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Role of Amino Acids in Translational Mechanisms Governing Milk Protein Synthesis in Murine and Ruminant Mammary Epithelial Cells

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Abstract The role of amino acids (AA) on translational regulation in mammary epithelial cells cultured under lactogenic conditions was studied. The rates of total protein synthesis and β-lactoglobulin (BLG) synthesis in mouse CID-9 cells were 2.1- or 3.1-fold higher, respectively, than in their bovine L-1 counterparts. Total AA deprivation or selective deprivation of Leu had a negative protein-specific effect on BLG synthesis that was more pronounced in bovine cells than in murine cells. Dephosphorylation of eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and S6 kinase (S6K1) on Thr^{389} but not on Ser^{411} was also more prominent in bovine cells. Noteably, deprivation of Leu had a less marked effect on BLG synthesis and 4E-BP1 or S6K1 phosphorylation than deprivation of all AA. In AA-deprived CID-9 cells, Leu specifically restored BLG synthesis from pre-existing mRNA whereas AA also restored total protein synthesis. This restoration was associated with a more pronounced effect on 4E-BP1 and S6K1 phosphorylation in bovine versus murine cells. Rapamycin specifically reduced Leu- and AA-stimulated BLG translation initiation in a dose-dependent manner. A further reduction was observed for Leu-treated cells in the presence of LY294002, a PI3K (phosphatidylinositol 3-kinase) inhibitor, which also reduced total protein synthesis. These findings suggest that direct signaling from AA to the translational machinery is involved in determining the rates of milk protein synthesis in mammary epithelial cells. J. Cell. Biochem. 98: 685–700, 2006.

Key words: mammary gland; milk proteins; β-lactoglobulin; translation; 4E-BP1; S6K1

Regulation of milk protein synthesis in the mammary gland has been associated with anatomical and spatial adaptation to the reproductive cycle. Starting at mid-pregnancy, extracellular stimuli initiate a staged program of milk protein gene expression that progresses during lactation and terminates upon weaning [Rosen et al., 1999]. A defined set of transcription factors bind specific *cis*-regulatory sequences in milk protein genes and contributes to the precise control of this cyclic process [Marti et al., 1999; Groner, 2002; Coletta et al., 2004].

Whereas transcriptional regulation of milk protein gene expression has been extensively studied, a critical role for translation control has only recently been demonstrated. In differentiated CID-9 mammary epithelial cells, insulin and prolactin were shown to be both necessary and sufficient to induce cytoplasmic elongation of the poly(A) tract of β-casein mRNA and consequent increase in translational efficiency, whereas the mRNA of glyceraldehyde-3-phosphate dehydrogenase, a typical non-milk protein, is unaffected [Choi et al., 2004]. This provides a mechanism for the in vivo observations that the length of the poly(A) tract of the β-casein mRNA shortens upon weaning but elongates when pups are allowed to nurse [Kuraishi et al., 2000].

The notion that post-transcriptional mechanisms govern milk protein expression is supported

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by the disappointing increase (8%–20%) in β-casein concentration in the milk of transgenic cows having additional copies of β- and κ-casein genes [Brophy et al., 2003]. Furthermore, the fact that transgenic mice with the same genes express these proteins at much higher levels suggests a quantitative limitation on protein synthesis in the bovine gland [Persuy et al., 1992; Rijnkels et al., 1995]. By contrast, non-transgenic cattle treated with insulin clamps and infused with casein as a source of amino acids (AA) exhibited a transient but significant change in milk protein production [Griinari et al., 1997]. Cows receiving both treatments had 28% higher levels of total milk protein compared to 10% for casein infusion alone and 4% for insulin clamps alone. The increase in milk protein content was associated with a dramatic 54% decrease in the concentration of essential AA in the circulation, suggesting the activation of mechanisms that regulate protein synthesis. Since a large proportion of incoming AA is released from the mammary epithelial cell back into the bloodstream [Maas et al., 1998], this further implies that the bovine mammary gland is not functioning at maximum capacity.

The anabolic properties of AA have long been appreciated, and stimulation of protein synthesis in response to food intake has been associated with the activation of translation initiation and the rapid re-aggregation of polysomes [Kimball and Jefferson, 2000]. Yet it has only recently become apparent that AA act as signaling molecules to regulate protein synthesis. Branched-chain AA, particularly Leu, can reproduce the stimulatory effect of AA on protein synthesis by regulating translation initiation [Kimball and Jefferson, 2004; Proud, 2004]. In L6 myoblasts, AA principally regulate translational initiation at the level of Met-tRNAi binding to the 40S ribosomal subunit, which is controlled by the activity of eIF2B [Kimball et al., 1998; Kimball, 2002]. In other cell lines and tissues, signaling through the mammalian target of rapamycin (mTOR) is impaired by deprivation of AA [Kimball and Jefferson, 2004; Proud, 2004].

mTOR is activated by hormones, growth factors, and nutrients. It is directly phosphorylated by protein kinase B (PKB; a.k.a Akt) and may represent the point of convergence for separate pathways stimulated by insulin and AA. mTOR regulates mRNA binding to the 43S initiation complex by affecting the phosphorylation state of 4E-BP1. Phosphorylation of 4E-BP1 at several sites is hierarchical [Wang et al., 2005] and ultimately releases the initiation factor eIF4E to start a cascade of events resulting in the stimulation of cap-dependent translation [Gingras et al., 2001].

S6 kinase 1 (S6K1) phosphorylates the 40S ribosomal protein S6. It has been suggested that AA signal to S6K1 via mTOR [Hara et al., 1998]. A correlation has been demonstrated between S6K1 activity and translation efficiency of 5′-TOP mRNAs, which possess an uninterrupted run of pyrimidine residues adjusted to the 5′-cap structure. 5′-TOP mRNAs encode many proteins that are involved in translation such as the ribosomal proteins, eIF1A, eIF2, and the poly(A) binding protein. Yet, nutritional regulation of 5′-TOP mRNA translation was shown to be independent of S6K1 phosphorylation or activity [Tang et al., 2001]. It has also been recently postulated that an intracellular AA sensor may be linked to a small G-protein, Rheb, a positive regulator of mTOR signaling that functions in *Drosophila* downstream of tuberous sclerosis complex (TSC)2 and upstream of mTOR in controlling cell growth and regulating S6K1 activity [Kimball and Jefferson, 2004].

AA regulation of mRNA translation has been studied primarily in skeletal muscle and liver, tissues that are subjected to rapid anabolic processes after periods of starvation. The mammary gland also undergoes massive production of proteins, especially milk proteins, which could be regulated at the translation initiation step during the lactation period. In the current work, we have studied translational regulation of milk protein expression in differentiated mouse and bovine cell cultures. We report a specific effect for Leu on the initiation of β-lactoglobulin (BLG) mRNA translation and a negative correlation between the lower capability of the bovine cells to synthesize BLG and their sensitivity to total AA and Leu manipulations.

MATERIALS AND METHODS

Cell Culture and Transfections

CID-9 cells were grown in DMEM:F12 (1:1 Gibco-BRL, Paisely, UK) containing 5% heat-inactivated fetal calf serum (FCS, Sigma, St. Louis, MO), insulin (bovine, 5 μg/ml, Sigma) gentamicin (50 μg/ml, Sigma), and combined

antibiotics (penicillin, 100 units/ml and streptomycin, 0.1 mg/ml, BioLabs, Jerusalem, Israel). L-1 cells, a cloned cell line derived from lactating bovine mammary gland [German and Barash, 2002], were grown in DMEM:F12 (1:1) containing 10% FCS, insulin (bovine, 5 μg/ml), hydrocortisone (1 μg/ml, Sigma), epidermal growth factor (mouse EGF, 10 ng/ml, Sigma), gentamicin (50 μg/ml), and combined antibiotics.

For extracellular matrix (ECM)-dependent activation, cells were plated on Matrigel (Collaborative Bio-Medical Products, Bedford, MA) in a 6-well dish (0.5×10^6 cells/well) or in a 24-well dish (0.2×10^6 cells/well). DMEM:F12 containing 2% FCS, insulin (5 μg/ml), hydrocortisone (3 μg/ml), and prolactin (ovine, 3 μg/ml, provided by the NIDDK program) was added for 24 h. The medium was then switched to one without FCS. Stable transfections of the β-lactoglobulin (BLG) cDNA constructs into CID-9 or L-1 cells were carried out with lipofectamine reagent (Gibco-BRL) as previously described [Barash et al., 1999]. Selection with G418 was initiated after 3 days and continued for 3 weeks. Surviving colonies (>100) were pooled and stocks were grown.

mRNA Isolation and Northern Blotting

Total RNA was isolated from mammary epithelial cells using Trizol (Gibco); 15-μg aliquots were analyzed on 1.5% agarose gels and transferred to Nytran N nylon filters. The latter were baked for 2 h at 80°C and stained with methylene blue to locate the 18 S and 28 S ribosomal RNA. BLG mRNA was detected by hybridization at 65°C to a 404-bp ^{32}P-labeled PCR product, prepared from reverse-transcribed bovine mammary RNA with the primers 5′-CCTTGTGCTGGACACCGACTAC-3′ and 5′-CTTTATTGCTGAAGGAGGAGCGG-3′ and the oBLG cDNA [Shani et al., 1992] as template. Bovine and murine 16 S mRNAs were detected using a 1,055-bp ^{32}[P]-labeled probe prepared as a PCR fragment from the mouse 16S gene [Iavnilovitch et al., 2004].

Immunoblot Analysis

For the detection of 4E-BP1, pS6K1, and eIF4E, proteins were extracted from the epithelial cells by three freeze-thaw cycles in 10 mM Tris-HCl, pH 7.4, containing 150 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM 50 mM NaF, 1 mM Na_3Vo_4, 1 mM PMSF, and 1 μg/ml aprotinin [Barash, 1999]. Extracted proteins were fractionated by SDS–PAGE and transferred onto nitrocellulose filters. Equal amounts of loaded protein were confirmed by staining the blot with Ponceau S (Sigma). For 4E-BP1 detection, filters were blocked and reacted with rabbit anti-4E-BP1 antibody (Zymed Laboratories, San Francisco, CA; diluted 1:500 [Barash, 1999; German and Barash, 2002]. Detection of S6K1 phosphorylated on Thr^{389}, was performed with anti-phospho-p70 S6 kinase antibody (Cell Signalling, Beverly, MA) diluted 1:500. Goat anti-rabbit IgG complexed with HRP (Zymed) served as the second antibody. eIF4E was detected with anti-eIF4E antibodies (BD Bioscience, San Jose, CA) diluted 1:500. For the detection of S6K1 phosphorylated on Ser^{411}, filters were reacted with anti-phospho-p70^{S6K} antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:300. Goat anti-mouse IgG complexed with HRP served as the second antibody. Signals were generated with an ECL kit (Amersham, Buckinghamshire, UK) or Super Signal, West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) according to the manufacturer's protocol. Signals at the linear range of development, were quantitatied using the software Gel-Pro Analyser, ver.3.0 (MediaCybernetics, UK) and expressed relative to the value of the control non-deprived cells.

Metabolic Labeling

CID-9 and L-1 cells were cultured on Matrigel in 35-mm dishes and labeled for 1 h with 1 ml of DMEM containing 100 uCi/ml [^{35}S] Met as previously described [Barash et al., 1999; German and Barash, 2002]. Briefly, total incorporation of [^{35}S] Met to TCA-precipitable protein was measured in 5-μl aliquots pipetted onto nitrocellulose filters. For the determination of de novo BLG synthesis, aliquots (300–500 μl) of cell lysate, containing equal amounts of TCA-precipitable protein, were pre-treated with Protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden), then reacted with anti-BLG antibodies (Nordic Immunology, Tilburg, The Netherlands, diluted 1:250) and precipitated with Protein A-Sepharose. Proteins were separated by SDS–PAGE, and the gels were exposed to XAR Kodak film following fluorography. Signals at the linear range of development were quantitatied using the software Gel-Pro Analyser, ver.3.0 (MediaCybernetics, UK) and

expressed relative to the value of the control, non-deprived cells.

Labeling AA-deprived cells was performed in Earle's Balanced Salts solution containing Met (17.24 g/ml, representing its level in DMEM:F12). This was to maintain equal Met pools in control and deprived cells. Insulin, prolactin, and hydrocortisone were present during labeling.

S6K1 Activity

Cells were extracted in 50 mM Tris-HCl containing 150 mM NaCl, 1% NP-40, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1 μg/ml aprotinin. The kinase activity in cell lysates was assayed using a $p70^{S6K}$ activity kit (Upstate Biotechnology, Waltham, MA) in immunoprecipitates according to the manufacturer's protocol. Incorporation of ^{32}P into the synthetic substrate (AKRRRLSSLRA) was measured by precipitation of 10-μl aliquots pipetted onto phosphocellulose filters and washed in 0.75% phosphoric acid.

Real-Time PCR

RNA from L-1 and CID-9 cells was isolated using Trizol. Reverse transcription of 1 μg RNA was performed at 37°C for 50 min in 25 μl of 50 mM Tris-HCl, pH 8.3, containing 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, dATP, dTTP, dCTP, and dGTP (Stratagene, La Jolla, CA, 10 mM each), 0.5 μg of oligo(dT) primer (Promega), and 200 U of SUPERSCRIPT II (Gibco/BRL). RNase-free DNase I (1 μl) was added to eliminate residual genomic DNA contamination. Real-time PCR was performed in an ABI Prism 7700 in 20-μl reactions containing 1 μl of the reverse transcription reaction mixture (10 ng cDNA), 10 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 10 μM primers and 0.3 μl of Taq polymerase. The thermal-cycle conditions consisted of 10 min at 95°C, and 40 cycles of 15 s at 95°C, and 1 min at 60°C. The primers were designed so that the PCR would yield a single product without any primer dimerization, the product being verified using a dissociation protocol. The amplification curves of the BLG and 16S were parallel. The results were calculated as $\Delta\Delta C_T$, as described in User Bulletin no. 2 for the ABI prism 7700 sequence detection system. The primers were 5′-CTGCTGCAGAAATGGGAGAAC-3′ and 5′-TTCTCATTCAAGGCATCGATCTT-3′ for BLG, 5′-AGGGATAACAGCGCAATCCTATT-3′ and 5′-CTTTGATAGCGGTTGCACCAT-3′ for bovine 16S rRNA, and 5′-GCTAAACGAGGGTCCAACTGTCT-3′ and 5′-GCTCCATAGGGTCTTCTCGTCTT-3′ for mouse 16S rRNA.

RESULTS

BLG Protein Synthesis in Bovine Cells Is More Sensitive to AA or Leu Withdrawal Than in Mouse Cultures

The mouse CID-9 and bovine L-1 cell lines were both derived from continuously proliferating, non-transformed mammary epithelial cell populations that lack tumorigenic properties [Danielson et al., 1984; German and Barash, 2002]. Both differentiate on Matrigel to produce mammospheres in which milk proteins are synthesized after induction with lactogenic hormones. To analyze the expression of similar specific milk protein genes, we stably transfected CID-9 cells with the ruminant ovine BLG gene [Shani et al., 1992] which is homologous to the bovine. Although the BLG gene is absent from the murine genome, upon stable introduction, it acquires the tissue and hormonal responsiveness characteristic of milk protein genes both in vivo and in vitro (see plsmid 585 in; [Baruch et al., 1995, 1998]). Preliminary analyses indicated that the murine CID-9 cells synthesize the transfected BLG at 3.1 ± 0.4-fold higher levels than the bovine L-1 cells (Fig. 1, inset). Transfection of L-1 cells with additional copies of the BLG gene only marginally affected the levels of BLG synthesis as measured by immunoblot analysis (data not shown). The presence of reliable antibodies to BLG enabled its analysis in both bovine and murine cells. (Antibodies that react with other ruminant milk proteins, such as caseins, are not commercially available.)

We first established the time frame for the effect of AA depletion on protein and RNA synthesis under conditions resembling the lactation state of the mammary gland. Cells were cultured on Matrigel in serum-free medium for 5 days in the presence of insulin, hydrocortisone, and prolactin. They stopped proliferating, differentiated into mammospheres, and began to produce milk proteins. Then, the medium was replaced with one lacking either the full set of AA or lacking only Leu for various time periods. During the last hour of incubation, the cells were labeled with

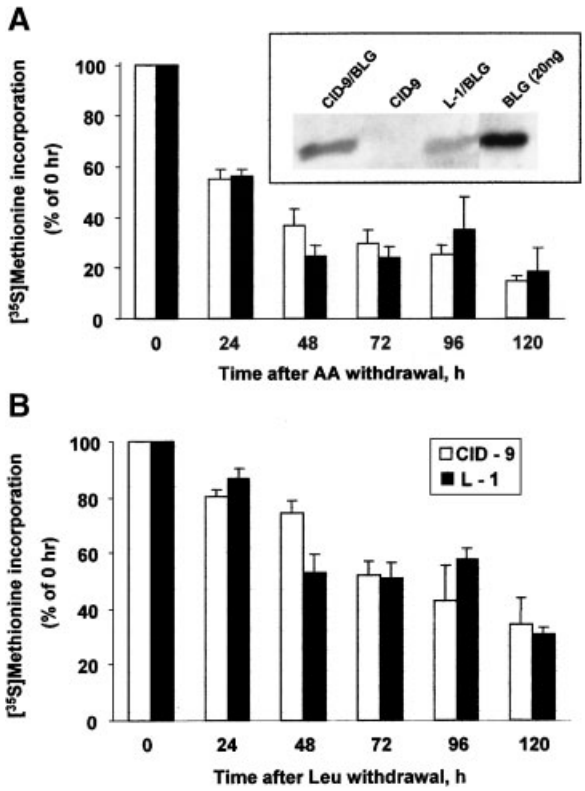

Fig. 1. Total protein synthesis is similarly affected by AA and Leu deprivation in murine and bovine cell cultures. Epithelial cells were allowed to differentiate on Matrigel for 5 days in the presence of insulin, hydrocortisone, and prolactin. Cells were washed and incubated in medium lacking either the full set of AA (**A**) or just Leu (**B**) for the indicated times. [^{35}S] Methionine was added to the medium for the final hour and its incorporation into TCA-perciptible proteins was measured in cell lysates. Bars represent mean ± SEM of relative incorporation from at least three independent experiments. Inset, immunoblot analysis of BLG expression in transfected CID-9 and L-1 cells. Equal amounts of protein/sample from cell lysate of L-1 and CID-9 cells were analyzed on SDS–PAGE, blotted onto membrane and reacted with anti-BLG antibodies. Representative blot of three independent analyses.

[^{35}S] Met. Hormones were present during the depletion and labeling periods.

The total rate of protein synthesis was 2.1-fold higher in differentiated, hormonally treated CID-9 cells compared to their bovine L-1 counterparts ($2.6 \pm 0.4 \times 10^6$ TCA precipitable cpm vs. $1.2 \pm 0.1 \times 10^6$ per 1×10^5 cells, respectively). In both cell lines, protein synthesis decreased during the first 48 h of AA depletion to a level 25 to 30% of controls (Fig. 1A). Longer periods of AA deprivation had no further effect on [^{35}S] Met incorporation. Cells of both origins cultured in medium lacking Leu exhibited a more gradual decline in total protein synthesis, mainly during the first 24 h of Leu deprivation (Fig. 1B). No significant differences were noted between AA and Leu deprivation on CID-9 versus L-1 cells ($P < 0.05$).

The effect of AA or Leu deprivation on the synthesis of BLG was measured by immunoprecipitation of proteins from control and deprived cells (Fig. 2). BLG synthesis declined faster and to a greater extent in the AA-deprived L-1 cells compared to CID-9 cells (Fig. 2C vs. A). Twenty-four hours after AA withdrawal, L-1 cells synthesized BLG at 40% of control levels whereas CID-9 cells were only slightly affected. By 72 h of AA deprivation, L-1 cells synthesized BLG at ~8% of controls whereas synthesis in CID-9 cells remained at ~40%. Similarly, withdrawal of Leu affected BLG synthesis in L-1 cells more severely than in CID-9 cells. The major difference occurred over the first 24 h. During that period, [^{35}S] Met incorporation was reduced by ~60% in L-1 cells compared to ~20% in CID-9 cells. The more severe effect of both AA and Leu withdrawal on L-1 cells was maintained for at least 72 h (Fig. 2C,D vs. A,B).

We wished to distinguish between effects on levels of BLG mRNA (transcription, splicing, nuclear export, degradation) versus efficiency of BLG mRNA translation. We therefore measured BLG mRNA levels by both Northern blotting and real-time PCR as a function of AA withdrawal (Table I). The time course of deprivation was limited to 72 h, after which time protein synthesis was only marginally affected. BLG mRNA levels decreased for both CID-9 and L-1 cells, but for both cell lines, the decrease in mRNA was less rapid than the decrease in BLG synthesis (Fig. 2). For instance, after 24 h the decrease in BLG mRNA was undetectable in L-1 cells but the decrease in BLG synthesis was more than 60%.

Effect of AA or Leu Deprivation on the Levels or Activities of eIF4E, 4E-BP1, and S6K1 in L-1 and CID-9 Cells

The foregoing results indicate that a change translational efficiency of BLG mRNA rather than a change in BLG mRNA levels is responsible for the decrease in BLG synthesis following AA withdrawal, and that the translational effect is greater in L-1 than CID-9 cells. To determine which changes in the translational machinery might be responsible, we tested several factors and signal transduction pathways known to affect protein synthesis.

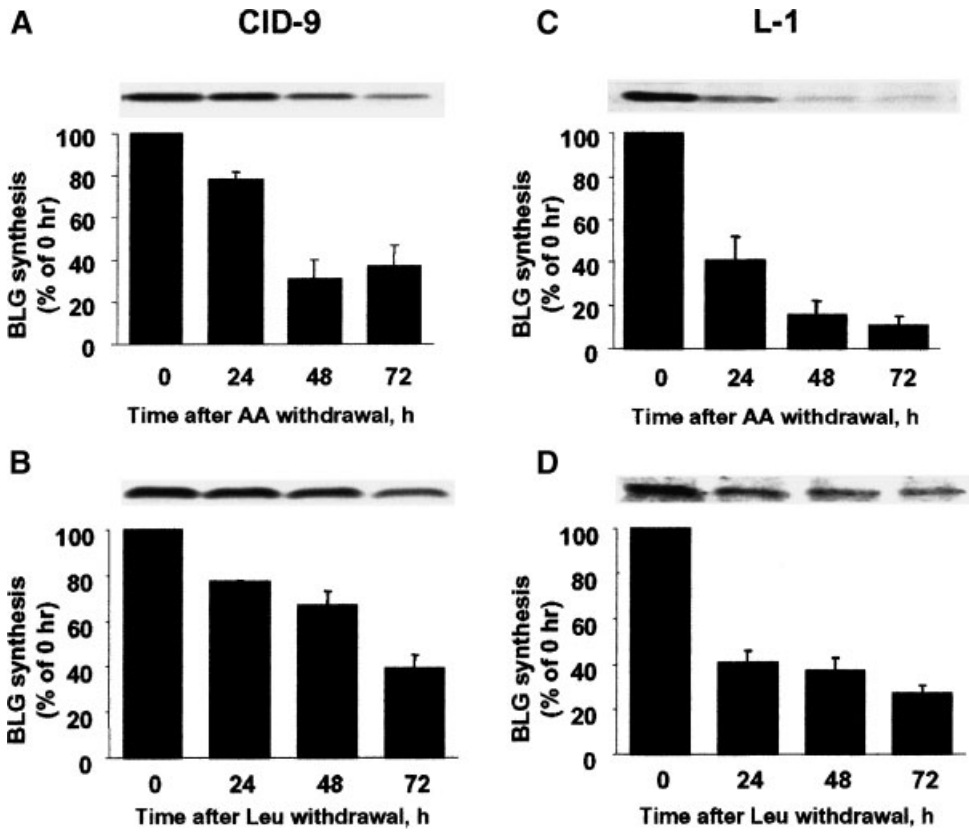

Fig. 2. AA, particularly Leu, deprivation in bovine L-1 cells suppresses BLG synthesis more rapidly than in their murine CID-9 counterparts. Differentiated, hormonally treated mammary epithelial cells were deprived of either total AA (**A**, **C**) or Leu (**B**, **D**) for the indicated times. [^{35}S] Met was added to the medium for the final hour. De novo BLG synthesis was measured by immunoprecipitation in aliquots containing equal levels of TCA-perciptible protein as described in Materials and Methods. Samples were subjected to SDS–PAGE followed by autoradiography. In each analysis, the values of the quantitated signals (insets) were related to that of the non-deprived cells and are presented in the bar graphs as an average ± SEM of more than three independent experiments.

TABLE I. Deprivation Effect on BLG RNA Expression in CID-9 or L-1 Cells

	Time after AA withdrawal, h			
	0	24	48	72
CID-9				
Northern blot	1.0	0.9	0.8	0.8
Real-time PCR	1.00 ± 0.15			0.85 ± 0.3
L-1				
Northern blot	1.0	1.0	0.9	0.8
Real-time PCR	1.00 + 0.02			0.65 ± 0.2

Epithelial cells were allowed to differentiate on Matrigel in the presence of insulin, hydrocortisone, and prolactin for 5 days. Cells were washed, and incubated for 72 h in medium lacking AA for the indicated times. Total RNA (10 μg) prepared from intact and deprived cells was fractionated, transferred to a nylon filter, and hybridized with the ^{32}[P]-labeled PCR product of the BLG cDNA. Filters were stripped and re-hybridized with the labeled PCR product of 16 S. The relative intensity of the BLG/16 S signals is presented. In addition, real-time PCR was performed on RNA samples prepared from intact and deprived cells as described in Experimental. Relative mean values ± SEM of $\Delta\Delta C_T$ from three independent experiments are presented.

Total AA deprivation of murine and bovine cells did not alter eIF4E levels over the short term (Fig. 3A) or for up to 120 h (not shown). It has been shown that AA deprivation blocks S6K1 activity in CHO and HEK293 cells, although there are only minor effects in hepatoma cells [Hara et al., 1997; Wang et al., 1998]. To study the effect of AA in regulating S6K1 phosphorylation and activity in mammary epithelial cells, we measured S6K1 phosphorylation on Ser^{411} and Thr^{389}, located in the pseudosubstrate and rapamycin-sensitive domains, respectively. No effect of AA deprivation on Ser^{411} phosphorylation was observed in either L-1 or CID-9 cells after 8 h (Fig. 3A) or up to 120 h (data not shown). By contrast, a significant decrease was observed in Thr^{389} phosphorylation (Fig. 3A,B). The decrease was more rapid in L-1 (~ 50% after 30 min) than in CID-9 cells.

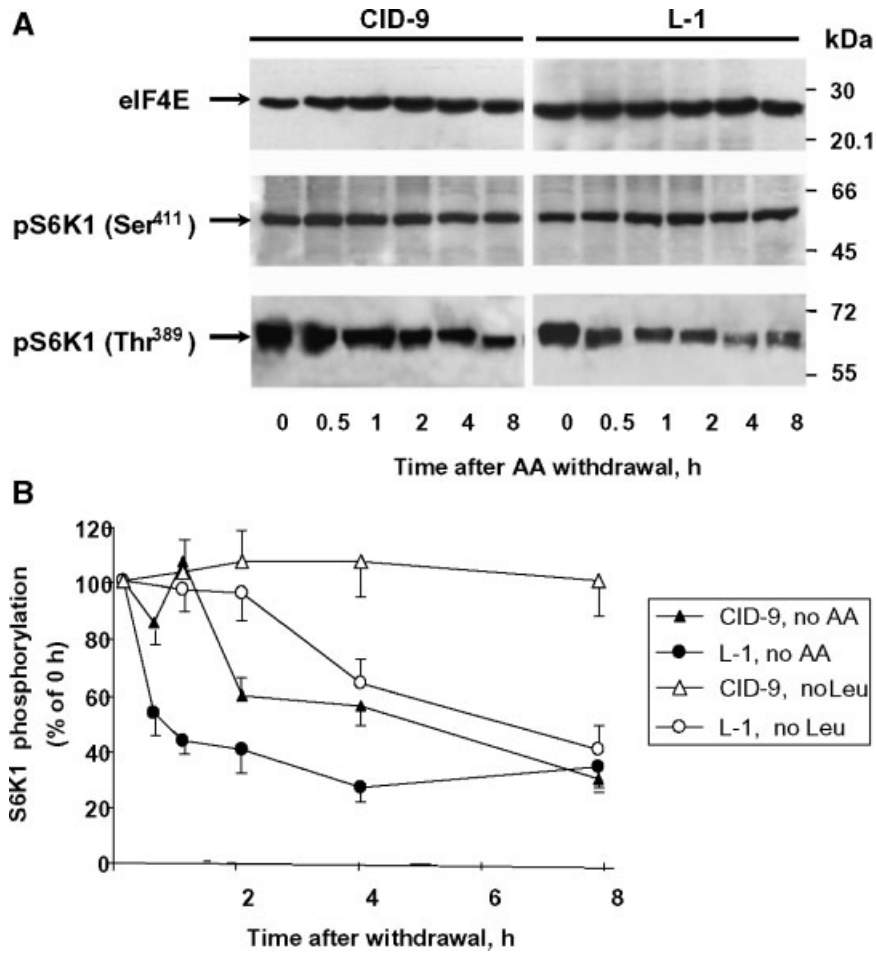

Fig. 3. The level of phosphorylated S6K1(Thr^{389}) decreases more rapidly in AA- and Leu-deprived bovine cells relative to their murine counterparts. **A**: S6K1 phosphorylation on Thr^{389}, but not on Ser^{411} is affected by AA deprivation. Differentiated, hormonally treated cells were deprived of AA for the indicated times. eIF4E, pS6K1(Ser^{411}), and pS6K1(Thr^{389}) were detected by immunoblot analysis. **B**: Densitomatric analysis of signals obtained from S6K1(Thr^{389}) immunoblot of AA- or Leu-deprived cells. Signals in the linear range of their development were quantitated as described in Materials and Methods. In each analysis, values from deprived cells were related to their non-deprived controls. Averaged values ± SEM from three independent analyses are presented.

Differences between the two cell lines were also noted for Leu deprivation (Fig. 3B). Whereas S6K1 phosphorylation in CID-9 was hardly affected after 8 h, it was reduced to less than 50% of the control in L-1 cells. The loss of Thr^{389} phosphorylation was slower for Leu deprivation than AA deprivation.

The amount of eIF4E available for translation is regulated, in part, by sequestration in an inactive complex with 4E-BP1, being released by phosphorylation of the latter [Lin et al., 1994; Pause et al., 1994]. We compared 4E-BP1 status in both mouse and bovine cell lines starved for AA for 6 h (Fig. 4A,C) or 24 h (Fig. 4B,D). 4E-BP1 was detected in its three isoforms: non-phosphorylated (α), partly phosphorylated (β), and fully phosphorylated (γ). In CID-9 cells, there was little change in the γ form over 6 h of AA withdrawal, but it decreased over 24 h, indicating that eIF4E is progressively becoming unavailable for translation (Fig. 4A,B). By contrast, the loss of the γ form was much more rapid in L-1 cells, detectable after only 30 min, reaching 85% loss by 6 h (Fig. 4C), and essentially complete by 24 h (Fig. 4D). Withdrawal of Leu caused similar but less pronounced effects on 4E-BP1 phosphorylation with little effect in CID-9 cells (Fig. 5). Dephosphorylation progressed more slowly than in the AA-deprived cells, but more than 50% loss was still observed in L-1 cells.

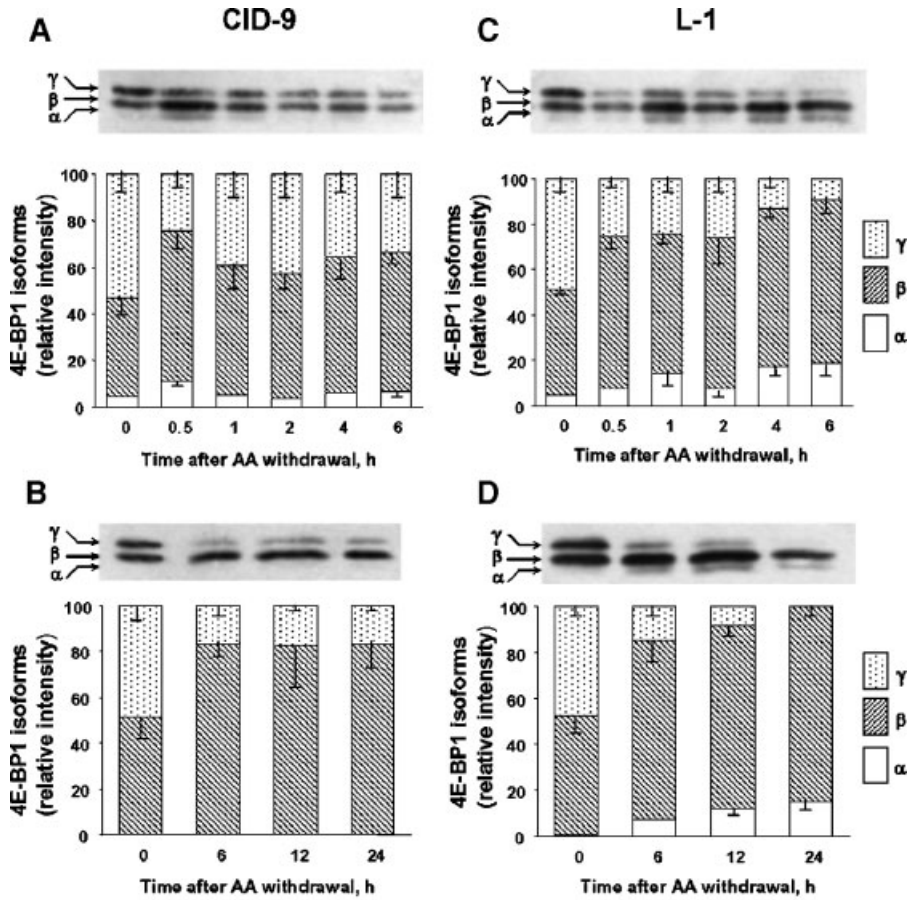

Fig. 4. Dephosphorylation of 4E-BP1 occurs faster in murine cells than in bovine cells following AA deprivation. Differentiated, hormonally treated CID-9 and L-1 cells were incubated in medium lacking AA for a short (**A**, **C**) and longer (**B**, **D**) time. 4E-BP1 was determined in cell extracts by immunoblot analysis and the intensity of the signals was measured by densitometry. Signals of individual 4E-BP1 subunits from three independent cultures in the linear range of their development were quantitated as described in Materials and Methods, averaged, and expressed as mean ± SEM, relative to their combined intensity.

Activated S6K1 directly phosphorylates the S6 ribosomal protein. In AA-deprived CID-9 cells, the decrease in S6K1(Thr^{389}) phosphorylation correlated with decreased S6K1 activity in phosphorylating an artificial substrate with AA sequences derived from the human S6 ribosomal protein (Fig. 6A). Within 0.5 h, S6K1 activity was significantly decreased ($P < 0.05$) and by 8 h, it was only 45% of control. Longer deprivation produced no further effect. In fact, some recovery could be observed. Surprisingly, AA deprivation did not affect S6K1 activity in L-1 cells over the short term but caused a 40% decrease after 4 days. Leu deprivation caused similar but less severe effects. In CID-9 cells there was a rapid but transient decrease in S6K1 activity whereas L-1 cells were hardly affected (Fig. 6B).

Restoring Protein Synthesis by the Addition of AA or Leu to AA-Deprived Cells

The potential of all AA or Leu alone to rescue protein synthesis from deprived cells was studied in differentiated CID-9 and L-1 cells cultured for 72 h in hormonally supplemented, AA-deficient medium. In these cells, total and BLG protein synthesis was decreased to about 25% of control levels (Fig. 7A), whereas BLG mRNA levels remained higher relative to non-deprived cells (Table I). Restoration of protein synthesis was limited to the translation of pre-existing mRNA by the addition of 5 μg/ml actinomycin D, an inhibitor of DNA-dependent RNA synthesis [Loreni et al., 2000]. By 2 h after the addition of AA to the deprived cells, protein synthesis was increased 2.5-fold relative to the

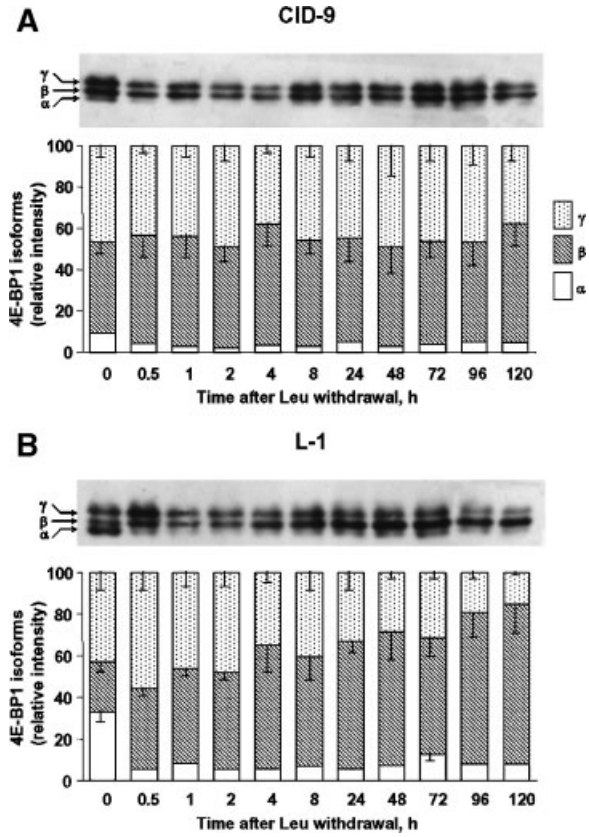

Fig. 5. Leu withdrawal results in dephosphorylation of 4E-BP1 in L-1, but not CID-9 cells. Differentiated, hormonally treated CID-9 (**A**) and L-1 (**B**) cells were incubated in medium lacking Leu for the indicated time periods. 4E-BP1 was determined in cell extracts by immunoblot analysis and the intensity of the signals was measured by densitometry. Signals of the individual 4E-BP1 forms are presented as percentage of the total intensity of the three forms. Signals of individual 4E-BP1 subunits from three independent cultures in the linear range of their development were quantitated as described in Materials and Methods, averaged, and expressed as mean ± SEM, relative to their combined intensity.

depleted controls in both cell lines (Fig. 7A). Nevertheless, the non-starved level of synthesis was only partially recovered. By contrast, Leu alone did not stimulate synthesis of total proteins during this period.

BLG synthesis in CID-9 cells was restored by AA re-addition and reached a level 1.8-fold higher than the control deprived cells after 2 h (Fig. 7B). Surprisingly, Leu alone induced BLG synthesis to a level comparable to that of the complete set of AA and with a shorter lag time—1 h after its addition, a significant induction of BLG synthesis was observed. Unfortunately, an accurate analysis of rescued BLG-synthesis in L-1 cells was not possible due to its low level.

The effect of AA or Leu restoration on 4E-BP1 phosphorylation was also studied in CID-9 and L-1 cells deprived of AA for 6 h (Fig. 8). Withdrawal of AA caused a decrease in the relative level of the fully phosphorylated γ-form to about 10% of the total amount of the protein. As early as 1 min after AA were restored, an increase in the γ form was detected in both cell lines (Fig. 8A,C). Ten minutes later, the control, non-starved level was reached. Readdition of Leu stimulated phosphorylation of 4E-BP1 to control levels in the bovine L-1 cells, whereas CID-9 cells were less affected (Fig. 8B,D).

A similar approach was taken to study the consequences of AA or Leu readdition to AA-deprived cells on S6K1 phosphorylation and activities (Fig. 9). Withdrawal of AA for 8 h resulted in decreased phosphorylation of S6K1(Thr^{389}) to about 30% of control levels in the murine and bovine cells. Partial restoration, up to 80% of control levels, was obtained within 5–10 min of AA readdition to CID-9 cells (Fig. 9A). Leu administration induced S6K1 phosphorylation to up to 60% of the respective controls in both cell lines (Fig. 9B). Exceptionally high phosphorylation levels, up to four times the control, were recovered within 10 min in L-1 cells treated with AA (Fig. 9A). The time course of the restoration of S6K1 activities by AA or Leu in CID-9 cells resembled that of its phosphorylation (Fig. 9C, D). L-1 S6K1 activity, which was not affected by AA deprivation during this time period, was also resistant to the restorative effect.

To study signaling pathways involved in AA regulation of translation, cells were depleted of AA for 72 h and protein synthesis was initiated from pre-existing RNA in the presence of rapamycin, an mTOR inhibitor, LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, or cordycepin, an inhibitor of cytoplasmic polyadenylation (Fig. 10). These inhibitors were added together with actinomycin D 0.5 h before AA or Leu was restored, and cells were cultured for an additional 2 h. The effects of the various inhibitors are shown relative to the level of total protein or BLG synthesis determined at the termination of the incubation period in intact cells.

Rapamycin did not significantly ($P > 0.05$) affect the capability of AA or Leu to restore total protein synthesis in either CID-9 or L-1 cells (Fig. 10a,d). In contrast, at 100 ng/ml, it reduced their potential to rescue BLG synthesis

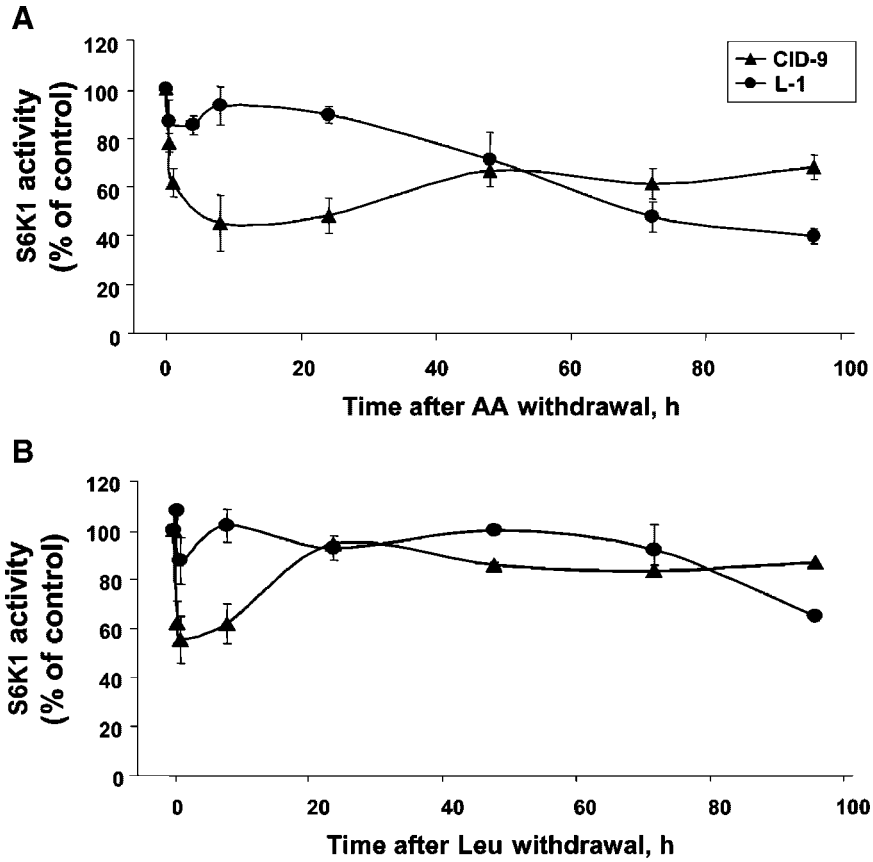

Fig. 6. S6K1 activity is rapidly decreased by AA deprivation in CID-9 cells. Differentiated, hormonally treated L-1 and CID-9 cells were deprived of all AA (**A**) or Leu (**B**) for the indicated time periods. S6K1 activity was determined by analyzing ^{32}[P] incorporation into S6 as described in Materials and Methods. The data represent mean ± SEM of at least three independent experiments.

in CID-9 cells to close to that of the AA-depleted cells (Fig. 10g,j). More prominent effects were observed for LY294002. At 15 μg/ml, total protein synthesis could be rescued to only 45% or 35% of controls by restoration of AA or Leu, respectively (Fig. 10b,e). For AA-treated cells, this represents the baseline of deprived cells, whereas for Leu-treated cells in which total protein synthesis was not restored, this is 65% below basal levels.

The rescue of BLG synthesis by AA or Leu was measured in aliquots containing equal radioactivity of total protein. This required higher levels of extract for cells treated with LY294002. Consequently, the data presented in Figure 10h,k indicate a stronger inhibitory effect of LY294002 on BLG synthesis than on total protein synthesis. Interestingly, the effect of LY294002 was more pronounced on the translation of RNA from cells treated with Leu alone than from those treated with the complete set of AA.

AA-stimulated rescue of total protein synthesis was inhibited by cordycepin in a dose-dependent manner to baseline, AA depleted, levels in the AA-treated cells, and to 15% or 35% of baseline level in the Leu-treated L-1 and CID-9 cells, in which total synthesis was not rescued (Fig. 10c,f). Similar to LY294002, BLG synthesis was more susceptible to the inhibitory effect than total proteins as it still decreased when equal levels of proteins were analyzed (Fig. 10i,l). Finally, at 200 μg/ml, the inhibitory effect of cordycepin was more pronounced on Leu-induced synthesis than on that induced by the total complement of AA. The results with cordycepin indicate that cytoplasmic polyadenylation is required for AA- and Leu-stimulated total protein synthesis and particularly for BLG synthesis.

DISCUSSION

Nutrient supply has long been recognized as essential for maintaining milk-protein levels,

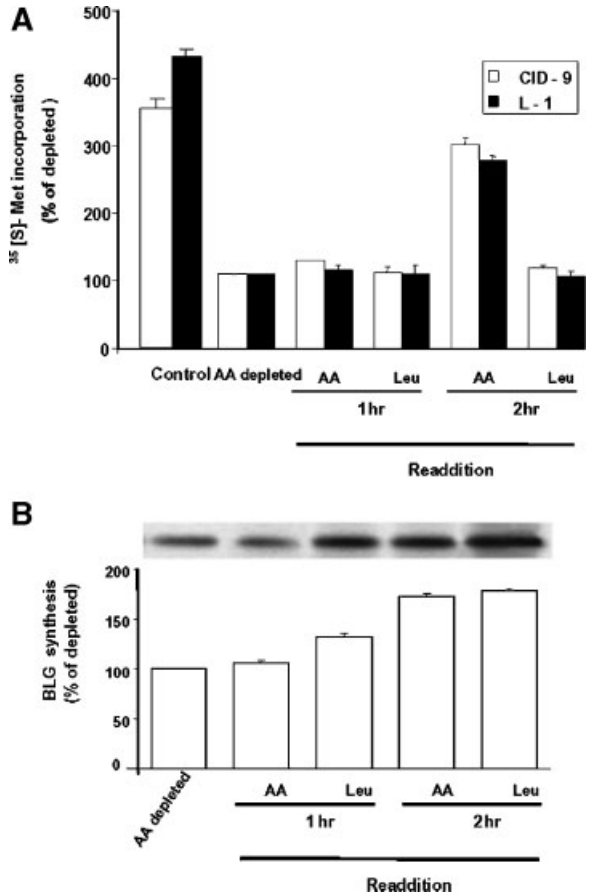

Fig. 7. BLG translation is induced by Leu, independent of its notable effect on total protein synthesis. **A**: Differentiated, hormonally treated CID-9, and L-1 cells were deprived of AA for 72 h. AA or Leu was restored for 1 or 2 h, and metabolic labeling was performed during the first or second hour of restoration. [^{35}S]Met incorporation relative to that determined at the end of starvation, averaged from nine independent experiments, is presented (**B**). BLG was immunoprecipitated from equal amounts of TCA-perciptible proteins and analyzed by SDS–PAGE. Signals at the linear range of their development were quantitated as described in Materials and Methods and related to starved-cell values. Data represent mean ± SEM of five independent experiments.

and nutritional studies aimed at examining the role of individual AA in regulating milk-protein synthesis have been performed in vivo [Maas et al., 1997, 1998; Shennan and Peaker, 2000] or in the perfused mammary gland [Mepham et al., 1979]. However, an accurate evaluation of the direct role of single amino acid in the control of milk-protein synthesis has never been possible due to the different uptakes of the individual AA by the gland. Furthermore, to the best of our knowledge, translational control of milk protein synthesis by AA has never been studied. In the current work, we have studied the effect of AA manipulation on translational regulation of the milk-protein BLG in two cell lines, which differ in their capabilities to synthesize total proteins or the milk-specific protein BLG. This difference may not merely result from a random disparity among cell lines, since both cultures were prepared by a similar methodology. Although L-1 cells have undergone one more step and represents a single clone, these cells synthesize BLG comparably to most other clones. Indeed, lower BLG expression in L-1 cells compared to the murine CID-9 cell population was detected even after transfection with additional copies of the BLG gene. To some extent, these differences may represent the intrinsic properties of epithelial cells in the mammary glands of the two species, resulting in lower levels of milk-protein content in bovine versus rodents.

Despite the higher rate of protein synthesis in the murine epithelial cells, AA and Leu deprivation of L-1 and CID-9 cultures resulted in comparable rates of decrease in the synthesis of total proteins by the differentiated cultures of the two cell lines. However, synthesis of the milk protein BLG in L-1 cells was more susceptible to the lack of total AA or of the individual amino acid Leu. The sharper and more pronounced decrease in BLG protein synthesis in bovine cells was associated with dephosphorylation of 4E-BP1 and S6K1 (Thr^{389}). While other factors, such as eIF2B activity, eIF4E phosphorylation, or the rate of peptide elongation, may also be involved [Kimball and Jefferson, 2000], we suggest that the changes in 4E-BP1 and S6K1(Thr^{389}) phosphorylation reflect a faster and more pronounced operation of the signaling apparatus in the bovine cells, and this achieves a more rapid shutdown of translation in the face of AA insufficiency. The concept of exploiting S6K1 or 4E-BP1 phosphorylation status as an indicator of the cell's inclination to shut down translation has been previously presented using CHO-IR cells [Hara et al., 1998]. The current study validates this concept by correlating the stringency of 4E-BP1 and S6K1 regulation of phosphorylation in two comparable mammalian cells to their in vivo function, that is, the ability to synthesize milk protein. Interestingly, within a given cell culture, some differences in the AA effects on the individual translation factors were observed. The best example of this is the fourfold induction of S6K1 (Thr^{389}) phosphorylation in AA-rescued L-1 cells, which

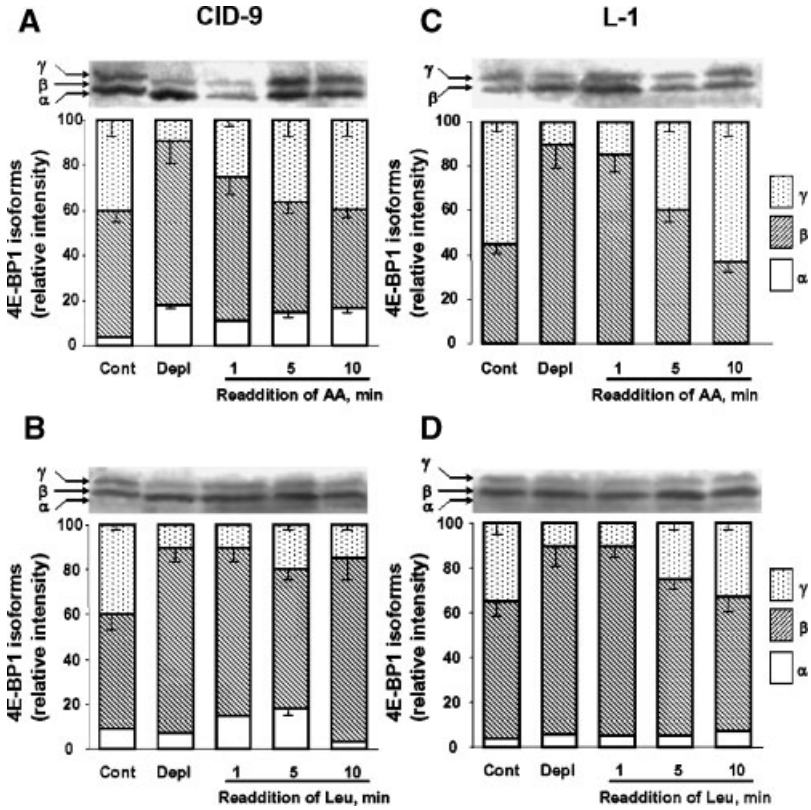

Fig. 8. 4E-BP1 phosphorylation is better restored by AA or Leu in bovine L-1 cells compared to their murine CID-9 counterparts. Differentiated, hormonally treated CID-9 (**A**, **B**), and L-1 (**C**, **D**) cells were deprived of AA for 6 h. AA (A, C) or Leu (B, D) were then restored for up to 10 min and 4E-BP1 was analyzed in the cell lysate by immunoblot. Signals of individual 4E-BP1 subunits from three independent cultures at the linear range of their development were quantitated as described in Materials and Methods, averaged, and expressed as mean ± SEM, relative to their combined intensity.

was associated with recovery of 4E-BP1 phosphorylation to only the control baseline. The difference between the rates of the 4E-BP1 and S6K1 reactions may result from multiple links between mTOR and its downstream targets [Wang et al., 2005] or from different sensitivities to hydrocortisone [Liu et al., 2004], which in combination with insulin and prolactin form the lactogenic hormone mixture.

S6K1 activity has generally been correlated with the phosphorylation of Thr^{389}, which is located at a rapamycin-sensitive phosphorylation site in the C-terminal linker domain. Nevertheless, other sites have also been suggested as relevant. Here we demonstrate that in neither cell line does AA deprivation involve phosphorylation of Ser^{411}, which together with Ser^{418}, Thr^{421}, and Ser^{424} lie in a serine-proline-rich region within the pseudosubstrate region. Phosphorylation at this site is thought to activate S6K1 via relief of pseudosubstrate suppression [Pullen et al., 1998; Dufner and Thomas, 1999]. However, S6K1 activity is not always correlated with Ser^{411} phosphorylation as shown in mitotic HeLa cells [Shah et al., 2003]. In the current study, we were only able to correlate S6K1 phosphorylation on Thr^{389} with its activity in CID-9 cells. The reason for the delayed decrease in S6K1 activity compared to its phosphorylation (Thr^{389}) in the AA-deprived L-1 cells is unknown.

It is worth noting that both the murine and bovine epithelial cell cultures synthesize BLG for at least 72 h in the absence of extracellular AA. This continuous rather than immediate decrease in BLG synthesis supports the concept of an intracellular nutrient sensor [Proud, 2004]. In that defined ex vivo environment, it senses a gradual decrease in the internal pool of AA rather than their complete elimination from the medium and regulates cellular anabolic processes accordingly. Upon restoration, however, this putative sensor is able to rapidly signal to translation initiation. Data collected

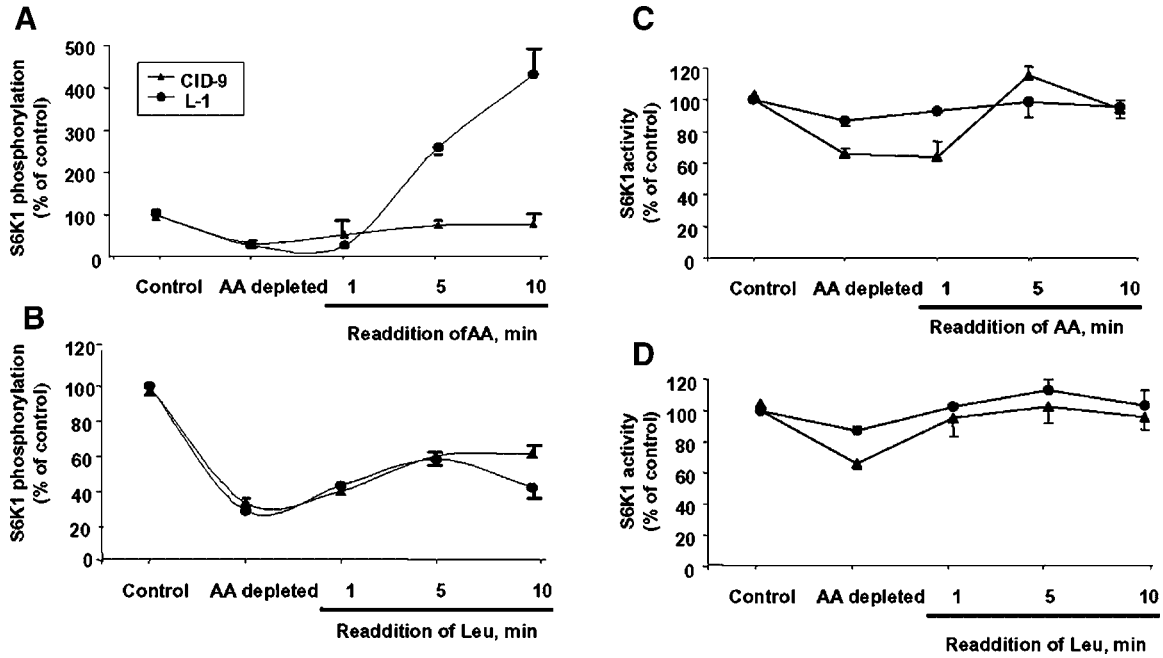

Fig. 9. Rescue of S6K1 phosphorylation and activity by AA or Leu supplementation to AA-deprived CID-9 and L-1 mammary cells. **A**: Differentiated, hormonally treated CID-9, and L-1 cells were deprived of AA for 8 h. AA or Leu were then restored for up to 10 min and S6K1 phosphorylation (on Thr^{389}) (**A**, **B**) or activity (**C**, **D**) was analyzed in the cell lysate. Signals from three analyses of S6K1 phosphorylation at the linear range of their development were quantitated as described in Materials and Methods and expressed relative to intact control cells. S6K1 activity was measured in three independent analyses and expressed as mean ± SEM.

from *Xenopus* oocytes microinjected with Leu [Christie et al., 2002] suggest that this AA sensor is associated with Rheb, a positive regulator of mTOR signaling, which serves as a component of the mTOR complex [Kimball and Jefferson, 2004].

Lack of Leu had a less marked effect on BLG synthesis than that detected in the absence of total AA. This implies that additional AA might be involved in controlling translation suppression in the mammary gland during deprivation. The more gradual decrease in BLG synthesis in Leu-deprived cells does not necessarily indicate translational regulation, since a clear delineation between the roles of AA in translation signaling and as substrates for synthesis of the polypeptide chain could not be made. The less dramatic effect of Leu on 4E-BP1 and S6K1 phosphorylation supports the view that additional AA participate in translational regulation during deprivation in mammary epithelial cells. The rate of activation of translational initiation by Leu differs significantly between muscle, liver, PC12 cells, CHO cells, and other tissues [Hara et al., 1997; Kleijn and Proud, 2000; Anthony et al., 2001]. In most tissues studied, and particularly muscle, Leu is the most effective AA in regulating protein synthesis. Nevertheless, other AA, such as Gln, could play roles as well [Kimball and Jefferson, 2002].

The analysis of translational control in the deprived L-1 and CID-9 cells was complemented by restoration experiments conducted in actinomycin D-treated cultures for shorter times in order to focus on translational control. Restoration of BLG synthesis from its pre-existing RNA was inferred from the difference between relatively high levels of BLG mRNA and decreased BLG protein in AA-deprived cells. This discrepancy most likely results from stabilizing BLG mRNA with prolactin and hydrocortisone [Chomczynski et al., 1984, 1986; Eisenstein and Rosen, 1988] and suppressing the synthesis of short-lived cytosolic protein(s) involved in the degradation of BLG mRNA during AA deprivation [Yokota et al., 1995; Hesketh et al., 1998]. Sufficient amounts of BLG RNA in the AA-deprived CID-9 cells enabled Leu to rapidly and specifically stimulate BLG mRNA translation without affecting total protein synthesis. This constitutes evidence for specific translational regulation of

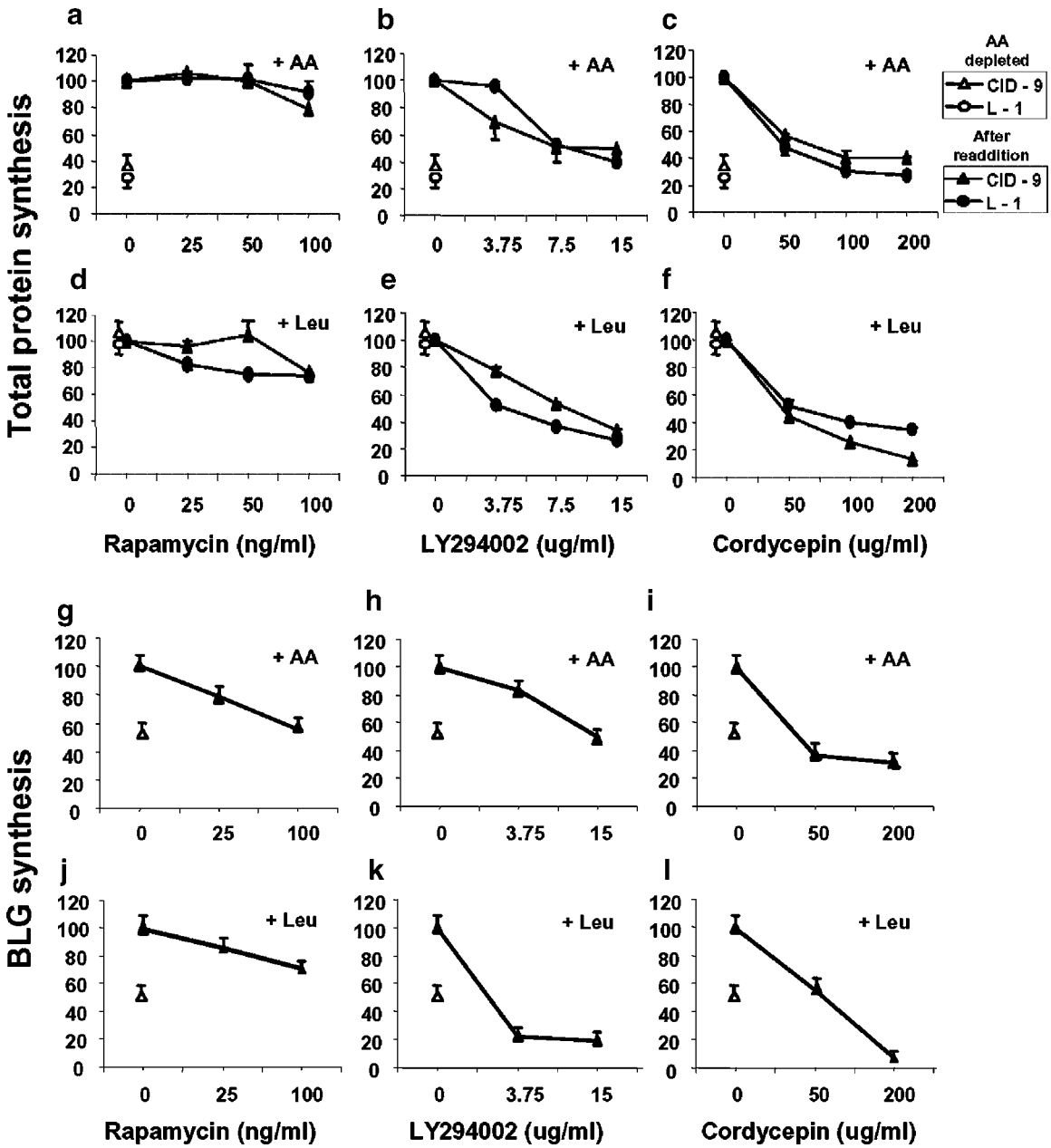

Fig. 10. Metabolic pathways involved in regulating the translation of BLG mRNA. Differentiated, hormonally treated CID-9, and L-1 cells were deprived of AA for 72 h. Then, AA or Leu were restored for 2 h in the presence of rapamycin, LY294002 or cordycepin. To prevent transcription initiation, the culture medium also contained actinomycin D. Total protein synthesis was determined by metabolic labeling of cells with [^{35}S] Met during the second hour following the addition of AA (**a–c**), or Leu (**d–f**). Radioactive TCA-perciptible proteins were counted and data were related to that measured in control intact cells. BLG synthesis, restored by AA (**g–i**) or Leu (**j–l**), was measured by immunoprecipitation of CID-9 cell lysate containing equal amounts of TCA-perciptible proteins. The intensity of the signals originated from the three independent analyses was quantitated the linear range of their development as described in Materials and Methods, averaged and presented as mean ± SEM relative to that measured in control, intact cells.

milk proteins by AA. Apparently, the potential of Leu to regulate BLG synthesis depends on particular environmental conditions. In fact, our studies with deprived mammary epithelial cells indicate that protein synthesis as well as 4E-BP1 and S6K1 phosphorylation are more affected by the elimination of all AA relative to that of Leu alone. Conversely, the similar levels of BLG synthesis rescued by Leu and the full set of AA suggest that upon restoration, BLG

mRNA translation depends on additional or rate-limiting factor(s), such as those involved in controlling the elongation phase of translation [Proud, 2000], which participate, along with 4E-BP1 and S6K1, in setting the final translation rate.

AA convey their signal via mTOR but in a PI3K-dependent manner [Gingras et al., 2001]. Insulin and prolactin mediate PI3K activity via IRS-1 [Myers et al., 1994; Berlanga et al., 1997; Drakas et al., 2004], but the synergism between these hormones, which is needed for lactogenic activity, involves polyadenylation of milk protein mRNAs [Choi et al., 2004]. We found that interfering with the latter mechanism results in severe inhibition of BLG synthesis. Our data indicate that initiation of BLG mRNA translation by AA or Leu alone relies mainly on a direct effect via mTOR, but in the absence of the complete set of AA, BLG translation is also susceptible to the inhibition of the PI3 signaling pathway. The high sensitivity of BLG to AA manipulation of translation highlights this delicate mechanism of regulating milk protein expression.

ACKNOWLEDGMENTS

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REFERENCES

Anthony TG, Reiter AK, Anthony JC, Kimball SR, Jefferson LS. 2001. 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.

Barash I. 1999. Prolactin and insulin synergize to regulate the translation modulator 4E-BP1 via mitogen-activated protein kinase-independent but wortmannin- and rapamycin-sensitive pathway. Mol Cell Endocrinol 155:37–49.

Barash I, Faerman A, Richenstein M, Kari R, Damary GM, Shani M, Bissell MJ. 1999. In vivo and in vitro expression of human serum albumin genomic sequences in mammary epithelial cells with beta-lactoglobulin and whey acidic protein promoters. Mol Reprod Dev 52:241–252.

Baruch A, Shani M, Hurwitz DR, Barash I. 1995. Developmental regulation of the ovine beta-lactoglobulin/human serum albumin transgene is distinct from that of the beta-lactoglobulin and the endogenous beta-casein genes in the mammary gland of transgenic mice. Dev Genet 16:241–252.

Baruch A, Shani M, Barash I. 1998. Insulin and prolactin synergize to induce translation of human serum albumin in the mammary gland of transgenic mice. Transgenic Res 7:15–27.

Berlanga JJ, Gualillo O, Buteau H, Applanat M, Kelly PA, Edery M. 1997. Prolactin activates tyrosyl phosphorylation of insulin receptor substrate 1 and phosphatidylinositol-3-OH kinase. J Biol Chem 272:2050–2052.

Brophy B, Smolenski G, Wheeler T, Wells D, L'Huillier P, Laible G. 2003. Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. Nat Biotechnol 21:157–162.

Choi KM, Barash I, Rhoads RE. 2004. Insulin and prolactin synergistically stimulate beta-casein messenger ribonucleic acid translation by cytoplasmic polyadenylation. Mol Endocrinol 18:1670–1686.

Chomczynski P, Qasba P, Topper YJ. 1984. Essential role of insulin in transcription of the rat 25,000 molecular weight casein gene. Science 226:1326–1328.

Chomczynski P, Qasba P, Topper YJ. 1986. Transcriptional and post-transcriptional roles of glucocorticoid in the expression of the rat 25,000 molecular weight casein gene. Biochem Biophys Res Commun 134:812–818.

Christie GR, Hajduch E, Hundal HS, Proud CG, Taylor PM. 2002. Intracellular sensing of amino acids in Xenopus laevis oocytes stimulates p70 S6 kinase in a target of rapamycin-dependent manner. J Biol Chem 277:9952–9957.

Coletta RD, Jedlicka P, Gutierrez-Hartmann A, Ford HL. 2004. Transcriptional control of the cell cycle in mammary gland development and tumorigenesis. J Mammary Gland Biol Neoplasia 9:39–53.

Danielson KG, Oborn CJ, Durban EM, Butel JS, Medina D. 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc Natl Acad Sci USA 81:3756–3760.

Drakas R, Tu X, Baserga R. 2004. Control of cell size through phosphorylation of upstream binding factor 1 by nuclear phosphatidylinositol 3-kinase. Proc Natl Acad Sci USA 101:9272–9276.

Dufner A, Thomas G. 1999. Ribosomal S6 kinase signaling and the control of translation. Exp Cell Res 253:100–109.

Eisenstein RS, Rosen JM. 1988. Both cell substratum regulation and hormonal regulation of milk protein gene expression are exerted primarily at the posttranscriptional level. Mol Cell Biol 8:3183–3190.

German T, Barash I. 2002. Characterization of an epithelial cell line from bovine mammary gland. In Vitro Cell Dev Biol Anim 38:282–292.

Gingras AC, Raught B, Sonenberg N. 2001. Regulation of translation initiation by FRAP/mTOR. Genes Dev 15: 807–826.

Griinari JM, McGuire MA, Dwyer DA, Bauman DE, Barbano DM, House WA. 1997. The role of insulin in the regulation of milk protein synthesis in dairy cows. J Dairy Sci 80:2361–2371.

Groner B. 2002. Transcription factor regulation in mammary epithelial cells. Domest Anim Endocrinol 23:25–32.

Hara K, Yonezawa K, Kozlowski MT, Sugimoto T, Andrabi K, Weng QP, Kasuga M, Nishimoto I, Avruch J. 1997. Regulation of eIF-4E BP1 phosphorylation by mTOR. J Biol Chem 272:26457–26463.

Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J. 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J Biol Chem 273:14484–14494.

Hesketh JE, Vasconcelos MH, Bermano G. 1998. Regulatory signals in messenger RNA: Determinants of nutrient-gene

interaction and metabolic compartmentation. Br J Nutr 80:307–321.
Iavnilovitch E, Cardiff RD, Groner B, Barash I. 2004. Deregulation of Stat5 expression and activation causes mammary tumors in transgenic mice. Int J Cancer 112: 607–619.
Kimball SR. 2002. Regulation of global and specific mRNA translation by amino acids. J Nutr 132:883–886.
Kimball SR, Jefferson LS. 2000. Regulation of translationinitiation in mammelian cells by amino acids. In: Sonenberg NH, Hershey JWB, Mathews MB, editors. Translation control of gene expression. New York: Cold Spring Harbor Laboratory Press. pp 561–579.
Kimball SR, Jefferson LS. 2002. Control of protein synthesis by amino acid availability. Curr Opin Clin Nutr Metab Care 5:63–67.
Kimball SR, Jefferson LS. 2004. Molecular mechanisms through which amino acids mediate signaling through the mammalian target of rapamycin. Curr Opin Clin Nutr Metab Care 7:39–44.
Kimball SR, Horetsky RL, Jefferson LS. 1998. Implication of eIF2B rather than eIF4E in the regulation of global protein synthesis by amino acids in L6 myoblasts. J Biol Chem 273:30945–30953.
Kleijn M, Proud CG. 2000. Glucose and amino acids modulate translation factor activation by growth factors in PC12 cells. Biochem J 347:399–406.
Kuraishi T, Sun Y, Aoki F, Imakawa K, Sakai S. 2000. The poly(A) tail length of casein mRNA in the lactating mammary gland changes depending upon the accumulation and removal of milk. Biochem J 347:579–583.
Lin TA, Kong X, Haystead TA, Pause A, Belsham G, Sonenberg N, Lawrence JC Jr. 1994. 4E-BP1 as a link between mitogen-activated protein kinase and translation initiation. Science 266:653–656.
Liu Z, Li G, Kimball SR, Jahn LA, Barrett EJ. 2004. Glucocorticoids modulate amino acid-induced translation initiation in human skeletal muscle. Am J Physiol Endocrinol Metab 287:E275–E281.
Loreni F, Thomas G, Amaldi F. 2000. Transcription inhibitors stimulate translation of 5′ TOP mRNAs through activation of S6 kinase and the mTOR/FRAP signalling pathway. Eur J Biochem 267:6594–6601.
Maas JA, France J, McBride BW. 1997. Model of milk protein synthesis. A mechanistic model of milk protein synthesis in the lactating bovine mammary gland. J Theor Biol 187:363–378.
Maas JA, France J, Dijkstra J, Bannink A, McBride BW. 1998. Application of a mechanistic model to study competitive inhibition of amino acid uptake by the lactating bovine mammary gland. J Dairy Sci 81:1724–1734.
Marti A, Lazar H, Ritter P, Jaggi R. 1999. Transcription factor activities and gene expression during mouse mammary gland involution. J Mammary Gland Biol Neoplasia 4:145–152.
Mepham TB, Peters AR, Alexandrov S. 1979. Effects of restriction of amino acid supply to the isolated perfused guinea-pig mammary gland. J Dairy Res 46:69–73.
Myers MG, Jr., Grammer TC, Wang LM, Sun XJ, Pierce JH, Blenis J, White MF. 1994. Insulin receptor substrate-1 mediates phosphatidylinositol 3′-kinase and p70S6k signaling during insulin, insulin-like growth factor-1, and interleukin-4 stimulation. J Biol Chem 269:28783–28789.
Pause A, Belsham GJ, Gingras AC, Donze O, Lin TA, Lawrence JC, Jr., Sonenberg N. 1994. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. Nature 371:762–767.
Persuy MA, Stinnakre MG, Printz C, Mahe MF, Mercier JC. 1992. High expression of the caprine beta-casein gene in transgenic mice. Eur J Biochem 205:887–893.
Proud C. 2000. Control of the elongation phase of protein synthesis. In: Sonenberg NH, Hershey JWB, Mathews MB, editors. Translation control of gene expression. New York: Cold Spring Harbor. pp 719–739.
Proud CG. 2004. mTOR-mediated regulation of translation factors by amino acids. Biochem Biophys Res Commun 313:429–436.
Pullen N, Dennis PB, Andjelkovic M, Dufner A, Kozma SC, Hemmings BA, Thomas G. 1998. Phosphorylation and activation of p70s6k by PDK1. Science 279:707–710.
Rijnkels M, Kooiman PM, Krimpenfort PJ, de Boer HA, Pieper FR. 1995. Expression analysis of the individual bovine beta-, alpha s2- and kappa-casein genes in transgenic mice. Biochem J 311:929–937.
Rosen JM, Wyszomierski SL, Hadsell D. 1999. Regulation of milk protein gene expression. Annu Rev Nutr 19:407–436.
Shah OJ, Ghosh S, Hunter T. 2003. Mitotic regulation of ribosomal S6 kinase 1 involves Ser/Thr, Pro phosphorylation of consensus and non-consensus sites by Cdc2. J Biol Chem 278:16433–16442.
Shani M, Barash I, Nathan M, Ricca G, Searfoss GH, Dekel I, Faerman A, Givol D, Hurwitz DR. 1992. Expression of human serum albumin in the milk of transgenic mice. Transgenic Res 1:195–208.
Shennan DB, Peaker M. 2000. Transport of milk constituents by the mammary gland. Physiol Rev 80:925–951.
Tang H, Hornstein E, Stolovich M, Levy G, Livingstone M, Templeton D, Avruch J, Meyuhas O. 2001. Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Mol Cell Biol 21:8671–8683.
Wang X, Campbell LE, Miller CM, Proud CG. 1998. Amino acid availability regulates p70 S6 kinase and multiple translation factors. Biochem J 334:261–267.
Wang X, Beugnet A, Murakami M, Yamanaka S, Proud CG. 2005. Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. Mol Cell Biol 25:2558–2572.
Yokota T, Kanamoto R, Hayashi S. 1995. c-myc mRNA is stabilized by deprivation of amino acids in primary cultured rat hepatocytes. J Nutr Sci Vitaminol (Tokyo) 41:455–463.