Amino acid deprivation induces translation of branched-chain α-ketoacid dehydrogenase kinase

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Doering, Christopher B., and Dean J. Danner. Amino acid deprivation induces translation of branched-chain α-ketoacid dehydrogenase kinase. Am J Physiol Cell Physiol 279: C1587–C1594, 2000.—Leucine, isoleucine, and valine are used by cells for protein synthesis or are catabolized into sources for glucose and lipid production. These branched-chain amino acids influence protein synthesis, hormone release, and cell cycle progression along with their other metabolic roles. The branched-chain amino acids play a central role in regulating cellular protein turnover by reducing autophagy. These essential amino acids are committed to their catabolic fate by the activity of the branched-chain α-ketoacid dehydrogenase complex. Activity of the branched-chain α-ketoacid dehydrogenase complex is regulated by phosphorylation/inactivation of the α-subunit performed by a complex-specific kinase. Here we show that elimination of the branched-chain amino acids from the medium of cultured cells results in a two- to threefold increased production of the branched-chain α-ketoacid dehydrogenase kinase with a decrease in the activity state of the branched-chain α-ketoacid dehydrogenase complex. The mechanism cells use to increase kinase production under these conditions involves recruitment of the kinase mRNA into polyribosomes. Promoter activity and the steady-state concentration of the mRNA are unchanged by these conditions.

posttranscriptional regulation; polyribosomes; regulation of catabolism

CATABOLIC STATES, INCLUDING starvation, diabetes, infections, trauma, and cancer, all decrease plasma amino acid concentrations. In response, the body accelerates protein degradation in skeletal muscle as a means to maintain plasma amino acid concentration (36, 42). Amino acids produced from autophagy can be used directly for energy production, ketone body formation, gluconeogenesis, or new protein biosynthesis. If protein breakdown persists, a cachectic condition ensues (28, 44). Various essential amino acids have been shown to attenuate protein catabolism (39). The branched-chain amino acids (BCAA), particularly leucine, are important in controlling protein turnover in most tissues (2, 4, 22, 30, 34, 35, 42). Several roles of leucine in this process have been described. Leucine stimulates the phosphorylation of 4E-BP1, causing its release from eukaryotic initiation factor (eIF)-4E to allow formation of the eIF-4F initiation complex for protein synthesis (10). When cells are deprived of leucine, 4E-BP1 remains bound to eIF-4E and global protein synthesis decreases, whereas polysome fractions are diminished (10, 45). In contrast, leucine starvation results in the L-system transporter protein concentration being increased to promote uptake of extracellular leucine. The increase in transporter results from changes in translation rate (24).

Conservation of BCAA by cells is important in cell preservation. Within cells, the BCAA have two fates, incorporation into protein or irreversible degradation. Oxidative decarboxylation of the branched-chain α-ketoacids by branched-chain α-ketoacid dehydrogenase (BCKD) commits these amino acids to their catabolic fate (5). BCKD is a nuclear encoded multienzyme complex located in the mitochondria of all mammalian cells. In response to changing needs for BCAA, the activity of BCKD is regulated to conserve these essential amino acids. The activity state of BCKD is decreased when the E1α subunit of the complex is phosphorylated by a complex-specific kinase (16). The activity state of BCKD is the percentage of complex that is unphosphorylated and active relative to the total amount of complex present. The amount of kinase protein present within a cell varies such that tissues with a low activity state, such as skeletal muscle, contain high levels of kinase protein. In contrast, liver and kidney contain less kinase protein and therefore maintain a high activity state of BCKD (7). When rats are fed a diet low in protein, the activity state of BCKD is decreased in liver, presumably to conserve protein (12). This response was shown to require an increase in kinase protein (41). Together, these observations suggest that activity state is not simply a balance between the specific activity of the kinase and its countering phosphatase but more a function of the amount of kinase protein present within a cell varies such that tissues with a low activity state, such as skeletal muscle, contain high levels of kinase protein. In contrast, liver and kidney contain less kinase protein and therefore maintain a high activity state of BCKD (7). When rats are fed a diet low in protein, the activity state of BCKD is decreased in liver, presumably to conserve protein (12). This response was shown to require an increase in kinase protein (41). Together, these observations suggest that activity state is not simply a balance between the specific activity of the kinase and its countering phosphatase but more a function of the amount of kinase protein present within any time. Therefore, decreases in the activity state of BCKD would necessitate an increased amount of kinase protein. To further investigate the cellular mechanisms regulating the BCKD activity state, we determined the effects of
BCAA depletion on BCKD activity and BCKD kinase expression in cultured cells. Here we show that depriving cells of the BCAAs decreases the BCKD activity state with a concomitant increase in the amount of kinase protein. The latter response results from a post-transcriptional event involving enhanced recruitment of mRNA in the polyribosome fraction.

MATERIALS AND METHODS

Cell lines and culture conditions. Ham’s F-12-K and RPMI 1640 lacking the BCAA were prepared by Atlanta Biologicals based on the Gibco formulas. PBS was dialyzed (6,000–8,000 molecular weight cut off) against three changes of eight volumes of 1× PBS to reduce free amino acid concentrations below detectable levels (6) and was confirmed in these studies by HPLC analysis (14). When stated, individual BCAA were supplied to the depleted media at the final concentration defined for each medium (RPMI 1640: I and L at 3.8 × 10⁻⁴ M, V at 1.7 × 10⁻⁴ M; F-12-K: I at 3 × 10⁻⁵ M, and L and V at 1 × 10⁻⁴ M; see Ref. 23). Rat liver cells (clone 9 (C9) from ATCC) were maintained in Ham’s F-12-K medium supplemented with 10% FBS at 37°C in 5% atmospheric CO₂. DG75, an EBV-negative human B cell line, and human lymphoblasts (EM280) derived by EBV transformation of human lymphocytes were maintained in RPMI 1640 supplemented with 15% FBS at 37°C in 5% atmospheric CO₂. During the 5-day culture in BCAA-depleted medium, C9 cells did not have a medium change, whereas DG75 and EM280 had one change of medium after 3 days. HPLC analysis of conditioned medium was performed to ensure equal loading. GAPDH has been shown as a housekeeping gene to be equally expressed in all conditions.

Enzyme assays and Western blots. BCKD activity assays were done as previously described (32). To inhibit endogenous BCKD kinase activity and fully activate the complex, cells were incubated with 1 mM n-butyrosuccinate (BCKD kinase activity and fully activate the complex), was done as previously described (32). To inhibit endogenous BCKD kinase activity and fully activate the complex, cells were incubated with 1 mM n-butyrosuccinate (BCKD kinase activity and fully activate the complex), were done as previously described (32). To inhibit endogenous BCKD kinase activity and fully activate the complex, cells were incubated with 1 mM n-butyrosuccinate (BCKD kinase activity and fully activate the complex), were done as previously described (32). To inhibit endogenous BCKD kinase activity and fully activate the complex, cells were incubated with 1 mM n-butyrosuccinate (BCKD kinase activity and fully activate the complex), were done as previously described (32). To inhibit endogenous BCKD kinase activity and fully activate the complex, cells were incubated with 1 mM n-butyrosuccinate (BCKD kinase activity and fully activate the complex), were done as previously described (32). To inhibit endogenous BCKD kinase activity and fully activate the complex, cells were incubated with 1 mM n-butyrosuccinate (BCKD kinase activity and fully activate the complex), were done as previously described (32).

Nucleic acid analysis. Total RNA was isolated using TriReagent (Sigma) following the manufacturer’s instructions and was quantified by measuring the absorbance at 260 nm. The ratios of absorbance at 260 nm to that at 280 nm were consistently 1.7–1.9. For Northern blot analysis, 20 μg total RNA from each sample was resolved in a 1% agarose-formaldehyde gel at 80 V for 4 h. RNA was transferred to a Hybond N⁺ nylon membrane by capillary action overnight in 20× saline-sodium citrate (SSC). Northern blots were done as described (1) with the following modifications. Hybridization buffer contained 5× SSC, 50% formamide, 7% SDS, 5% polyethylene glycol, and 50 μg denatured salmon sperm DNA/ml. The membrane was prehybridized for 4 h at 42°C before adding the [γ⁻³²P]dCTP-labeled cDNA probe for overnight incubation at 42°C. The membrane was washed one time with 2× SSC and 0.5% SDS at 55°C for 20 min, two times with 0.5× SSC and 0.5% SDS at 65°C for 20 min, and one time with 0.2× SSC and 0.2% SDS at 65°C for 20 min.

Hybridizing components were detected by autoradiography using Biomarin Blue film (Marsh Biomedical Products, Rochester, NY). Ethidium bromide staining of the gel and hybridization using radiolabeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA as probes were performed to ensure equal loading. GAPDH has been shown previously not to change as a result of complete amino acid deprivation (17, 26).

Luciferase assay. The luciferase reporter gene assays were done in C9 cells using rat BCKD kinase promoter constructs (20) driving firefly luciferase production. Three different promoter regions were tested that contained nucleotides upstream of the AUG codon to positions −58, −128, and −449. Cells were transfected by FuGENE 6 (Boehringer Mannheim) using a ratio of 50:1 test vector to pRL-cytomegalovirus control renilla luciferase plasmid. After 8 h in complete medium, cells were changed to fresh medium either with or without the BCAA and were cultured for 4 days. Cells were harvested, and luciferase activities were measured using the Dual Luciferase Assay system (Promega) according to the manufacturer’s protocol. Luminescence was determined with a Turner model TD20/20 luminometer. Firefly luciferase values were corrected for transfection efficiency by dividing by the corresponding renilla luciferase activity.

Polyribosome analysis. Preparation of polyribosomes for analysis of BCKD kinase mRNA translation was done essentially as described (9). The EM280 cell line was cultured for 5 days with or without BCAA in the medium. In an attempt to obtain similar cell numbers at the end of 5 days, only 30 × 10⁶ cells were seeded in + BCAA medium, whereas 70 × 10⁶ cells were seeded in − BCAA medium. At the end of 5 days, all cells were washed with 1× PBS and resuspended in fresh medium containing 100 μg cycloheximide for 15–20 min. Cells were then transferred to a buffer containing 75 mM NaCl, 4 mM MgCl₂, 20 mM Tris-HCl, pH 7.5, and 0.75% Triton X-100 and were allowed to lyse for 3–5 min on ice. The solution was clarified by centrifugation at 10,000 g for 10 min, and the supernatant was used for polyribosome fractionation. Fractionation was on a 15–45% linear gradient of sucrose in 80 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 5 mM MgCl₂ by centrifugation at 39,000 g for 90 min in an SW41 Ti rotor. Fractions were collected in 0.5-ml aliquots, and RNA profiles were determined by monitoring absorbance at 254 nm. Total RNA was prepared from 350 μl of each fraction using TRIzol (Sigma). The RNA was resuspended in 50 μl of 0.5% SDS and 1 mM EDTA, added to 150 μl RNA denaturing solution, and heated at 65°C for 15 min (21). Denatured RNA was loaded on a slot blot fitted with a Hybond N⁺ membrane. Membrane hybridization was done as for the Northern blot methods described in Nucleic acid analysis using both BCKD kinase and GAPDH as probes.

RESULTS

Activity state and BCAA amino acid metabolism. Cells cultured in medium lacking the BCAA respond by decreasing the activity state of the BCKD complex. This response was demonstrated with hepatocytes and lymphoblasts (Table 1). After each cell type was incubated in BCAA-depleted media for 5 days, the activity state of BCKD was reduced by at least 50% relative to the active state in the same cells cultured in complete medium. As shown for DG75 cells, this response was time dependent (Fig. 1A). Total BCKD activity in the cells grown in BCAA-depleted medium was the same.
As demonstrated with the C9 cells (Fig. 2), branched-chain α-keto acid dehydrogenase activity in cells grown in complete medium is elevated two- to threefold. Deprivation of BCAA resulted in a marked increase in the BCKD kinase protein level above that found in cells grown in complete medium, for this same time, not significantly different (P = 0.325). Beyond 3 days in BCAA-free medium cell growth halts (Fig. 1B), presumably due to G1 cell cycle arrest caused by isoleucine depletion (13, 27, 48). The change in BCKD activity and growth at 5 days was not exclusively due to cell death. Trypan blue exclusion analysis showed that >75% of the cells were living (Fig. 1C), and the cells grew normally when returned to complete medium after this time (data not shown). Assuming the slight increase observed in cell death could help to replenish the depleted media with BCAA, we performed HPLC analysis on the conditioned media after the 5-day culture period (Table 2). The BCAA-deprived media contained no detectable BCAA by HPLC, whereas the control media contained BCAA concentrations similar to fresh media. HPLC also failed to detect intracellular free BCAA in these same cells cultured for 5 days in BCAA-deprived media.

**Effects on BCKD kinase protein levels.** During BCAA deprivation, there was an increase in the BCKD kinase protein concentration present in the mitochondria. The amount of kinase protein in EM280, DG75, and C9 cells cultured without BCAA was markedly increased over that found in cells grown in complete medium (Fig. 2A). As demonstrated with the C9 cells (Fig. 2B) by quantitative Western blot analysis (31), the kinase protein level increased two- to threefold. Deprivation of leucine alone over this same time period did not result in elevated BCKD kinase protein levels above that found in control medium (data not shown).

**Measurements of promoter activity and steady-state mRNA.** Synthesis of proteins depends on the sequential activity of gene transcription, RNA stability, and translation. Increased production of a protein can be the result of an altered rate of any of these processes. Two experimental approaches show that neither in-
Increased promoter activity nor RNA stability contributed to the observed increase in kinase protein. First, luciferase reporter assays with the kinase promoter driving production of luciferase showed no significant difference between cells grown in the two culture conditions (Fig. 3). BCKD kinase basal promoter activity requires only the first 58 bp upstream of the start site (21), and lengthening this to include an additional 391 bp upstream did not alter the response. Second, Northern blots also did not show an increase in steady-state mRNA concentration for the kinase transcripts between control and BCAA-deprived cells at days 1 or 5 (Fig. 4A). Densitometry quantification of data from three independent experiments consistently showed that the steady-state BCKD kinase mRNA concentration did not increase with 5 days of BCAA deprivation (Fig. 4B). In fact, a slight decrease in BCKD kinase mRNA was observed after 5 days of culture in BCAA-depleted medium compared with cells grown in complete medium. This is consistent with the finding that total RNA harvested per cell is almost 30% lower in BCAA-deprived cells vs. control cells (data not shown).

Alteration of translation state. To determine if the translation rate of BCKD kinase mRNA is increased, polyribosomes were isolated from cells grown in the presence and absence of the BCAA for 5 days. The EM280 cell line was used for these experiments due to ease of culturing large cell numbers and the fact that they showed the greatest response observed by Western blot (Fig. 2A). As seen in Fig. 5A, the profiles of the polyribosome traces from each experimental condition were not substantially different except for the fact that less total RNA was present in the fractions from the BCAA-depleted cells. Hybridization to total RNA prepared from these polyribosome fractions with BCKD kinase and GAPDH-radiolabeled probes demonstrates that more of the BCKD kinase mRNA is found in the higher-order polyribosome (heptasome and larger) fractions (11–13, 15) from cells grown in the BCAA-deprived medium (Fig. 5B and Table 3). BCKD kinase transcript was not detected in fractions that did not contain ribosomes (fractions 1–4) from cells grown in either media.

Examples of translational control often involve the nucleotide sequence and/or secondary structure of the 5'-untranslated region (UTR) of the specific mRNA (18, 37). In an attempt to assess functionality of the 5’-UTR, the cDNA for mouse BCKD kinase was engineered to contain either 107 or 21 nt of 5’-UTR along with the first 70 of 3’-UTR. When the constructs were subjected to in vitro transcription/translation (TNT; Promega), the amount of immunodetectable kinase protein produced from the 107-base 5’-UTR construct was not different from the amount synthesized from the 21-base construct (data not shown).

Table 2. Concentration of BCAA in dialyzed fetal bovine serum, culture medium, and cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Leucine (mM)</th>
<th>Isoleucine (mM)</th>
<th>Valine (mM)</th>
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</thead>
<tbody>
<tr>
<td>Dialyzed FBS</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Medium −BCAA</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Medium +BCAA</td>
<td>414.6</td>
<td>119.9</td>
<td>360.7</td>
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<td>ND</td>
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<td>ND</td>
</tr>
<tr>
<td>Cells +BCAA</td>
<td>70.9</td>
<td>15.2</td>
<td>47.0</td>
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</tbody>
</table>

Units are μM. ND, below detection level.

**Fig. 2.** Western blot for mitochondrial BCKD kinase reveals an increase in BCKD kinase upon BCAA depletion. A: lymphoblasts [DG75 and EM280 (280) cell lines] were grown in suspension culture for 5 days in media with (+) or without (−) BCAA. Mitochondrial protein (20 μg) was resolved on 10% SDS-PAGE and processed for Western blot with BCKD kinase antisera. B: C9 rat hepatocytes were treated as above for Western blot analysis. The amount of mitochondrial protein loaded on the gel is included at bottom. All blots were reprobed with BCKD complex antisera and were observed to contain similar levels of each subunit, attesting to equal loading of the samples (data not shown).

**Fig. 3.** Luciferase activity driven by the rat BCKD kinase promoter remains unchanged after BCAA depletion. Hepatocytes were transfected with a plasmid containing 1 of 3 different lengths of promoter region DNA (−449, −128, or −58) linked to the firefly luciferase reporter gene and a control renilla luciferase plasmid. Cells were maintained for 4 days in medium with (filled bars) or without (open bars) BCAA before measuring luciferase activity. Results are means ± SD of 6 independent determinations.

**Fig. 4A** and **B**. Western blot for mitochondrial BCKD kinase reveals an increase in BCKD kinase upon BCAA depletion. A: lymphoblasts [DG75 and EM280 (280) cell lines] were grown in suspension culture for 5 days in media with (+) or without (−) BCAA. Mitochondrial protein (20 μg) was resolved on 10% SDS-PAGE and processed for Western blot with BCKD kinase antisera. B: C9 rat hepatocytes were treated as above for Western blot analysis. The amount of mitochondrial protein loaded on the gel is included at bottom. All blots were reprobed with BCKD complex antisera and were observed to contain similar levels of each subunit, attesting to equal loading of the samples (data not shown).
DISCUSSION

BCAA concentrations modulate many cellular processes, including protein synthesis, proteolysis, and hormonal responses whether measured in whole animals or cultured cells (19, 34, 38, 42). Although a wide range of cellular functions are influenced by BCAA concentration, the mechanisms that regulate the catabolism of these essential amino acids are poorly understood. To determine the effect of BCAA deprivation on BCAA degradation, we measured BCKD activity state and BCKD kinase levels in three types of cells in culture and defined the mechanism these cells used to achieve this regulation.

Marten et al. (29) examined the transcription of 19 genes in cultured hepatocytes for a response to amino acid deprivation. They found that one-third showed increased expression, and one-third did not respond to the culture conditions. Other investigators have demonstrated amino acid starvation to alter translation of certain proteins (37). Observed decreases in protein synthesis result from a loss of mRNA movement into and dissociation from polyribosomes (45). These responses were observed in perfused livers and in cultured hepatocytes and Chinese hamster ovary cells (37). Further investigations attributed amino acid effects on translation to changes in the phosphorylation state of specific translation factors (15, 25, 47). Recent reports showed that leucine is the primary amino acid responsible for changing the phos-
phorylation state of the translational repressor 4E-BP1 and thus altering translation (10, 46). In limited cases, amino acid deprivation results in induction of specific proteins that tend to be involved in maintenance of cellular amino acid concentrations (17).

The concentration of free BCAA within a cell is a function of dietary intake, cell transport, incorporation into protein, and catabolism. Because degradation of BCAA by BCKD is an irreversible process, shifts in BCKD activity state represent the major mechanism for regulating BCAA concentration. In rats, when dietary protein or BCAA are limited, BCKD activity is suppressed (3, 8, 33). This reduction in BCKD activity has been attributed to changes in the phosphorylation state of the complex resulting from increased BCKD kinase activity. These changes occurred only after the animals were kept on the protein-deprived diet for 5–12 days. Because BCAA or protein deprivation in intact animals affects many tissues and metabolic pathways, we used cultured cells to model only the cellular response. Within cell culture, the extracellular environment is controlled easily, and single entities can be varied. Similar to the whole animal studies, we found that the maximal effect of BCAA deprivation required at least 5 days. The cells responded to these conditions by decreasing the BCKD activity state by at least 50% (Table 1). The decrease in BCKD activity state is in direct response to a two- to threefold increase in BCKD kinase protein (Fig. 2). No corresponding increase was observed for BCKD kinase steady-state mRNA levels or kinase promoter activity, as determined by luciferase reporter activity. These findings do not implicitly demonstrate that there is not some level of change in mRNA half-life or transcription rate but when taken together argue against either mechanism being substantially responsible for the response observed during the course of our study. The increase in BCKD kinase protein does appear to be the result of increased translation of the steady-state BCKD kinase mRNA, as evidenced by a shift of these transcripts into higher-order polyribosomes (Fig. 5 and Table 3). The experiments also demonstrate that, under both the control and BCAA-deprived state, all detectable BCKD kinase transcripts are bound by ribosomes. Therefore, the response is not simply a general shift of free kinase mRNA to ribosomally bound kinase mRNA but a specific change in the number of ribosomes bound to each transcript. The >20% increase in the percentage of kinase transcripts bound to higher-order polyribosomes appears to account for the two- to threefold increase in kinase protein over a 5-day period of accumulation.

Because extended times are needed to elicit the response to BCAA deprivation, it was not possible to use protein synthesis inhibitors to block the response. The prolonged times also worked against using transient transfections of the cells to study in vivo roles of the 5’- and 3’-UTR of this mRNA. Although our results approximate those measured in protein-deprived rats, the mechanism in rats was attributed to increased BCKD kinase mRNA concentration (41). This difference remains to be resolved.

The mechanism these cultured cells use to shift BCKD kinase mRNA into polyribosomes was not defined. Structural features of the 5’-UTR regions of mRNAs have been shown to play an important role in regulation of translation (18). Because the mRNA sequences for human (accession no. AF026548), rat (accession no. M93271), and mouse (accession no. AF043070) BCKD kinase are available, we performed qualitative analysis of these for potential regulatory regions. The length of each appears to be sufficient to contain information for regulation. The human 5’-UTR contains 273 nucleotides, whereas the rat and mouse have 121 and 106 bases, respectively. Sequence comparisons reveal a similarity index of 78.3 for rats and mice and a 48.5 index between rats and humans, implying conservation and importance of this region. All contain potential in-frame upstream open reading frames (uORF) and tracts of pyrimidines and can fold into secondary structures, as assessed by Lasergene software analysis (Table 4). The inclusion of uORFs has also been attributed to the instability of mRNAs, but that does not appear to be a condition observed here, since the steady-state concentration of the kinase mRNA is not changed (11). Although these putative features exist, we were not able to demonstrate a change in protein production by in vitro translation experiments using cDNA constructs with a deleted 5’-UTR compared with the full-length cDNA. The enriched environment in reticulocyte lysates for translation is likely to mask in vivo conditions.
There are inherent properties of BCKD kinase that prohibit using standard methods to continue examination of potential mRNA regulatory sequences and to address potential changes in kinase protein turnover rates that may occur during BCAA deprivation. First, construction of a tagged kinase protein is complicated by the need for the mitochondrial targeting peptide at the amino terminal end that is lost after import into the matrix, and the carboxy terminal end of the protein contains the catalytic domain. A carboxy terminal V5-His<sup>8</sup>-tagged version of the human BCKD kinase is catalytically inactive although it is correctly imported and processed in cells that are transfected with plasmid directing its synthesis (unpublished observation).

Second, the presence of BCKD kinase within all tissues containing mitochondria significantly complicates experiments involving comparisons of kinase expression by the need for the mitochondrial targeting peptide at the matrix, and the carboxy terminal end of the protein contains the catalytic domain. A carboxy terminal V5-His<sup>8</sup>-tagged version of the human BCKD kinase is catalytically inactive although it is correctly imported and processed in cells that are transfected with plasmid directing its synthesis (unpublished observation).

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A 50% decrease in BCKD activity state has a substantial effect on BCAA metabolism within these cells. By decreasing the activity state without changing total activity, the cells are effectively limiting the catabolism of BCAA to one-half of the normal rate. This may be enough to prevent substantial losses in total cellular protein in the absence of normal cellular leucine concentrations. In our studies, total cellular protein did not decrease in cells grown in the absence of BCAA despite the fact that there are no detectable BCAA either inside or outside the cell. In fact, total protein har-

vested was slightly higher on a per cell basis in the BCAA-deprived cells (540 vs. 420 ng/cell in control cells). One possibility to account for the lack of detectable free BCAA is that all potentially free BCAA are rapidly bound as charged aminoacyl-tRNAs and are incorporated into newly synthesized proteins. Irrespective of this model, the ability of cells to adapt and retain cellular protein levels in the absence of these essential amino acids reflects the importance of BCKD regulation.

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