stimulated S6K1 activity (19). These studies collectively demonstrate that activation of hepatic protein synthesis and translation initiation in response to food intake is not dependent on an increase in plasma insulin. Although published evidence does not support a primary role for insulin in the present study, we cannot rule out a partial role for insulin on the basis of available data.

Recently, we showed that recruitment of rp mRNAs into polysomes is associated with activation of the S6K1 signaling pathway but discordant with global rates of hepatic protein biosynthesis after oral administration of leucine (4). In that study, feeding leucine was sufficient to encourage rp mRNA translation in association with increased phosphorylation of S6K1 and rpS6. That investigation was the first to directly test the effects of amino acid availability on rp mRNA translation in vivo. In the present study, we were interested in extending our previous findings by examining the effect of removing singular amino acids from the diet on S6K1 signaling and rp mRNA translation. The results of this study suggest that dietary restriction of one or more essential amino acids inhibits the translation of hepatic rp mRNAs concomitant with attenuated S6K1 signaling.

Although our experimental protocol also produced a decline in global rates of protein synthesis, the repression of rp mRNA translation was observed to be greater than that of non-rp mRNAs. This is likely due to the presence of a TOP sequence at the 5′ end of the rp transcripts. Disruption of the 5′TOP sequence prevents the inhibitory effects of rapamycin (an inhibitor of S6K1 phosphorylation) on rp mRNA translation in NIH 3T3 cells (22), highlighting the importance of this regulatory element in the selective control of this class of genes. Selective modulation of these transcripts, in accord or discord with global rates of protein synthesis, may serve the purpose of temporally regulating ribosome biogenesis under conditions of altered growth rate. Furthermore, the present results emphasize the fact that global rates of protein synthesis do not necessarily reflect the translation of all mRNAs in the cell to the same extent.

Whereas a correlation between S6K1 activation and the specific upregulation of TOP mRNA translation is reported in vitro (22) and in vivo (4), this is not the case for 4E-BP1 phosphorylation. Studies using S6K1 mutants resistant to rapamycin demonstrate the macro-lide to repress 4E-BP1 phosphorylation despite continued S6K1 activation and rp mRNA translation (22, 46). In addition, treatment of L6 myoblasts with rapamycin represses 4E-BP1 phosphorylation but not global rates of protein synthesis (28), an observation that questions the role of 4E-BP1 in the global regulation of capped mRNAs. It has been proposed that changes in eIF4E availability via 4E-BP1 phosphorylation modulate the synthesis of a family of mRNAs that possess significant secondary structure in their 5′-untranslated regions, such as ornithine decarboxylase (28, 31). Although the translation of mRNAs with highly structured 5′-untranslated regions may have been selectively regulated under the current experimental conditions, examples of this class of mRNAs were not examined in the
current study. Further investigation into the effect of amino acid deprivation on the translation of mRNAs with highly structured 5′-untranslated regions is warranted.

In summary, feeding diets deficient in one or more essential amino acids produces reductions in global rates of protein synthesis as well as the selective down-regulation of mRNA translation. These effects were associated with changes in translation initiation and S6K1 signaling. Although the mechanism leading to changes in eIF2B activity requires further investigation, the relationship between S6K1 signaling and the preferential translation of rp mRNAs was confirmed.

We are grateful to Sharon Rannels and Lynne Hugendubler for technical assistance and to Dr. Thomas C. Vary for determining the specific radioactivity of serum phenylalanine.

This work was supported by N.I.H. Institutes of Health Grants DK-13499 (L. S. Jefferson), GM-39277 (T. C. Vary), and GM-08619 (J. C. Anthony). T. G. Anthony is also supported by an American Diabetes Association Postdoctoral Fellowship (L. S. Jefferson).

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