Preservation of Liver Protein Synthesis during Dietary Leucine Deprivation Occurs at the Expense of Skeletal Muscle Mass in Mice Deleted for eIF2 Kinase GCN2*

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In eukaryotic cells, amino acid depletion reduces translation by a mechanism involving phosphorylation of eukaryotic initiation factor-2 (eIF2). Herein we describe that mice lacking the eIF2 kinase, general control nonderepressible 2 (GCN2) fail to alter the phosphorylation of this initiation factor in liver, and are moribund in response to dietary leucine restriction. Wild-type (GCN2+/+) and two strains of GCN2 null (GCN2−/−) mice were provided a nutritionally complete diet or a diet devoid of leucine or glycine for 1 h or 6 days. In wild-type mice, dietary leucine restriction resulted in loss of body weight and liver mass, yet mice remained healthy. In contrast, a significant proportion of GCN2−/− mice died within 6 days of the leucine-deficient diet. Protein synthesis in wild-type livers was decreased concomitantly with increased phosphorylation of eIF2 and decreased phosphorylation of 4E-BP1 and S6K1, translation regulators controlled nutritionally by mammalian target of rapamycin. Whereas translation in the liver was decreased independent of GCN2 activity in mice fed a leucine-free diet for 1 h, protein synthesis in GCN2−/− mice at day 6 was enhanced to levels measured in mice fed the complete diet. Interestingly, in addition to a block in eIF2 phosphorylation, phosphorylation of 4E-BP1 and S6K1 was not decreased in GCN2−/− mice deprived of leucine for 6 days. This suggests that GCN2 kinase activity can also contribute to nutritional regulation of the mammalian target of rapamycin pathway. As a result of the absence of these translation inhibitory signals, liver weights were preserved and instead, skeletal muscle mass was reduced in GCN2−/− mice fed a leucine-free diet. This study indicates that loss of GCN2 eIF2 kinase activity shifts the normal maintenance of protein mass away from skeletal muscle to provide substrate for continued hepatic translation.

Mammals require an adequate supply of dietary essential amino acids (i.e. those that cannot be synthesized de novo) to grow and thrive. Deficiency in one or more essential amino acids in the diet of rodents results in anorexia and loss of body protein (1, 2). Loss of body proteins in growing animals is due in large part to a depression in protein synthesis at the initiation stage of mRNA translation (3, 4).

Translation initiation is an intricate process coordinated by a family of protein factors called eukaryotic initiation factors (eIFs) (for review, see Ref. 5). eIF2 plays a central role in regulating global translation rates via phosphorylation of serine 51 on its α subunit (6). Under diverse conditions of cell stress, phosphorylation of eIF2α inhibits guanine nucleotide exchange on eIF2 and restrains mRNA translation. At the same time, the phosphorylation of eIF2α serves to enhance gene-specific translation important for regulating the expression of genes that manage cellular insults (7–9). A family of four known protein kinases have been described to phosphorylate eIF2α in response to different cell stressors (6, 7, 10–12). Coined the “integrated stress response” (13), this process places eIF2α phosphorylation as the decisive event in regulating the ability of the cell to cope with environmental stress.

One of the eIF2α kinases, termed general control nonderepressible 2 (GCN2), is activated under conditions of nutrient deprivation or UV irradiation (14–16). The role of GCN2 in response to amino acid starvation has been characterized extensively in yeast (10, 17, 18). In yeast, starvation for any single amino acid results in intracellular accumulation of uncharged tRNAs, which binds to GCN2 on a domain homologous to histidyl-tRNA synthetases. This binding event triggers a conformational change in GCN2, enhancing phosphorylation of eIF2α. Whereas eIF2α phosphorylation serves to slow global rates of translation, at the same time it enhances translation of the transcriptional activator, GCN4. Increased levels of GCN4 leads to induction of genes that encode amino acid biosynthetic enzymes and other related metabolic proteins in an attempt to correct for the nutritional deficiency. Whereas GCN4 is not present in mammalian systems, several transcription factors have been shown to be induced in response to phosphorylation of eIF2α by amino acid starvation in mammalian cells, including members of the activating transcription factor and CCAAT/enhancer-binding protein (C/EBP) family of transcriptional transactivators (8, 9).

Another signaling event altered in response to nutrients involves the mammalian target of rapamycin (mTOR) protein.
kinase, a downstream effector of the phosphatidylinositol 3-kinase/Akt (protein kinase B) signaling pathway. Signaling downstream of mTOR is implicated in many aspects of cell growth including cell cycle control and ribosome biogenesis. mTOR activates both the ribosomal p70 S6 kinase (S6K1) and the mRNA cap-binding protein inhibitory protein, 4E-BP1, and its pharmacological inhibition causes G1 phase cell cycle arrest (19, 20). Recently, there have been reports of potential cross-talk between GCN2 and the TOR signaling pathway in yeast (19, 20). Specifically, rapamycin releases TOR-directed phosphorylation of yeast GCN2, contributing to enhanced eIF2α kinase activity (21, 22). Although this finding has not yet been extended to mammals, it suggests that events downstream of mTOR, namely the phosphorylation of 4E-BP1 and S6K1, may be coordinated with eIF2α phosphorylation via GCN2. Previous work by one of the authors (4) demonstrates that dietary leucine deprivation results in both the increased phosphorylation of eIF2α and the concomitant reduced phosphorylation of 4E-BP1 and S6K1 in rat liver.

The mammalian form of GCN2 was identified several years ago (23, 24), and soon thereafter a GCN2 knockout mouse was developed and partially described (25). Its phenotype was initially unremarkable, reportedly growing and reproducing normally under freely fed conditions. However, pregnant GCN2−/− dams fed a diet devoid of leucine bore fewer viable pups as compared with pregnant GCN2+/+ dams, suggesting that GCN2 is important for managing nutritional stress during embryogenesis. Currently, there are no studies addressing the role of GCN2 in managing postnatal stress in these mice. Thus, the focus of this investigation was to characterize the growth response of GCN2−/− mice to dietary amino acid restriction, and to determine whether GCN2 is important for coping with nutritional stress postnatally. We were also interested in understanding if GCN2 is the primary kinase involved in catalyzing the phosphorylation of eIF2α in response to amino acid deprivation in vivo, or if other eIF2α kinase family members also serve a role in sensing amino acid deprivation. Finally, we wished to examine whether dysregulation of eIF2α phosphorylation would impact signaling downstream of mTOR.

EXPERIMENTAL PROCEDURES

Animals and Diets—The following study protocol was approved by the Institutional Care and Use Committee at the Indiana University School of Medicine, Evansville Center for Medical Education. Mice were obtained by in-house breeding or from Jackson Laboratories (Bar Harbor, ME). Four to 6-week-old male C57BL/6J (GCN2−/−) and GCN2−/− mice (either on a heterozygous hybrid background of C57BL/6J and TL1 129SvEvTac, as reported in (25) and designated GCN2−/−BC), or back-crossed onto the C57BL/6J background 8 generations (GCN2−/−BC) were maintained on a 12-h light/dark cycle and provided free access to commercial rodent chow (PMI International, Brentwood, MO) and tap water prior to the experiment. At the start of the feeding experiment, mice were acclimated to a nutritionally complete, control diet for a minimum of 3 days, and then randomly assigned to one of four dietary treatments as follows: AA, continued free access to the nutritionally complete, control diet; LEU, free access to a diet that was devoid of the essential amino acid leucine; GLY, free access to a diet that was devoid of the non-essential amino acid glycine; PF, restricted access to the AA diet that equaled the intake of mice freely consuming the LEU diet as previously detailed (4). Rodents, when fed a diet deficient in an essential amino acid, will reduce their intake (26, 27). Consequently, the PF group was used to compare the effects of amino acid versus calorie deprivation on signaling events regulating protein synthesis. PF mice received their daily food ration as one meal at the beginning of the light cycle. All diets were isocaloric and compositionally the same in terms of carbohydrate and lipid components as described previously (4). The LEU and GLY diets were isonitrogenous with the AA diet, with alanine, glutamate, and aspartate compensating for the missing amino acid. Animals in each group were offered their diets at the beginning of the light cycle following an overnight fast and euthanized at 1 h and 6 days (144 h) following commencement of feeding. Food intake and body weight was recorded daily and body and tissue wet weights (liver and skeletal muscle) were recorded at each point of euthanasia. Animals were killed by decapitation and trunk blood was collected for the determination of serum amino acids and insulin concentrations.

Serum Measurements—Serum was obtained by centrifugation of clotted blood and then snap-frozen and stored at −20 °C. Serum samples were sent to the Indiana University School of Medicine Quantitative Amino Acid Core Facility (under the direction of Edward Liechty) for the determination of amino acid profiles by the ninhydrin method, using standard ion exchange chromatography with a Beckman 6300 automated amino acid analyzer. Serum insulin was measured using a commercial radioimmunoassay kit (Linco, Inc., St. Louis, MO).

Protein Synthesis—Ten minutes before euthanasia, all mice were injected i.p. with a bolus solution of [3H]phenylalanine per kg of body weight for the measurement of tissue protein synthesis. Each tissue sample was processed to determine the enrichment of labeled phenylalanine in liver protein as previously described (28). Tissue 1-[3H]phenylalanine enrichments were measured in Stony Brook by monitoring the ions at m/z 336 and 341 of the tertiary butyldimethylsilyl derivative on a model MD800 GC-MS (Fisons Instruments) operated under electron impact (29). The intraperotonal route of injection to ensure constant precursor enrichment has been previously validated (30). Additionally, time course studies performed using the intraperotonal route of injection demonstrated constant enrichment of the free phenylalanine within the liver and linear incorporation of injected tracer into tissue protein for at least 20 min (data not shown).

Tissue Preparation for Immunoblot Analysis—Tissues were homogenized as previously described (4) using a Dounce glass homogenizer in 7 volumes of buffer consisting of (in mm) 20 HEPES (pH 7.4), 100 KCl, 0.2 EDTA, 2 EGTA, 1 dithiothretiol, 50 NaF, 50 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 benzamidine, and 0.5 sodium vanadate. The homogenates were immediately centrifuged at 10,000 × g for 10 min at 4 °C for analysis of the translation initiation factor phosphorylation state as described below.

Phosphorylation of eIF2α—Phosphorylation of eIF2α was measured as described (4) using an antibody that recognizes the protein only when it is phosphorylated at serine 51 (Cell Signaling Technology, Inc., Beverly, MA). Results were normalized for total eIF2α with an antibody that recognizes the protein irrespective of phosphorylation state (Santa Cruz Biotechnology, Santa Cruz, CA).

Phosphorylation of 4E-BP1—Phosphorylation of 4E-BP1 was measured as a change in mobility during SDS-polyacrylamide gel electrophoresis as detected by immunoblot analysis as described previously (4). Briefly, an aliquot of the 10,000 × g supernatant was boiled for 10 min and centrifuged at 10,000 × g for 30 min at 4 °C. The resultant supernatant was added to 1 volume of SDS sample buffer and then subjected to protein immunoblot analysis using a polyclonal 4E-BP1 antibody (Bethyl Laboratories, Montgomery, TX).

Phosphorylation of S6K1—Phosphorylation of S6K1 was measured as a decrease in mobility during SDS-polyacrylamide gel electrophoresis as described previously (4). Briefly, an aliquot of the 10,000 × g supernatant was added to 1 volume of SDS sample buffer. Immunoblot analysis was then performed using a polyclonal S6K1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistics—All data were analyzed by the STATISTICA statistical software package for the Macintosh, volume II (StatSoft, Tulsa, OK). Data were analyzed using two-way ANOVA to assess main effects, with mouse strain and diet treatment as the independent variables. When a significant overall effect was detected, differences among treatment groups were assessed with Duncan’s Multiple Range post-hoc test. The level of significance was set at p < 0.05 for all statistical tests. Data are reported as mean ± S.E.

RESULTS

GCN2−/−BC Mice Are More Fragile Than GCN2−/−/− or GCN2−/− Mice—A prior characterization of the GCN2 null mouse indicated that these mice grow normally with complete nutrition (25). GCN2−/− mice used in this earlier study were of a heterozygous hybrid background, a genetic mixture of the C57BL/6J and TL1 129 SvevTac strains (designated in this study as GCN2−/−/−). It is recognized that the genetic background of engineered mice can influence physiological response to challenge (31); therefore, GCN2−/−/− mice were backcrossed into the C57BL/6J lineage for 8 generations (designated in this study as GCN2−/−/−BC) to produce a mouse that is 99.6% genetically identical to a C57BL/6J mouse (designated in this study
as GCN2<sup>−/−</sup> mice. Average litter size of the GCN2<sup>−/−</sup> strain was greater than GCN2<sup>−/−<sub>BC</sub></sup> or GCN2<sup>+/+<sub>BC</sub></sup> (6.73 ± 0.26 versus 5.65 ± 0.29 versus 5.46 ± 0.45 pups per litter, respectively, p < 0.05). Although similar in initial litter size, GCN2<sup>−/−<sub>BC</sub></sup> mice were decidedly more fragile than GCN2<sup>+/+</sub> mice, as a subset of most litters died shortly (1–2 days) after birth. GCN2<sup>−/−<sub>HH</sub></sup> mice could be weaned at day 21, but weaning had to be delayed until day 28 with GCN2<sup>−/−<sub>BC</sub></sup> mice to prevent additional mortality, particularly when housed singly or on wire-bottom cages. By contrast, GCN2<sup>+/+</sub> and GCN2<sup>−/−<sub>HH</sub></sup> mice adapted to these environmental conditions upon weaning without incident. Growth curves from birth until day 28 of age demonstrated that GCN2<sup>−/−<sub>HH</sub></sup> mice grew larger than GCN2<sup>−/−<sub>BC</sub></sup> or GCN2<sup>+/+<sub>BC</sub></sup> mice (p < 0.05 at days 12–28; Fig. 1). GCN2<sup>−/−</sub> and GCN2<sup>−/−<sub>BC</sub></sup> mice grew similarly during the suckling period until day 27 when the body weights of GCN2<sup>−/−<sub>BC</sub></sup> mice began to plateau as compared with GCN2<sup>+/+</sub> mice, ending up statistically the smallest in size at day 28.

**GCN2<sup>−/−</sup> Mice Consume Less Food, Lose More Body Weight, and Have Increased Mortality When Fed a Leucine-devoid Diet**—Intake of a leucine-free diet caused the expected reduction in food intake in all strains (two-way ANOVA p < 0.05 main effect of −LEU on reducing intake) (Fig. 3B). However, GCN2<sup>−/−<sub>BC</sub></sup> mice consumed less food than GCN2<sup>−/−<sub>HH</sub></sup> mice independent of dietary treatment (two-way ANOVA p < 0.05 for change in food consumption). Intake of the leucine-devoid diet in both GCN2<sup>−/−<sub>HH</sub></sup> strains was less than that in GCN2<sup>−/−<sub>BC</sub></sup> mice (~30% in GCN2<sup>−/−<sub>BC</sub></sup> versus ~34% in GCN2<sup>−/−<sub>HH</sub></sup> versus ~22% in GCN2<sup>+/+</sub> compared with respective AA intake). Consumption of the −GLY diet was similar to intake of the AA diet in each respective strain, whereas the PF intake successfully matched the intake of the −LEU diet.

Although GCN2<sup>−/−<sub>HH</sub></sup> mice grew better than GCN2<sup>−/−<sub>BC</sub></sup> mice, they also lost more body weight on a leucine-devoid diet. GCN2<sup>−/−<sub>HH</sub></sup> mice fed −LEU lost 23% of their starting body weight by day 6, whereas GCN2<sup>−/−<sub>BC</sub></sup> mice lost 14.3% (Fig. 3A). GCN2<sup>−/−<sub>HH</sub></sup> mice further became scrawny and lethargic in appearance by day 6 (Fig. 2), yet none died. In contrast, GCN2<sup>−/−<sub>BC</sub></sup> mice fed −LEU experienced a substantial rate of death before the end of the experiment (Table I). Forty percent (8 of 20) of the initial starting number of GCN2<sup>−/−<sub>BC</sub></sup> mice assigned to a leucine-devoid diet would die within 3–5 days. GCN2<sup>−/−<sub>BC</sub></sup> mice that remained viable throughout the 6-day experimental period also appeared sickly and experienced loss of body weight (~19.4%) that was slightly greater than GCN2<sup>−/−<sub>BC</sub></sup> mice fed the −LEU diet, and statistically intermediate between the GCN2<sup>−/−<sub>HH</sub></sup> and GCN2<sup>−/−<sub>BC</sub></sup> strains. Mice fed a glycin-devoid diet generally gained weight throughout the study period, although GCN2<sup>−/−<sub>BC</sub></sup> mice gained less weight than GCN2<sup>−/−<sub>HH</sub></sup>. This is likely because of reduced intake of the −GLY diet by GCN2<sup>−/−<sub>BC</sub></sup> mice (Fig. 3B). All PF mice lost weight because of reduced caloric intake equal to GCN2<sup>−/−<sub>BC</sub></sup> mice fed a leucine-devoid diet. Thus, leucine deficiency in GCN2<sup>−/−<sub>BC</sub></sup> mice resulted in greater weight loss than that because of caloric restriction alone. A small number of GCN2<sup>−/−<sub>BC</sub></sup> mice fed the other diets also died before the end of the study period (Table I). Closer examination of the expired GCN2<sup>−/−<sub>BC</sub></sup> AA, −GLY, and PF mice revealed their starting body weights to be smaller than the average group weights (below 12 versus 15 g average). Therefore, similar to younger GCN2<sup>−/−<sub>BC</sub></sup> mice, their demise may be related to an inability to adapt to being housed singly in a wire-bottom cage, as noted above.

**GCN2<sup>−/−<sub>BC</sub></sup> Mice Experience Sparing of Liver Mass during Dietary Leucine Starvation—**Leucine deprivation reduced wet weight of liver in all strains. However, when expressed relative to body weight, only GCN2<sup>−/−<sub>BC</sub></sup> mice fed on the leucine-devoid diet experienced significant loss of liver relative to body mass (~20% in −LEU, p < 0.05 as compared with AA; Fig. 3C). In contrast, both GCN2<sup>−/−</sub> strains were resistant to losing a significant percentage of their liver mass. Interestingly, food restriction (PF group) significantly reduced liver mass in both GCN2<sup>−/−<sub>BC</sub></sup> (~15%) and GCN2<sup>−/−<sub>BC</sub></sup> (~22%) mice. A glycin-free diet did not alter liver mass in any strain compared with the AA diet.

**Dietary Leucine Starvation Reduces Skeletal Muscle Mass in GCN2<sup>−/−</sup> Mice**—The wet weights of hind limb muscle (gastrocnemius plus plantaris) were reduced in all mice in response to caloric restriction (PF) or leucine deprivation. However, when expressed as a ratio to body weight, skeletal muscle from GCN2<sup>−/−</sup> mice generally remained in proportion to body weight irrespective of dietary treatment (Fig. 3D). By contrast, GCN2<sup>−/−<sub>HH</sub></sup> and GCN2<sup>−/−<sub>BC</sub></sup> mice demonstrated proportionately greater loss of muscle mass by feeding −LEU (~20.5% and −17.5%, respectively, p < 0.05) and GCN2<sup>−/−<sub>BC</sub></sup> strain.
demonstrated marked loss by PF (−16.5%, p < 0.05). Thus, loss of functional GCN2 resulted in greater loss of skeletal muscle in response to nutrient deprivation.

**Amino Acid Profiles Suggest GCN2−/− Mice Fed −LEU or PF for 6 Days Are More Catabolic Than GCN2+/+ Mice—** Serum concentrations of the 20 essential and non-essential amino acids were measured at 1 h and 6 days following consumption of experimental diets (Tables II and III). Data in the two GCN2 null strains were pooled, because values were similar. At 1 h, serum leucine concentrations were lower and serum isoleucine and valine concentrations were higher, in all mice fed −LEU (main effect of leucine-free diet by two-way ANOVA, p < 0.05) (Table I). In all mice fed −GLY, serum concentrations of glycine and serine were lower, irrespective of mouse strain (main effect of glycine-free diet by two-way ANOVA, p < 0.05). There were no changes in the concentrations of any other amino acids.

Six days later, serum concentrations of the same amino acids were measured again (Table III). Mice fed the −LEU diet continued to have reduced leucine concentrations as compared with mice fed the AA diet (two-way ANOVA main effect of −LEU diet, p < 0.05), but circulating levels of leucine in GCN2−/− mice fed −LEU were 42% lower than GCN2+/+ mice fed −LEU. In GCN2+/+ mice fed the −LEU diet, serum concentrations of isoleucine and valine were equal to GCN2−/− mice fed AA. Conversely, isoleucine and valine concentrations in GCN2−/− mice fed the −LEU were 2.7- and 2.1-fold higher, respectively, than GCN2−/− mice fed the AA diet. In addition, concentrations of isoleucine, valine, alanine, and serine in GCN2−/− mice fed −LEU were equal to PF mice. The PF mice, which were food-restricted −30% in daily energy, displayed elevated concentrations of leucine, isoleucine, valine, alanine, and asparagine at day 6 as compared with same strain using the AA diet. The PF amino acid profiles suggest increased catabolism of body proteins in response to acute food deprivation, consistent with an adopted “meal feeding” pattern of intake where most of the daily rations were consumed in the early hours following presentation of food. Furthermore, GCN2−/− PF mice displayed additional elevations in circulating concentrations of serine (+186%), lysine (+156%), threonine (+221%), and arginine (+173%) as compared with GCN2−/− mice fed AA, signifying further catabolism of body proteins. Mice fed the −GLY diet maintained marked to significant reductions in serum glycine and serine, but did not demonstrate changes in the concentration of any other amino acid.

Serum insulin was measured at both 1 h and 6 days following experimental diet intake, and again, data from the two GCN2 null strains were combined (Tables II and III). At both time points, mice fed −LEU demonstrated reduced circulating concentrations of insulin (two-way ANOVA main effect of a leucine-devoid diet, p < 0.05) likely due in part to reduced intake of food during these periods. There was no effect of mouse strain or other dietary treatments on serum insulin values.

**Reductions in Relative Protein Synthesis by Dietary Leucine Deprivation Are Maintained in GCN2−/− but Not GCN2+/− Mice—** Protein synthesis was measured as the enrichment of phenylalanine labeled with deuterium into liver protein, using the “flooding dose” method to saturate and equilibrate all tissue precursor pools of tracer amino acid. Data in the two GCN2 null strains were pooled, as values were similar. One hour following commencement of feeding, mice fed −LEU demonstrated significant reductions in labeling of liver protein that were similar between strains (GCN2+/+: −29% versus GCN2−/−: −33%; two-way ANOVA main effect of leucine-devoid diet, p < 0.05) (Fig. 4A). There was no effect of feeding −GLY to the labeling of liver protein in either strain. On day 6, there was a differential effect of dietary leucine deprivation between wild-type and null strains (Fig. 4B). Hepatic tracer enrichment remained suppressed in GCN2−/− mice fed −LEU as compared with GCN2+/+ AA (−29%, p < 0.05). In contrast, enrichment of...
phosphorylation of eIF2α and downstream signaling events in translation initiation in response to GCN2 and Dietary Amino Acid Deprivation.

**Table II**

| AA | GLY | PF |
|----|-----|----|
| Insulin (ng/ml) | 2.14 ± 0.26 | 1.71 ± 0.13 | 1.95 ± 0.18 |
| Leucine (μmol/liter) | 224.33 ± 10.35 | 68.82 ± 5.68 | 215.20 ± 17.38 |
| Isoleucine (μmol/liter) | 150.15 ± 4.75 | 222.76 ± 23.47 | 144.04 ± 15.64 |
| Valine (μmol/liter) | 585.33 ± 23.13 | 733.90 ± 86.16 | 500.58 ± 35.32 |
| Glycine (μmol/liter) | 1512.85 ± 142.69 | 1403.16 ± 125.25 | 350.54 ± 77.27 |
| Serine (μmol/liter) | 322.43 ± 10.65 | 326.64 ± 24.38 | 191.84 ± 11.09 |

* Main effect of –leucine diet (by two-way ANOVA), p < 0.05.
† Main effect of –glycine diet (by two-way ANOVA), p < 0.05. There was no significant effect of mouse strain on parameters shown.

**Table III**

| AA | LEU | GLY | PF |
|----|-----|-----|----|
| Inulin (mg/ml) | 0.56 + 0.01 | 0.10 ± 0.05 | 0.58 ± 0.07 | 0.50 ± 0.08 |
| Leucine (μmol/liter) | 133.55 ± 9.11 | 76.40 ± 6.26 | 140.43 ± 21.48 | 311.29 ± 53.20 |
| Isoleucine (μmol/liter) | 81.67 ± 7.34 | 89.06 ± 8.47 | 114.61 ± 15.61 | 237.32 ± 30.04 |
| Valine (μmol/liter) | 313.93 ± 18.21 | 325.64 ± 30.49 | 329.63 ± 45.53 | 726.90 ± 114.73 |
| Glycine (μmol/liter) | 1235.62 ± 76.18 | 838.64 ± 55.54 | 216.78 ± 40.23 | 1445.49 ± 30.90 |
| Serine (μmol/liter) | 276.10 ± 15.96 | 276.31 ± 28.00 | 171.06 ± 17.06 | 245.75 ± 10.43 |
| Alanine (μmol/liter) | 501.86 ± 43.91 | 561.74 ± 25.69 | 600.14 ± 81.07 | 1522.23 ± 96.85 |
| Lysine (μmol/liter) | 680.36 ± 39.93 | 668.74 ± 34.16 | 692.73 ± 78.21 | 636.09 ± 106.59 |
| Methionine (μmol/liter) | 140.54 ± 16.94 | 112.19 ± 6.81 | 128.65 ± 22.41 | 319.80 ± 36.13 |
| Threonine (μmol/liter) | 485.43 ± 40.25 | 467.10 ± 27.73 | 442.88 ± 71.82 | 630.50 ± 52.56 |
| Asparagine (μmol/liter) | 69.08 ± 7.42 | 55.60 ± 4.54 | 64.66 ± 7.58 | 125.68 ± 13.94 |
| Arginine (μmol/liter) | 154.10 ± 11.16 | 140.81 ± 7.92 | 172.49 ± 27.56 | 151.50 ± 7.71 |

For 4E-BP1, the γ-form is the most highly phosphorylated species, labeled as α, β, and γ (Figs. 6 and 7). Phosphorylation of S6K1 was calculated as a ratio of the density of the upper bands (β + γ forms) over the total density of all bands present. The primary and dominant eIF2α kinase that responds to nutrient deprivation.

To examine the possibility that eIF2α phosphorylation might alter other events in translation initiation, the phosphorylation states of 4E-BP1 and S6K1, two downstream effectors of mTOR that are involved in regulating the translation of growth-related mRNAs were also examined. Phosphorylation of both proteins was examined by migration following SDS-PAGE and immunoblot analysis. During electrophoresis, 4E-BP1 and S6K1 are resolved into multiple phosphorylated species, labeled as α, β, and γ (Figs. 6 and 7). For 4E-BP1, the γ-form is the most highly phosphorylated species, labeled as α, β, and γ (Figs. 6 and 7). Phosphorylation of S6K1 was calculated as a ratio of the density of the upper bands (β + γ forms) over the total density of all bands present. The primary and dominant eIF2α kinase that responds to nutrient deprivation.
tion in all mice. These results indicate that loss of functional GCN2 precludes maximal inhibition of mTOR signaling by amino acid deprivation.

**DISCUSSION**

Herein we report the following novel observations: 1) GCN2 is the dominant kinase involved in the phosphorylation of eIF2α in the liver in response to amino acid deprivation, because GCN2−/− mice do not display increased eIF2α phosphorylation in the liver following 1 h and 6 days of feeding a leucine-devoid diet; 2) GCN2 is important for viability under nutritional stress. GCN2−/− mice that consumed a leucine-devoid diet became scruffy in appearance and either lost significantly more weight than GCN2+/+ mice, or died before the end of the study period; 3) mice lacking functional GCN2 had reduced abilities to chronically down-regulate hepatic protein synthesis in response to leucine starvation over time, resulting in preservation of liver mass relative to body size; 4) the inability to adequately restrain protein synthesis in the liver results in increased loss of skeletal muscle to supply needed substrate (leucine); and 5) loss of GCN2 reduces dephosphorylation of 4E-BP1 and S6K1 in response to leucine deprivation, suggesting a role for this eIF2α kinase in the regulation of the mTOR-directed pathway. Together, these results demonstrate that GCN2 contributes to the regulation of protein balance and the coordination of translation initiation during dietary amino acid deprivation.

**GCN2 Is the Primary eIF2α Kinase Activated in Liver during Amino Acid Limitation**—GCN2 is one member in a family of eIF2α kinases. Previously, it was reported that while eIF2α phosphorylation was completely dependent on GCN2 in mouse embryonic fibroblasts subjected to leucine starvation for up to 6 h, extended periods of starvation induced one or more alternative eIF2α kinases (25). In the current study, compensatory phosphorylation of eIF2α did not occur (Fig. 5). The difference between our findings in vivo and those in cultured cells are most likely because of the fact that complete starvation of a single amino acid cannot be produced by dietary manipulation alone, because breakdown of body proteins can produce a continued supply of the deficient amino acid. Thus, it can be argued that amino acid starvation in cultured cells represents a more extreme situation that may not be encountered in animal tissues. A possible exception to this idea involves treatment of amino acid-depleting enzymes to treat cancer. During treatments using anti-cancer drugs such as asparaginase, the circulating concentration of the targeted amino acid is depleted to such a low level that the resulting response may be comparable with amino acid-free culture conditions (32). This concept remains to be fully investigated.

Whereas genetic diversity impacted the gross phenotype in

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2 R. L. Byerley, B. J. McDaniel, M. A. McNurlan, D. L. Durden, R. C. Wek, and T. G. Anthony, unpublished observations.
this study (Fig. 2 and Table I), common themes remained in both GCN2 null strains at the tissue, cellular, and subcellular levels. Directional changes in serum and tissues were generally consistent in both GCN2 null strains, even if the response was more marked in one null strain versus the other. And although higher rates of mortality only occurred in the GCN2 null BC strain, both null strains appeared generally unwell compared with GCN2+/+ mice fed the leucine-devoid diet. Nevertheless, this study substantiates previous reports indicating that the genetic background of a rodent can influence its phenotypic response to challenge (31).

Loss of GCN2 Triggers Death in Nutritionally Stressed Animals—The precise reason for the premature death of the GCN2 null BC mice remains ambiguous. It is noted that some GCN2 null mice completely stop eating within 1–3 days after presentation of the -LEU diet; thus, their declining health may be related to starvation. It is known that rodents develop a taste aversion to amino acid imbalanced diets, leading to a reduction in intake (1, 2). Work by Gietzen’s (26) group demonstrated that a specific area in the brain, the anterior piriform cortex, is reactive to amino acid imbalance, demonstrating increased phosphorylation of eIF2α and expression of c-Jun. Interestingly, GCN2 is abundantly expressed in brain (24). However, no study to date has identified GCN2 activation as being central to the regulation of food intake. Alternatively, the failing health of the GCN2 null BC mice fed -LEU may relate to altered glucoregulation in response to self-starvation. Kaufman and co-workers (33) created a mouse in which serine 51 on eIF2α was mutated to an alanine, preventing all phosphorylation at this site. This S51A eIF2α “knock-in” mouse was born viable but died within 18 h because of hypoglycemia associated with defective gluconeogenesis (33). Thus, the lack of eIF2α phosphorylation in the liver of nutritionally stressed GCN2 null mice may result in the altered ability to regulate blood glucose during these conditions. Preliminary studies of GCN2 null and GCN2 null lactating dams fed a leucine-free diet for 6 days demonstrated hypoglycemia in GCN2 null pups (65 ± 8 mg/dL) but not GCN2 null pups (101 ± 3 mg/dL).3 Further study addressing the role of GCN2 in glu-

3 T. G. Anthony, B. J. McDaniel, R. L. Byerley, B. C. McGrath, D. R. Cavener, M. A. McNurlan, and R. C. Wek, unpublished observations.
inhibition of protein synthesis at the level of translation initiation. Indeed, rats fed diets lacking one or more essential amino acids demonstrate hepatic protein synthesis to be lowered in association with reduced eIF2B activity (4), supporting the general idea that phosphorylation of eIF2α regulates global protein synthesis (39). Whereas our study shows that GCN2 is the major kinase that phosphorylates eIF2α during dietary leucine restriction, it also demonstrates that increased eIF2α phosphorylation is not required for immediate reduction of general translation (Figs. 4A and 5A). These results, which may appear contrary to contemporary descriptions in the literature, are in fact consistent with a previous report characterizing GCN2−/− mice (25). In the earlier study, in situ perfusion of histidinol induced eIF2α phosphorylation and restrained eIF2B activity in livers from GCN2−/− but not GCN2−/−/H mice (25). Nevertheless, liver protein synthesis was equally suppressed by histidinol in both strains of mice. Collectively, these data suggest that phosphorylation of eIF2α may function in conjunction with other regulators of translation, and emphasizes a significant role for eIF2α kinases in regulation of genespecific translation.

To determine whether regulatory mechanisms other than eIF2α phosphorylation are altered by loss of GCN2 function, we addressed whether signaling events downstream of mTOR were reduced in GCN2−/− mice fed LEU. Hyperphosphorylation of both 4E-BP1 and S6K1 are associated with the increased translation of specific classes of mRNAs important for growth (4, 40). In the current study, phosphorylation of both 4E-BP1 and S6K1 was reduced in GCN2−/− mice fed LEU at both 1 h and 6 days, supporting an inhibitory role for these translation regulators in global protein synthesis (Figs. 6 and 7). However, in GCN2−/− mice deprived of leucine, the phosphorylation of 4E-BP1 and S6K1 in liver was not decreased. These observations support the idea that GCN2 contributes to mTOR regulation of 4E-BP1 and S6K1 in response to amino acid depletion, but not to general caloric reduction because there was no difference in the phosphorylation levels of these translation regulators between PF fed GCN2−/− and GCN2−/− mice. Furthermore, increased phosphorylation of 4E-BP1 and S6K1 in the GCN2−/− mice deprived of leucine would not be conductive to dampened protein synthesis. Thus, it appears that signaling via mTOR does not exhibit major control of hepatic protein synthesis in the absence of the primary eIF2α kinase GCN2.

In the combined absence of changes in the phosphorylation of eIF2α, S6K1, and 4E-BP1 at 1 h, alternative or novel mechanisms may serve to initially repress hepatic protein synthesis in GCN2−/− mice during leucine deprivation. One possibility involves the phosphorylation of eIF2B on its ε subunit, associated with reductions in eIF2B guanine nucleotide exchange activity in muscle (41). However, decreased eIF2B activity does not appear to coincide with general reductions in protein synthesis in the liver of GCN2−/− mice (25). Another possibility is the suppression of elongation by means of eIF2 phosphorylation. Whereas the kinase that phosphorylates and inhibits eIF2 is regulated in part by a rapamycin-sensitive manner (19), the activity of eIF2 is also regulated by cellular energy levels via an mTOR-independent mechanism (42). It is reported that pharmacological activation of the AMP-activated protein kinase reduces general protein synthesis rates in the skeletal muscle of rodents (43), but it is unknown what effect AMPK activation has on liver protein synthesis. Further exploration in this area is warranted.

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REFERENCES

1. Beverly, J., Gietzen, D., and Rogers, Q. (1990) Am. J. Physiol. 258, R716–R723
2. Said, A., and Hegsted, D. (1970) J. Nutr. 100, 1363–1375
3. Pronczuk, A., Rogers, Q., and Munro, H. (1970) J. Nutr. 100, 1249–1258
4. Anthony, T., Reiter, A., Anthony, J., Kimball, S., and Jefferson, L. (2001) Am. J. Physiol. 281, E439–E459
5. Hershey, J., and Merrick, W. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J., and Mathews, M., eds) pp. 33–88, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
6. Dever, T. (2002) Cell 108, 545–556
7. Harding, H., and Ron, D. (2002) Diabetes 51, S455–S461
8. Harding, H., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) Mol. Cell 6, 1099–1108
9. Jiang, H.-Y., Wek, S., McGrath, B., Lu, D., Hai, T., Harding, H., Wang, X., Ron, D., Cavena, D., and Wek, R. (2004) Mol. Cell. Biol. 24, 1365–1377
10. Hinnebusch, A. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J., and Mathews, M., eds) pp. 185–244, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
11. Kaufman, R. (2000) in Translational Control of Gene Expression (Sonenberg, N., Mathews, M., and Hershey, J., eds) pp. 503–528, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
12. Chen, J.-J. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J., and Mathews, M., eds) pp. 529–546, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Harding, H., Zhang, Y., Zeng, H., Novoa, I., Lu, P., Calon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D., Bell, J., Hettmann, T., Leiden, J., and Ron, D. (2003) Mol. Cell 11, 619–633
14. Yang, R., Wek, S., and Wek, R. (2000) Mol. Cell. Biol. 20, 2706–2717
15. Wek, S. A., Zhu, S., and Wek, R. C. (1995) Mol. Cell. Biol. 15, 4497–4506
16. Deng, J., Harding, H., Raught, B., Gingras, A., Berlanga, J., Scheuner, D., Kaufman, R., Ron, D., and Sonenberg, N. (2002) Curr. Biol. 12, 1279–1286
17. Kimball, S., Anthony, T., Cavena, D., and Jefferson, L. (2004) in Topics in Current Genetics: Nutrient-induced Responses in Eukaryotic Cells (Winderickx, P., and Taylor, J., eds) Vol. 7, pp. 113–130, Springer-Verlag, Berlin
18. Wek, R., Staschke, K., and Narasimhan, J. (2004) in Topics in Current Genetics: Nutrient-induced Responses in Eukaryotic Cells (Winderickx, P., and Taylor, J., eds) Vol. 7, pp. 171–199, Springer-Verlag, Berlin
19. Proud, C. (2004) Biochem. Biophys. Res. Commun. 313, 429–436
20. Panwalkar, A., Verstoekse, S., and Giles, F. (2004) Cancer 100, 657–666
21. Kobata, H., Obata, T., Ota, K., Sasaki, T., and Ito, T. (2003) J. Biol. Chem. 278, 20457–20460
22. Cherkasova, V., and Hinnebusch, A. (2003) Genes Dev. 17,859–872
23. Berlangs, J., Santoyo, J., and De Haro, C. (1999) Eur. J. Biochem. 265, 754–762
24. Sood, R., Porter, A., Olsen, D., Cavener, D., and Wek, R. (2000) Genetics 154, 787–801
25. Zhang, P., McGrath, B., Reinert, J., Olsen, D., Lei, L., Gill, S., Wek, S., Vattem, K., Wek, R., Kimball, S., Jefferson, L., and Cavener, D. (2002) Mol. Cell. Biol. 22, 6681–6688
26. Gietzen, D., Ross, C., Has, S., and Sharp, J. (2004) J. Nutr. 134, 717–723
27. Harper, A., Benevenga, N., and Wohlhueter, R. (1970) Physiol. Rev. 50, 428–558
28. Caso, G., Ford, G., Nair, K., Garlick, P., and McNurlan, M. (2002) Proc S, 1–44
29. McNurlan, M., Garlick, P., Steigbigel, R., DeCristofaro, R., Frest, R., Lang, C., Johnson, R., Santasier, A., Cabahug, C., Furhier, J., and Gelato, M. (1997) Clin. Invest. 100, 2125–2132
30. Lundhagen, K., Ternell, M., Zachrisson, H., Moldaewer, L., and Lindstrom, L. (1991) Acta Physiol. Scand. 141, 207–219
31. Kahn, C. (2003) Exp. Diabesity Res. 4, 169–182
32. Woodward, C., Sur, P., Capizzi, R., and Modest, E. (1988) Biochem. Med. Metab. Biol. 30, 199–207
33. Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R. (2001) Mol. Cell 7, 1165–1176
34. Mortimore, G., and Khurana, K. (1990) Int. J. Biochem. 22, 1075–1080
35. Kobayashi, H., Borsheim, E., Anthony, T., Traber, D., Badalamenti, J., Kimball, S., Jefferson, L., and Wolfe, R. (2003) Am. J. Physiol. 284, E488–E498
36. Mortimore, G., and Poo, A. (1987) Am. Rev. Nutr. 7, 539–564
37. Talloccy, Z., Jiang, W., Virgin, H., Leib, D., Scheuner, D., Kaufman, R., Eskeinen, E., and Levine, B. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 190–195
38. Kanazawa, T., Taneike, I., Akasbi, R., Yoshizawa, F., Fuijura, N., Fujimura, S., and Kodowaki, M. (2004) J. Biol. Chem. 279, 8452–8459
39. Jefferson, L., and Kimball, S. (2003) J. Nutr. 133, 2046–2051
40. Kimball, S., Shantz, L., Horetsky, R., and Jefferson, L. (1999) J. Biol. Chem. 274, 11647–11652
41. Jefferson, L., Fabian, J., and Kimball, S. (1999) Int. J. Biochem. Cell Biol. 31, 191–200
42. Proud, C. (2004) Curr. Top. Microbiol. Immunol. 279, 215–244
43. Bolster, D., Crozier, S., Kimball, S., and Jefferson, L. (2002) Mol. Cell. Biol. 22, 6681–6688
44. Panwalkar, A., Verstoekse, S., and Giles, F. (2004) Cancer 100, 657–666
45. Proud, C. (2004) Biochem. Biophys. Res. Commun. 313, 429–436
46. Panwalkar, A., Verstoekse, S., and Giles, F. (2004) Cancer 100, 657–666
47. Proud, C. (2004) Curr. Top. Microbiol. Immunol. 279, 215–244
48. Bolster, D., Crozier, S., Kimball, S., and Jefferson, L. (2002) J. Biol. Chem. 277, 23977–23980
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