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ORIGINAL ARTICLE

Specificity of amino acid regulated gene expression: analysis of genes subjected to either complete or single amino acid deprivation

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Abstract Amino acid deprivation activates the amino acid response (AAR) pathway that enhances transcription of genes containing an amino acid response element (AARE). The present data reveal a quantitative difference in the response to deprivation of individual amino acids. The AAR leads to increased eukaryotic initiation factor 2α (eIF2α) phosphorylation and ATF4 translation. When HepG2 cells were deprived of an individual essential amino acid, p-eIF2α and activating transcription factor 4 were increased, but the correlation was relatively weak. Complete amino acid starvation in either Earle's balanced salt solution or Krebs–Ringer bicarbonate buffer (KRB) resulted in activation of transcription driven by a *SNAT2* genomic fragment that contained an AARE. However, for the KRB, a proportion of the transcription was AARE-independent suggesting that amino acid-independent mechanisms were responsible. Therefore, activation of AARE-driven transcription is triggered by a deficiency in any one of the essential amino acids, but the response is not uniform. Furthermore, caution must be exercised when using a medium completely devoid of amino acids.

Keywords SNAT2 · System A · Nutrient starvation · Amino acid transport · ATF4 · eIF2

Abbreviations

AAR	Amino acid response
AARE	Amino acid response element
ASNS	Asparagine synthetase
ATF3	Activating transcription factor 3
ATF4	Activating transcription factor 4
C/EBPβ	CCAAT/enhancer-binding protein beta
CAT-1	Cationic amino acid transporter
CHOP	C/EBP homology protein
EAA	Essential amino acids
EBSS	Earle's balanced salt solution
eIF2	Eukaryotic initiation factor 2
GCN2	General control non-derepressible
KRB	Krebs–Ringer bicarbonate buffer
MEM	Minimal essential medium
qPCR	Quantitative real-time PCR
RT-PCR	Reverse transcriptase-polymerase chain reaction
SNAT2	System A sodium-dependent neutral amino acid transporter 2
VEGF	Vascular endothelial growth factor

Introduction

Amino acids are involved in a variety of regulatory processes in both healthy and pathological states (Perta-Kajan et al. 2007; Suryawan et al. 2008) and their role as signaling molecules that regulate gene expression has received considerable interest in recent years (Hu et al. 2008; Liao et al. 2008). Amino acid availability influences

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transcription, mRNA maturation, translation, mRNA turnover, and autophagy, and, as a consequence, amino acids impose an integrative effect on cellular metabolism as a whole (Kilberg et al. 2005). Limiting one or all of the amino acids in vivo or in vitro is an experimental model that is used extensively to characterize the cellular transcriptional response to nutritional stress and an ever increasing number of amino acid regulated genes have been identified (Kilberg et al. 2005).

One of the initial responses to amino acid limitation is the activation of the general control non-derepressible 2 (GCN2) kinase, which in turn, catalyzes the phosphorylation of the alpha subunit of the eukaryotic initiation factor eIF2 (Harding et al. 2000; Kimball and Jefferson 2005; Zhang et al. 2002), although it is important to note that amino acid sensing mechanisms independent of GCN2-eIF2α appear to exist as well (Kimball and Jefferson 2006; Wang and Proud 2008). An immediate consequence of eIF2α phosphorylation is a general decrease in translation for the majority of mRNAs, but an enhancement in the translation of a subset of proteins involved in the adaptive response to nutritional stress. One of the primary targets of this enhanced translation is activating transcription factor 4 (ATF4) for which translation is minimal under amino acid-fed conditions, but highly induced from pre-existing mRNA following amino acid deprivation (Lu et al. 2004; Vattem and Wek 2004). Subsequently, ATF4 activates transcription of a spectrum of genes important for adaptation to the cell stress. For amino acid deprivation, this ATF4-dependent transcriptional activation occurs via a C/EBP-ATF composite site that functions as an amino acid response element (AARE) (Kilberg et al. 2005). Interestingly, there are multiple reports of differences across genes in the response to deprivation of all amino acids or to limitation of individual amino acids (Fernandez et al. 2003; Jousse et al. 2000; Lee et al. 2008) and therefore, additional levels of modulation of the ATF4 pathway cannot be excluded. Furthermore, evidence for the existence of ATF4-independent pathways has been reported as well (Gaccioli et al. 2006).

System A neutral amino acid transport activity is a sodium-dependent transporter that is expressed in most, if not all, nucleated mammalian cells (Bode 2001; Broer 2002; Brosnan 2003; Hyde et al. 2003). System A activity is relatively low in cells that are slowly dividing or in metabolic homeostasis, but its expression is responsive to a wide variety of hormones, cytokines, and to cell stress. With regard to the latter point, the activity is up-regulated in response to amino acid deprivation (Gazzola et al. 1972; Riggs and Pan 1972). Three System A-encoding genes have been identified and the nomenclature SNAT1, 2, and 4 (sodium-coupled neutral amino acid transporter) adopted (Mackenzie and Erickson 2004). It has been established that SNAT2 is the primary isoform that is induced at the level of transcription when mammalian cells are deprived of amino acids (Gazzola et al. 2001; Ling et al. 2001). The *SNAT2* gene represents an interesting model to investigate how changes in nutrient-regulated transcription are initiated and maintained. Furthermore, insight into the regulation of SNAT2-mediated transport may provide fundamental knowledge about how changes in dietary protein and the consequent plasma amino acid levels impact cellular metabolism. A C/EBP-ATF composite sequence (5′-ATTGCATCA-3′) was identified within the first intron of the *SNAT2* gene (nt +709/+717) that functions as an AARE and therefore, mediates the induction of *SNAT2* transcription by amino acid deprivation (Palii et al. 2004). Over-expression of exogenous ATF4 can activate transcription from a reporter gene driven by the *SNAT2* AARE (Palii et al. 2006), but total amino acid starvation of ATF4-deficient mouse embryonic fibroblasts still results in increased SNAT2 expression (Gaccioli et al. 2006).

The purpose of the present investigation was to further characterize the amino acid response (AAR) pathway under conditions of either single amino acid limitation or total amino acid starvation. Screening of 19 amino acid-regulated genes showed that there are differences in their response to limitation of individual amino acids. Exposure of HepG2 hepatoma cells to limiting amounts of any single essential amino acid resulted in the activation of the AAR pathway, as measured by phosphorylation of eIF2α (p-eIF2α) and ATF4 protein expression. As a consequence, transcription mediated through the *SNAT2* AARE was enhanced. Transcription from a luciferase reporter gene driven by a *SNAT2* genomic fragment was also increased when cells were incubated in a medium lacking a single amino acid or in either of two different media that were completely devoid of all amino acids. However, for one of these amino acid-free media, Krebs–Ringer buffer (KRB), the activation of transcription was not mediated by the AARE sequence, suggesting that the composition of the medium used to expose cells to an amino acid-free environment may trigger transcriptional activation mechanisms independent of an AARE.

Materials and methods

Cell culture

Mouse embryonic fibroblasts were maintained in Dulbecco's modified Eagles medium (DMEM) and HepG2 human hepatoma cells were cultured in minimal essential medium (MEM) (Mediatech, Herndon, VA, USA), supplemented to contain 1× non-essential amino acid solution, 4 mM glutamine, 100 mg/ml streptomycin sulfate, 100 units/ml

penicillin G, 0.25 mg/ml amphotericin B, and 5% (v/v) fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

Nutrient deprivation of HepG2 cells

To treat the HepG2 cells with amino acid-deficient medium, the complete MEM was removed by aspiration and replaced with the respective treatment medium: complete MEM, MEM lacking an individual amino acid, or a buffered salt solution lacking all amino acids. The complete amino acid deprivation media were either Earle's Balanced Salt Solution (EBSS) (Mediatech, Herndon, VA, USA) and KRB, and their salt compositions are listed in Table 1. All media were supplemented with 5% dialyzed fetal bovine serum during amino acid limitation. Individual amino acid deprivation media were prepared by supplementing EBSS with the appropriate amino acid stock solutions for the remaining 19 amino acids to equal their concentration in commercially available MEM, and then supplemented with 2 mM glutamine, 1× vitamin mix (Invitrogen, Carlsbad, CA, USA), 1× antibiotic–antimycotic mix (ABAM, Mediatech), and 5% dialyzed fetal bovine serum.

Plasmid constructs

The transcription reporter plasmids used were described previously (Palii et al. 2004), and contain the human *SNAT2* genomic wild type sequence from nt −512/+770, the −512/+770 sequence with the *SNAT2* AARE mutated, as well as the 3′ sequence deletion fragments −512/+59, −512/+400, and −512/+698, all driving the Firefly luciferase reporter gene in pGL3 (Promega, Madison, WI, USA).

Transient transfection and luciferase assays

Human HepG2 hepatoma cells were seeded in 24-well plates at the density of 1.5×10^5 cells/well in complete MEM and allowed to recover for 24 h. Transfection was performed with Superfect reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Typically, cells were transfected with 1.5 μg of plasmid DNA per well and 6 μl of reagent. After 3 h, cells were rinsed once with phosphate-buffered saline (PBS) and transferred to fresh MEM. At 16 h post-transfection, the medium was removed, the cells were rinsed once with PBS and incubated for 10 h in 1 ml/well of either complete MEM or the appropriate amino acid-deprived medium. After the completion of treatment, the cells were rinsed with PBS, lysed with 100 μl of 1× passive lysis buffer (Promega), and stored at −20°C overnight to assure complete disruption of the membranes following the freeze-thaw cycle. Luciferase activities were measured in a Sirius luminometer (Berthold Detection Systems, GmbH, Germany) with the Luciferase Reporter Assay System (Promega). The measurements were normalized against total protein content as evaluated by a Bradford assay (Bio-Rad, Hercules, CA, USA). For the luciferase assays presented, each graph was produced by averaging three independent experiments, each experiment consisting of six replicates. The values were compared to the appropriate MEM control value and statistical analysis was performed by Students *t*-test.

Table 1 Formulations for Krebs–Ringer Bicarbonate (KRB) and Earle's Balanced Salt Solution (EBSS)

Component	KRB (mM)	EBSS (mM)
$CaCl_2$ (anhydrous)	2.50	1.80
KCl	5.90	5.30
$MgSO_4$	1.20	0.81
NaCl	119.00	117.4
$NaHCO_3$	25.0	26.2
$NaH_2PO_4{\cdot}H_20$	–	1.01
K_2HPO_4	1.2	–
D-Glucose	5.60	5.55

Immunoblot analysis

Protein extracts were prepared upon completion of treatment and quantified by a Lowry assay (Kilberg 1989). Equal amounts of protein (30 μg) were separated on precast Criterion™ Tris/HCl polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and transferred to a Bio-Rad nitrocellulose membrane. Equal loading and transfer efficiency were monitored by staining the membranes with Fast Green. For ATF4 and eIF2α, the immunoblotting protocol was as described previously (Thiaville et al. 2008). For 4E-BP1, the membranes were blocked 5% bovine serum albumin and Tris-buffered saline/Tween [(TBS/T) (30 mM Tris base (pH 7.5), 200 mM NaCl and 0.1% Tween-20)] for 1 h at room temperature, with constant mixing. The primary antibody incubation (0.2–2 mg/ml) was performed overnight at 4°C with rotation in blocking solution. Afterwards the membrane was washed five times, 5 min each in 5% blocking solution on a shaker, followed by incubation with the appropriate peroxidase-conjugated secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) in 5–10% carnation non-fat dry-milk blocking solution for 1 h at room temperature with constant mixing. The membrane was washed five times in blocking solution and two times in TBS/T, 5 min each and the signal was detected with an Enhanced Chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA) by exposure of the membrane to Biomax® MR film

(Kodak, Rochester, NY, USA). For ATF4, equal loading was verified by probing for beta-actin (Abcam, Cambridge, MA, USA) at a dilution of 1:6,000. Antibodies for total eIF2α (cat no. 9722), p-eIF2α (cat no. 9721), 4E-BP1 (cat no. 9452), and p-4E-BP1 (cat no. 9455) were from Cell Signaling (Danvers, MA, USA).

Microarray analysis

Cell culture and RNA isolation

Mouse embryonic fibroblasts were incubated in amino acid complete F12/DMEM medium or F12/DMEM lacking the indicated amino acids. All media contained dialyzed and decomplemented serum.

Preparation of cDNA

Total RNA was isolated using an RNeasy mini kit (Qiagen). A 20 μg aliquot of total RNA and 5 μg of oligo-dT(20)VN were mixed to a final volume of 8 μl, incubated at 65°C for 5 min and snap-cooled on ice. Reverse transcription labeling mixture (12 μl) was then added to obtain a labeling reaction containing 0.5 mM dATP, dCTP, dGTP, 0.15 mM dTTP, 0.3 mM aminoallyl-dUTP, 400 U SuperScript II RNase H, 10 mM dithiothreitol, and 1× buffer. The mixture was incubated at 42°C for 2 h to generate aminoallyl cDNA. To hydrolyze the RNA template, 2 μl of 2.5 M NaOH were added to the reaction and incubated at 37°C for 15 min. The reaction was neutralized with 1 μl of 2 M HEPES and the unincorporated aminoallyl-dUTP was removed by the Qiaquick PCR purification kit following the instructions of the manufacturer except that three washes and two steps of elution were performed. Aminoallyl cDNA were precipitated at −80°C for 30 min by adding 20 μg glycogen, 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of 100% ethanol. After centrifugation (10,000×*g* for 15 min), the aminoallyl cDNA pellet was washed with 70% ethanol, suspended in 5 μl of nuclease-free H_2O and 3 μl 1 M sodium bicarbonate. The labeling reaction was performed by adding reactive dye (Molecular Probes), Alexa Fluor 555 for cDNA control ("fed" cells) and Alexa Fluor 647 for cDNA test (amino acid deprived cells), dissolved in 2 μl dimethyl sulfoxide for 1 h in the dark at room temperature. Uncoupled dyes were removed with the Qiaquick PCR purification kit, and the concentration of nucleic acid and labeling efficiency were evaluated by measuring the absorbance at 260 nm and 555 or 650 nm for Alexa Fluor 555 cDNA and Alexa Fluor 647 cDNA, respectively. A 25 pmol aliquot of each labeled cDNA target (from fed and amino acid deficient cells) was mixed and precipitated in the presence of 10 μg mouse cot 1 DNA, 10 μg poly A, and 5 μg yeast tRNA. The pellet was suspended in 20 μl DIG Easy Hyb solution (Roche).

Generation of micro arrays

Coding sequences of 19 selected genes were PCR amplified for microarray preparation and the products verified by loading on a 2% agarose gel and purified on Multiscreen PCR plate systems (Millipore). The cDNAs were prepared in 96-well plates to obtain 20 μl of a solution at 100 ng/μl in 3× SSC and 1.5 M betain. PCR products were printed on UltraGaps slides (Corning) using a MWG GMS 417 spotting robot in controlled environmental conditions (temperature of 18°C and humidity of 50%). The spot size was 125 μm^2 and average distance from spot-to-spot center was 300 μm. Printed arrays contained four blocks with 18 columns and 16 rows. All clones were spotted in triplicate. Selected housekeeping genes (Actb, Tuba1, B2m, Gapdh, Ppia, Rpl19, Tfrc) were also spotted in the four independent blocks. The samples were immobilized by ultraviolet irradiation (300 mJ) and excess DNA was removed by washing in 0.2% SDS at room temperature. Spotted slides were blocked in 3× SSC, 0.1% SDS, 0.1 mg/ml BSA at 50°C for 60 min, then washed in distilled water. Arrayed samples were denatured in water at 95°C for 2 min before hybridization. The quality of generated microarrays was evaluated by hybridization of one slide by batch production with Panomer 9 Random Oligodeoxynucleotides Alexa Fluor 555 conjugate following the instructions of the manufacturer (Molecular Probes).

Microarray hybridization and analysis

The labeled cDNAs were heated at 65°C for 2 min and applied to a 20 mm × 22 mm cover slip, which was placed on the microarray slides to form a thin layer. The slides were placed in CMT-hybridization chambers (Corning) and placed in a water bath at 37°C for 16 h. Slides were then removed from the chambers, washed three times for 10 min at 50°C in 1× SSC, 0.1% SDS, four times briefly in 1× SSC, rinsed in distilled water for 5 s and then dried by centrifugation at 1,000×*g* for 5 min. The slides were scanned with an Affymetrix 428 (Santa Clara, CA, USA) scanner using appropriate gains on the photomultiplier tube to obtain the highest intensity without saturation. A 16 bit TIFF image was generated for each channel and scanned images for Alexa Fluor 555 and Alexa Fluor 647 were then analyzed with GenePix Pro 4.1 software (Axon Instruments). Spots with intensities lower than twofold over the background were filtered out. Housekeeping gene normalization was then applied to correct for artifacts caused by different dye incorporation rates or scanner settings for the two dyes. The expression of genes used for normalization

was verified by RT-qPCR for each condition and shown to be unaffected by amino acid availability. The ratio of medians (background subtracted median pixel intensity at 635nm wavelength/background subtracted median pixel intensity at 532 nm wavelength) was utilized to estimate gene expression. Each clone was spotted three times on the same slide and the average of the induction ratios of the three independent spots was measured. The coefficient of variation was below 15% and if one value was aberrant it was removed.

Results

Microarray analysis of amino acid specificity

To survey the specificity of the amino acid responsiveness for a spectrum of amino acid-regulated genes, 19 genes that had been identified in preliminary studies were screened using an array approach (Table 2). Mouse embryonic fibroblasts were incubated for 6 h in control medium or in a medium lacking either a single amino acid (lysine, leucine, or histidine) or lacking all of the essential amino acids except methionine and cysteine. The results demonstrate that these genes are differentially regulated depending on the amino acid omitted from the culture medium. Some genes were induced to a greater extent by limitation of one amino acid compared to the collection of essential amino acids (e.g., Cxcl10) whereas other genes show the opposite expression pattern (e.g., Snord22, 1500012F01Rik). Furthermore, certain genes were more strongly induced by deprivation of a particular essential amino acid. For example, Cxcl10 was more strongly induced by histidine than by leucine or lysine limitation, whereas Chop (Ddit3) was more strongly induced by the lack of leucine or lysine than by the lack of histidine. These results demonstrate that the response to amino acid limitation varies according to the target gene and to the specific limiting amino acid.

Amino acid specificity of the eIF2α-ATF4 response

To monitor the activation of the AAR pathway by measurement of the phosphorylation of eIF2α and de novo ATF4 protein synthesis, HepG2 cells were incubated in

Table 2 Induction of gene expression in response to starvation in one individual amino acid

Gene symbol	Gene ID	Gene name	EAA/control fold-change	Lys-/control fold-change	His-/control fold-change	Leu-/control fold-change
1500012F01RIK	68949	RIKEN cDNA 1500012F01 gene	10.30 ± 0.84	3.75 ± 1.35	5.29 ± 0.38	3.99 ± 1.70
Ddit3	13198	DNA-damage-inducible transcript 3	6.46 ± 0.18	5.62 ± 0.42	4.12 ± 0.21	8.30 ± 2.68
Slc3a2	17254	Solute carrier family 3, member 2	6.26 ± 0.88	6.11 ± 1.03	4.64 ± 0.52	3.95 ± 1.48
Snord22	83673	Small nucleolar RNA, C/D box 22	6.18 ± 0.83	3.32 ± 1.16	4.34 ± 0.25	3.16 ± 0.90
Trib3	228775	Tribbles homolog 3 (drosophila)	5.87 ± 0.23	2.60 ± 0.06	4.88 ± 1.04	3.14 ± 0.64
Atf3	11910	Activating transcription factor 3	5.65 ± 0.53	5.85 ± 1.37	7.86 ± 2.59	4.09 ± 1.68
Ndrg1	17988	N-myc downstream regulated gene 1	4.64 ± 0.42	2.80 ± 0.48	2.29 ± 0.12	3.32 ± 1.02
Asns	27053	Asparagine synthetase	4.49 ± 1.94	3.47 ± 0.001	3.68 ± 0.24	2.93 ± 0.37
Slc7a5	20539	Solute carrier family 7, member 5	4.33 ± 0.44	3.02 ± 0.52	3.88 ± 1.47	3.58 ± 1.08
Ifrd1	15982	Interferon-related developmental regulator 1	3.37 ± 0.68	3.17 ± 0.21	2.64 ± 0.69	2.82 ± 0.10
Clic4	29876	Chloride intracellular channel 4	3.11 ± 0.04	1.95 ± 0.07	2.35 ± 0.64	2.13 ± 0.10
Ghitm	66092	Growth hormone inducible transmembrane protein	2.78 ± 0.68	2.41 ± 0.52	2.76 ± 0.46	2.92 ± 1.33
Fyn	14360	Fyn proto-oncogene	2.69 ± 0.84	1.74 ± 0.31	2.49 ± 0.85	1.66 ± 0.27
Cxcl10	15945	Chemokine (C-X-C motif) ligand 10	2.65 ± 0.33	6.23 ± 1.36	16.87 ± 3.10	3.67 ± 1.11
Slc1a5	20514	Solute carrier family1 (neutral amino acid transporter) member 5	2.64 ± 0.33	2.60 ± 0.52	3.55 ± 0.59	2.47 ± 0.82
Dusp16	70686	Dual specificity phosphatase 16	2.09 ± 0.17	1.73 ± 0.52	2.34 ± 0.75	1.85 ± 0.94
Eif3s8	56347	Eukaryotic tranlation initiation factor 3, subunit 8	1.99 ± 0.29	1.59 ± 0.23	1.97 ± 0.36	1.82 ± 0.42
Yars	107271	Tyrosyl tRNA synthetase	1.92 ± 0.24	1.91 ± 0.24	2.65 ± 0.96	2.09 ± 0.87
Gars	353172	Glycyl-tRNA synthetase	1.91 ± 0.38	2.05 ± 0.78	3.12 ± 1.45	2.87 ± 0.94

Fibroblasts were incubated for 8 h either in control or amino acid depleted medium. The depleted medium lacked either all essential amino acids (EAA) except methionine/cysteine or a single amino acid (leucine, lysine, or histidine). RNA was then extracted and microarray analysis performed as described in the Sect. "Methods." Three independent experiments were performed and the results shown are the average ± SEM

amino acid complete MEM or MEM lacking a single amino acid. The amino acids chosen were the ten classically defined "essential" or "indispensable" amino acids for growth of many mammals (Fig. 1). The p-eIF2α and ATF4 levels were measured after an incubation period of 2 h (Chen et al. 2004). Except for valine, each of the remaining amino acid depletions resulted in increased eIF2α phosphorylation (Fig. 1a). The increase in phosphorylation was approximately 2-fold to 10-fold compared to complete MEM, with leucine and threonine causing the largest increase in p-eIF2α, whereas isoleucine, lysine, methionine, and tryptophan elicited the weakest responses. Using the same protein samples as in Fig. 1a, ATF4 protein content was measured (Fig. 1b). Interestingly, there was not a strong correlation between p-eIF2α and ATF4 protein accumulation. For example, valine was the weakest amino acid with regard to p-eIF2α production, and yet, the abundance of ATF4 protein following valine removal was greater than several other amino acids that resulted in higher levels of p-eIF2α. Such differences are likely to be cell specific. For example, Abcouwer et al. (2002) observed that of 13 amino acids tested in their studies in retinal cells, valine depletion resulted in a lower level of vascular endothelial growth factor (VEGF) induction than the others. In the present studies, threonine deprivation resulted in the greatest increase in p-eIF2α, consistent with data reported by others for brain (Sharp et al. 2006), but its absence resulted in an increased ATF4 protein content that was near the average of the tested amino acids (Fig. 1b). Wang and Proud (2008) have suggested that eIF2α phosphorylation may not be the only triggering event in controlling protein synthesis during complete amino acid starvation. They present evidence that phosphorylation of eIF2B controls protein synthesis rates independent of eIF2α phosphorylation. While the data shown in Fig. 1 might argue that the linkage between p-eIF2α and ATF4 is not tight, given the inherent imprecision of immunoblot quantification and the fact that ATF4 runs as a rather broad band, the absolute values must be considered estimates. That said, the results do suggest that regulatory steps other than eIF2α phosphorylation impact the ATF4 synthetic pathway. Consistent with this concept, we have recently shown that there is cross-talk between the p-IF2α/ATF4 pathway and the MEK/ERK signaling pathway in HepG2 cells (Thiaville et al. 2008). In contrast to the AAR pathway that detects amino acid deficiency, the mammalian target of rapamycin (mTOR) pathway detects amino acid sufficiency and regulates protein synthesis accordingly (Kimball and Jefferson 2006). To establish that single amino acid limitation did not inactivate the mTOR pathway in the present studies, the abundance of the mTOR product phospho-4E-BP1 (p-4E-BP1) was monitored (Fig. 1c). As expected, total amino acid starvation decreased 4E-BP1 phosphorylation, whereas single amino acid deprivation did not. It is noted that total 4E-BP1 protein migrated as three distinct species, and that in the absence of amino acids (-AA) the ratio of the individual species favored increased expression of the two fastest migrating forms (Fig. 1c). Interestingly, the abundance of total 4E-BP1 protein was increased in response to nearly all of the single amino acid depletions. The significance of this observation is unclear, but the degree of phosphorylation (ratio of phosphorylated/total protein) remained relatively constant,

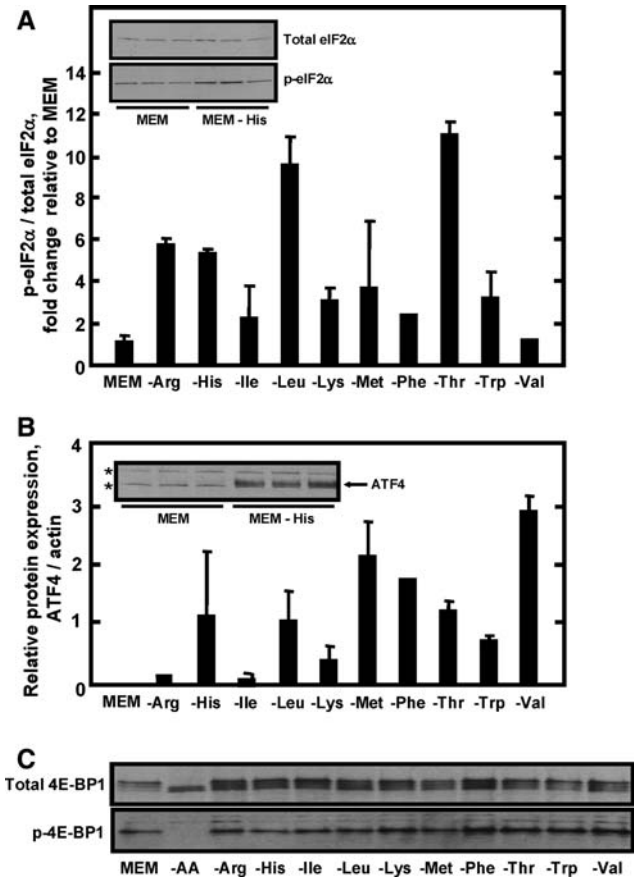

Fig. 1 Activation of the amino acid responsive pathway by limitation of individual amino acids. HepG2 hepatoma cells were incubated for 2 h in either amino acid complete MEM or MEM lacking the indicated amino acid. Whole cell protein extracts were subjected to immunoblotting for p-eIF2α, total eIF2α, ATF4, actin, total 4E-BP1, or p-4E-BP1, as described in the Sect. "Materials and methods." The degree of eIF2α phosphorylation is depicted relative to total eIF2 protein present and the data normalized to the value obtained for the amino acid complete MEM (Panel **a**). The ATF4 content is normalized to actin (Panel **b**). The abundance of ATF4 in the amino acid-complete MEM was below detection, so the results are presented as the relative values. The data depicted in Panels **a** and **b** are the average ± standard deviations for three or more independent experiments (except for Phe which is the average of two samples), and a representative blot is shown in the inset. The abundance of total 4E-BP1 and p-4E-BP1 is shown in Panel **c**. Total amino acid starvation (-AA) was achieved by incubation in EBSS

suggesting that the mTOR activity was relatively unchanged.

Transcriptional activation by single amino acid deprivation

To monitor the transcriptional response of an amino acid-regulated target gene, HepG2 hepatoma cells were incubated in complete MEM or MEM lacking a single amino acid prior to assaying for AARE-driven Firefly luciferase expression (Fig. 2a). A genomic fragment of the *SNAT2* gene, containing the promoter and the intronic AARE (nt −550/+770), was placed in front of the Firefly luciferase reporter gene and transiently expressed in HepG2 cells. From those tested for the AAR activation studies shown in Fig. 1, a subset of four amino acids were chosen to represent weak or strong activators of either p-eIF2α or ATF4. The increase in transcription ranged in magnitude from 2-fold (methionine) to 14-fold (histidine) (Fig. 2a). The methionine result was surprising in that its removal from the medium led to a relatively strong rise in both p-eIF2α and ATF4, and yet, there was a modest twofold induction of the reporter gene activity. It was established that the transcriptional activation by individual amino acid limitation was mediated by the *SNAT2* intronic AARE, as shown by the abrogation of the response when using a construct in which the AARE was mutated (Fig. 2a). As an illustration that the amino acid specification of increased *SNAT2* transcription is not linked to the SNAT2 transporter's own substrate specificity, amino acids that have little or no detectable transport via System A, such as arginine and leucine (Bracy et al. 1986), brought about adaptive transcriptional responses. These results, monitoring AARE-driven transcription, are consistent with the conclusion reached by analyzing the specificity of inducing System A transport activity in intact cells following amino acid withdrawal (Handlogten et al. 1982). Namely, that there is no linkage between transporter substrate preference and adaptive up-regulation.

Certain amino acid-free buffers may activate AARE-independent pathways

A number of previous studies investigating the effect of complete amino acid deprivation on System A transport activity were performed with KRB. In a previous study, we showed that *SNAT2* (called ATA2 at that time) transcription was induced by incubation in either MEM lacking the single amino acid histidine or in amino acid-free KRB (Bain et al. 2002). The AARE within the *SNAT2* gene had not yet been identified at the time of that report, so the genomic element(s) mediating those responses were unknown. To further investigate those observations,

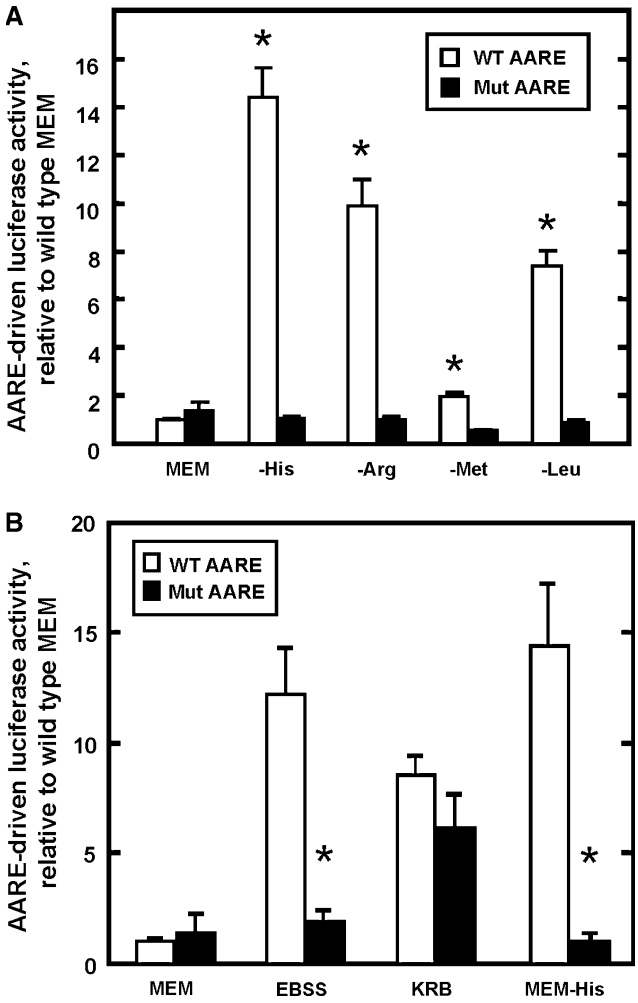

Fig. 2 Activation of the *SNAT2* AARE by single or total amino acid deprivation. Panel **a** HepG2 cells were transfected with a *SNAT2* genomic fragment (nt −512/+770) containing either a wild type (*WT*) or a mutated (*Mut*) AARE driving the Firefly luciferase reporter gene, as described in Sect. "Materials and methods." The cells were incubated in the amino acid deficient media for 10 h. The data are presented relative to control (WT construct, MEM value), which was set to 1. Statistically significant differences, relative to the MEM values, are indicated by *asterisks* ($P < 0.005$). Panel **b** HepG2 cells were transfected as described for Panel **a**, and then the cells were incubated for 10 h in either complete MEM, MEM lacking histidine (*MEM-His*), amino acid-free Krebs–Ringer bicarbonate buffer (*KRB*) or amino acid-free Earle's balanced salt solution (*EBSS*). The MEM value of the luciferase activity for the wild type control was set to 1. For each treatment, six replicates were performed and statistically significant differences between the WT and the mutated AARE constructs are indicated by *asterisks* ($P < 0.005$)

HepG2 cells transiently transfected with the wild type or mutated AARE sequence were incubated MEM, MEM lacking just histidine, or in two different media that are completely devoid of amino acids, KRB and EBSS (Fig. 2b). All three media that were amino acid deficient caused induced transcription of the reporter gene, although the magnitude of the increase in response to KRB was

slightly less than that for either EBSS or MEM lacking histidine (MEM-His). Surprisingly however, the increased transcription induced by incubation in either EBSS or MEM-His was completely blocked by mutation of the *SNAT2* AARE, whereas the activation by KRB was not significantly reduced compared to the wild type sequence (Fig. 2b). These results indicate that, in contrast to EBSS or single amino acid limitation, the KRB medium is triggering a transcriptional response from this *SNAT2* genomic fragment that does not require the AARE.

The SNAT2 intron 1 contains a KRB-responsive activity

To investigate the localization of the KRB-responsive activity within the *SNAT2* −512/+770 genomic fragment, multiple *SNAT2* deletion constructs were tested under conditions of histidine deprivation (MEM-His) or total amino acid starvation using KRB (Fig. 3). The −512/+770 *SNAT2* constructs containing the wild type and mutated AARE served as the controls and reflected the response observed in the previous experiments. Transfection with the promoter only construct, nt −512/+59, showed elevated reporter gene activity regardless of whether or not the medium was amino acid complete or deficient (Fig. 3). Constructs containing nt −512/+400 or nt −512/+698 resulted in a highly reduced basal (MEM) reporter activity and little or no induction of transcription by either histidine or complete amino acid deprivation (Fig. 3). These results demonstrate that the *SNAT2* sequence between +60 to +770 contains an element(s) that suppresses the basal *SNAT2* transcription under amino acid-fed conditions. Given that the −512/+770 fragment exhibited KRB responsive activity, whereas the −512/+698 construct did not, there is an AARE-independent, KRB-responsive element is within nt +698 to +770. Computer analysis of this region did not reveal the presence of putative binding sites for factors known to be associated with amino acid-dependent control of transcription.

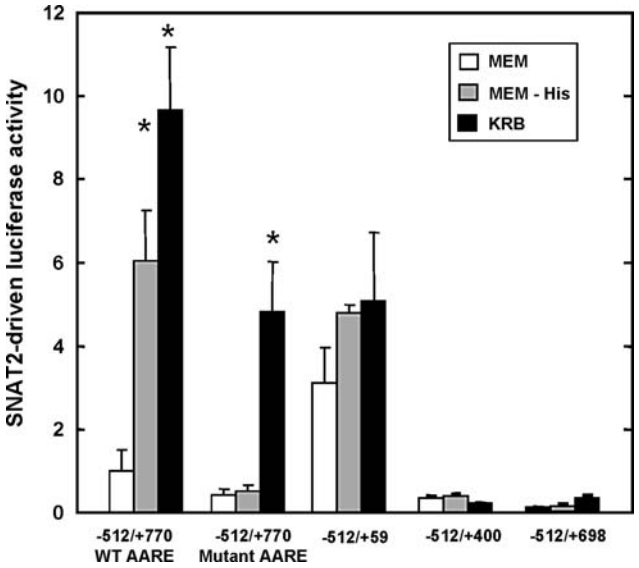

Fig. 3 The *SNAT2* AARE-independent, KRB-responsive element is in intron 1. HepG2 cells were transfected with the indicated *SNAT2* genomic fragments driving transcription for the Firefly luciferase reporter gene. Two different −512/+770 genomic fragments were tested for the ability to drive transcription, one containing the wild type AARE (*WT*, nt −709/−717) and one that contains a AARE mutated as described in the Sect. "Materials and methods." Sixteen hours after transfection the cells were treated with complete MEM medium, MEM lacking histidine (*MEM-His*), or KRB for 10 h. The control (MEM) value for the SNAT2 −512/+770 fragment WT was set to 1. Statistically significant differences, relative to the corresponding MEM control for each fragment, are indicated by *asterisks* ($P < 0.005$)

Discussion

The experiments described in the present report were designed to investigate the activation of gene transcription in response to limitation of individual amino acids or complete amino acid starvation. The following novel observations were made during these studies. (1) Individual amino acid-regulated genes respond differently to deprivation of individual amino acids, or to limitation of a collection of the essential amino acids. (2) Although variation in the absolute magnitude is observed between amino acids, the AAR pathway is activated in response to limitation of a wide spectrum of essential amino acids. (3) There was not a strong correlation between the degree of eIF2α phosphorylation and ATF4 protein accumulation. (4) Incubation of cells in an amino acid-free medium, either EBSS or KRB, resulted in increased transcription driven by a *SNAT2* genomic fragment, but in contrast to the results for EBSS, a significant portion of the transcription induced by KRB was not mediated through the AARE. (5) The AARE-independent activation of the *SNAT2* transcription was localized to a region within the first intron of the *SNAT2* gene.

The data generated by screening 19 selected amino acid-responsive genes showed that some genes are differentially regulated according to the limiting amino acid. There are several explanations that can be put forth to explain these data. The intracellular concentration of the limiting amino acid may differ according to the transport and metabolic use of that amino acid. Note that this variation in the decline in the intracellular concentration of a particular amino acid may be cell type specific. Alternatively, for a particular gene, more than one signaling pathway may contribute to the regulation of transcription and/or mRNA stability, and it could be hypothesized that certain amino

acids may preferentially trigger one pathway over another. These and other possibilities make it clear that the mechanisms involved in the sensing of amino acid availability are complex, and we have only begun to understand the basic aspects of these nutritionally important processes.

Transcription from the *SNAT2* amino acid transporter gene is increased following incubation of mammalian cells in amino acid deficient medium (Palii et al. 2004, 2006). The present studies utilizing complete amino acid starvation in combination with mutagenesis of the *SNAT2* sequence revealed a cryptic AARE-independent induction in the presence of KRB. Although the present experiments do not entirely rule out the possibility that this AARE-independent induction is still triggered by amino acid starvation, it seems unlikely given that the amino acid-free EBSS does not elicit the same response. Although *SNAT2* mRNA content has been demonstrated to be sensitive to osmotic changes (Gaccioli et al. 2006), total osmolarity does not appear to account for the difference in response to KRB and EBSS (313 mOsm versus 307 mOsm, respectively). It would appear more likely that differences in the chemical composition of EBSS and KRB accounts for this activation of the *SNAT2* gene. Compared to EBSS, KRB has 0.7 mM more calcium, 2.4 mM more potassium, 0.4 mM more magnesium, and 0.6 mM less sodium. Further experimentation will be required to determine whether or not ionic composition differences, or perhaps other changes such as pH, account for the differential activation between KRB and EBSS. Recently, simultaneous activation of the unfolded protein response and the AAR in HepG2 cells has been shown to override and block the induction of *SNAT2* by amino acid deprivation (Gjymishka et al. 2008). Regardless of the mechanism, the results indicate that incubation of HepG2 cells in KRB triggers an AARE-independent activation of the *SNAT2* gene.

Previous reports involving the *CHOP* and *ASNS* have shown that although both of these genes are amino acid regulated, they differ in their responses to individual amino acid limitation treatments (Jousse et al. 2000). In particular, it was documented that *CHOP* expression was minimally affected by histidine, asparagine, or cysteine limitation, consistent with the array data shown in Table 2, whereas the *ASNS* gene was strongly induced by the abundance of these amino acids. Conversely, methionine deprivation significantly increased CHOP mRNA content, while marginally affecting the ASNS mRNA level (Jousse et al. 2000). Current hypotheses propose that ATF4 represents the activating transcription factor for both of these AARE-containing genes following amino acid limitation. However, differences in AARE sequences and binding proteins (Averous et al. 2004) and co-activators (Chen et al. 2004; Cherasse et al. 2007) suggest that significant differences occur within the transcriptional mechanisms for the *ASNS* and *CHOP* genes. How amino acid specificity of the upstream detection process ultimately influences the signals at the level of the gene remains to be determined. Lee et al. (2008) compared the sensitivity to leucine versus cysteine limitation of HepG2-C3A hepatoma cells for a number of genes, including *CHOP* and *ASNS*, and observed that both were induced to a much greater degree by leucine deprivation. In contrast, *ATF3* and the *xCT* amino acid transporter were about five-times more sensitive to cysteine limitation. Fernandez et al. (2003) observed relatively minor differences in amino acid sensitivity of *Asns* and *Cat-1* induction in rat C6 glioma cells. As noted above, Abcouwer et al. (2002) tested the response of VEGF mRNA production in response to individual amino acid deprivation of a human retinal epithelial cell line (ARPE-19) and determined that most essential amino acids resulted in a twofold to threefold increase. However, limitation of the "non-essential" amino acid glutamine generated a six-fold induction, whereas depletion of the "essential" amino acid valine only produced a 33% increase in VEGF mRNA.

In summary, the variability in amino acid responsiveness reported in the literature and in the present analysis is quite varied. Among the many possible reasons for this are cell type variations, cell density, and medium volume differences which could lead to different rates of cytoplasmic depletion due to plasma membrane transport, metabolism, intracellular compartmentalization, and medium composition differences independent of the amino acid content. The latter possibility is underscored given the SNAT2 transcriptional difference observed in the present report for incubation in EBSS versus KRB. Clearly, there are many facets of the signaling pathways triggered by amino acid stress that remain to be elucidated.

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