Amino Acid Availability Controls TRB3 Transcription in Liver through the GCN2/eIF2α/ATF4 Pathway

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Abstract

In mammals, plasma amino acid concentrations are markedly affected by dietary or pathological conditions. It has been well established that amino acids are involved in the control of gene expression. Up to now, all the information concerning the molecular mechanisms involved in the regulation of gene transcription by amino acid availability has been obtained in cultured cell lines. The present study aims to investigate the mechanisms involved in transcriptional activation of the TRB3 gene following amino acid limitation in mice liver. The results show that TRB3 is up-regulated in the liver of mice fed a leucine-deficient diet and that this induction is quickly reversible. Using transient transfection and chromatin immunoprecipitation approaches in hepatoma cells, we report the characterization of a functional Amino Acid Response Element (AARE) in the TRB3 promoter and the binding of ATF4, ATF2 and C/EBPβ to this AARE sequence. We also provide evidence that only the binding of ATF4 to the AARE plays a crucial role in the amino acid-regulated transcription of TRB3. In mouse liver, we demonstrate that the GCN2/eIF2α/ATF4 pathway is essential for the induction of the TRB3 gene transcription in response to a leucine-deficient diet. Therefore, this work establishes for the first time that the molecular mechanisms involved in the regulation of gene transcription by amino acid availability are functional in mouse liver.

Introduction

Mammals have evolved a wide range of adaptative mechanisms to detect and respond to fluctuations in dietary nutrients. In particular they have to precisely regulate amino acid homeostasis taking into account two important characteristics of amino acid metabolism: (i) multicellular organisms are unable to synthesize all amino acids and (ii) there is no important dispensable amino acid store. Amino acidemia can be markedly affected by physiological or pathological conditions such as protein under-nutrition, imbalanced diet and various forms of stress (trauma, sepsis, etc.). Consequently, in order to adapt to amino acid availability, mammals have to adjust several physiological functions. One of the signal transduction pathways that is triggered in response to protein or amino acid starvation is referred to as the GCN2/eIF2α/ATF4 pathway [1]. The initial step in this pathway is the activation by uncharged tRNAs of the GCN2 kinase which phosphorylates the α subunit of translation initiation factor eIF2 (eIF2α on serine 51 [2,3]). This phosphorylation decreases the translation of most mRNAs by inhibiting the delivery of the initiator Met-tRNAi to the initiation complex. However, eIF2α phosphorylation also favors increased translation of a selected number of mRNAs, including that coding for the activating transcription factor 4 (ATF4). Once induced, ATF4 directly or indirectly induces transcription of a subset of specific target genes [4,5].

In cultured cell lines, several amino acid-responsive genes such as ASNS (Asparagine synthetase) [6,7,8] and CHOP (C/EBP homologous protein) [9,10,11] have been reported to contain AAREs (Amino Acid Response Elements) that mediate the enhanced transcription and function as enhancer elements [10]. The AARE sites have a 9 bp core element (5′-GTTCT/CATCA-3′) but the sequences can differ by one or two nucleotides between genes. It is now established that in amino acid-starved cells, a multiprotein complex is bound to the AARE sequences including a number of regulatory proteins such as ATF4 [7,12,13], CCAAT/enhancer binding protein β (C/EBPβ) [14], activating transcription factor 2 (ATF2) [15] or activating transcription factor 3 (ATF3) [13]. These factors are involved in either inducing or repressing transcription of target genes in response to amino acid starvation. Importantly, all of the known AARE sites bind ATF4, a master regulator of a number of amino acid-regulated genes. The binding activity and the role of other AARE binding factors appear to vary according to the AARE sequence and chromatin structure. For example, CHOP and ATF3 sequences also bind ATF2 whereas ASNS and SNAT2 sites do not [10,11,12,13]. Chromatin immunoprecipitation (ChIP) experiments have revealed that there is a highly coordinated time-dependent program of interaction between a precise set of ATF
subfamily members and coactivators leading to transcriptional activation of amino acid-regulated genes [8,15].

Trihibbles homolog 3 (TRB3) (also known as TRIB3, NIPK or SKIP3) is a pseudokinase which interacts with several transcription factors [16,17,18], protein kinases [19,20] and other proteins [21,22] and has been implicated in the control of stress response, cell viability and metabolic processes such as glucose or lipid metabolism. In particular, TRB3 has been identified as a feedback inhibitor of ATF4 involved in the transcriptional control of stress-regulated genes [16,23,24]. TRB3 has also been linked to pathophysiological conditions, including insulin resistance [19,25,26], cardiovascular disease [27] and diabetes [28]. TRB3 is an inducible gene whose expression is modulated by metabolic stresses including endoplasmic reticulum stress [17,29] and nutrient stress [24,30] and by insulin [31,32,33]. In the context of gene regulation by amino acid starvation, the role of TRB3 as a feedback inhibitor of ATF4 in amino acid-regulated transcription has been previously studied [24]. It was shown that (i) TRB3 overexpression inhibits CHOP and ASNS induction by leucine starvation, (ii) TRB3 is associated with ATF4 in the protein complex bound to the AARE sequence. It has been also documented that TRB3 is involved in the control of the basal level of gene expression under control/unstarved conditions [24]. At the level of gene expression, TRB3 appeared to be one of the most amino acid-induced transcripts in cultured cell lines. However, the mechanisms by which amino acids control TRB3 expression remained to be investigated.

Up to now, all the published data concerning the molecular mechanisms involved in the regulation of gene transcription by amino acid availability have been obtained in cultured cell lines. No result concerning these mechanisms was obtained in vivo in mammalian tissues. The present study aims to investigate the molecular mechanisms involved in the transcriptional activation of the TRB3 gene following amino acid limitation in mouse liver. Our results show that the TRB3 gene is up-regulated in the liver of mice fed a leucine-deficient diet. Using transient transfection and chromatin immunoprecipitation approaches in hepatoma cells, we report the characterization of an Amino Acid Response Element (AARE) in the TRB3 promoter and the binding of ATF4, ATF2 and C/EBPβ to this AARE sequence. We provide also evidence that the binding of ATF4 to the AARE plays a crucial role in the amino acid-regulated transcription of TRB3. In mouse liver, we demonstrate that the GCN2/eIF2α/ATF4 pathway is essential for induction of the TRB3 gene transcription in response to a leucine-deficient diet. Therefore, this work establishes that the molecular mechanisms involved in the regulation of gene transcription by amino acid availability are functional in mouse liver.

Results

Induction of hepatic TRB3 expression in response to one essential amino acid starvation

We first investigated the induction of TRB3 in the liver of mice fed a leucine-deficient diet for 2 h. When compared with the control diet, the consumption of chow lacking leucine leads to a large induction of TRB3 expression in the liver (Figure 1A) while a sharp decrease of leucine level (from 370 to 33 μM) was observed in the plasma (Figure 1B). Protein analysis shows that leucine deprivation induced phosphorylation of eIF2α, and expression of ATF4 and TRB3 (Figure 1C). Refeeding 2 h a control diet after a 2 h-hypoleucine diet was associated with a rapid increase of leucine plasma level and with marked decreases in eIF2α phosphorylation and ATF4, TRB3 expression.

To further characterize the mechanism of transcriptional activation of TRB3 following amino acid starvation, cultured hepatocyte-derived HepG2 cells were used. HepG2 cells were first incubated in leucine-free medium for 0–24 h and the mRNA levels for TRB3 were measured (Figure 2A). An initial increase in TRB3 mRNA content was observed after 2 h of leucine deprivation and reached a value of about 14-times higher than the control after 24 h. To investigate whether transcription contributed to the increase of TRB3 mRNA in HepG2 cells, the transcription activity of TRB3 was measured by analysing the synthesis of hnRNA [34]. Within 2 h after leucine removal, transcription was increased and continued to rise until reaching a peak of six times the control value at 6 h, and then rapidly declined (Figure 2B). These results show that increased transcription contributes to the elevation in TRB3 mRNA following amino acid limitation. The difference in absolute magnitude between the increase in transcription rate and the steady-state mRNA could be explained by the amino acid-dependent stabilization of the TRB3 mRNA as described for some other amino acid-regulated genes [1,35]. Furthermore, the effects on TRB3 protein expression were checked for some other individual amino acids. Figure 2C shows that starvation for essential amino acids such as lysine and methionine enhanced TRB3 expression whereas glutamine starvation (a non-essential amino acid) had no significant effect. This experiment also showed that ATF4 expression is induced by leucine, lysine or methionine starvation in HepG2 cells.

Identification of a functional Amino Acid Response Element in the TRB3 promoter

In the 5′-upstream region of the human TRB3 gene, from −7131 to −7033, three identical tandemly arranged repeats each consisting of 33-bp were previously described [17,29]. This promoter region was reported to contain an endoplasmic reticulum (ER) stress response element. To investigate further the contribution of these repeats to the amino acid-dependent increase in TRB3 transcription, a series of deletions in the promoter was created and fused to the Firefly luciferase reporter gene, in an assay for the response to amino acid deprivation. Figure 3A shows that a −7857 to −6940 promoter fragment (row 1) was able to mediate a level of induced response to amino acid deprivation which is consistent with the 4-fold increase in the transcription rate (see Figure 2B). Further TRB3 promoter deletions can be divided into two groups according to their level of amino acid inducibility. The first group includes two deletions that produced high levels of amino acid inducibility (rows 2 and 5) while the second group of deletions led to complete loss of amino acid inducibility (rows 3 and 4). These finding demonstrate that the 99-bp TRB3 promoter region from −7131 to −7033 including the three tandemly arranged 33-bp-long repeats is essential for amino acid regulation.

Close inspection of the sequence of the middle part of each 33-bp repeat reveals similarity with the Amino Acid Response Elements (AARE) core sequence identified in other amino acid regulated genes such as CHOP, ASNS, system A amino acid transporter (SNAT2) and ATF3 (Figure 3B). To examine whether this 99-bp TRB3 promoter sequence could, by itself, render a heterologous promoter amino acid responsive, one or two copies of this segment were cloned 5′ of the minimal herpes simplex virus promoter for thymidine kinase (TK). As shown in Figure 3C, a single copy of the TRB3 promoter sequence was able to regulate the basal promoter in response to leucine starvation (row 2). Furthermore, the leucine starvation-induced activity was more strongly enhanced by the presence of two copies of this TRB3 sequence (row 3) and the induced response to amino acid deprivation was greater than those observed with two copies of CHOP or ATF3 AARE sequences (rows 4 and 5). Figure 3D shows...
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that the transcriptional activity from the TRB3 AARE was enhanced by a decrease in leucine concentration in a dose-dependent manner in the range of those observed in the blood of leucine-starved animals (see Figure 1B). Altogether, these results demonstrate that the 99-bp sequence in the TRB3 promoter can be considered as an AARE that can be efficient at a physiological leucine concentration.

The induction of TRB3 transcription in response to amino acid limitation is dependent of GCN2 and independent of PERK

The GCN2 kinase has been shown to be involved in the up-regulation of a set of specific genes in response to amino acid starvation [36]. We first examined the role of GCN2 in the amino acid regulation of TRB3 transcription in MEF cells. The effect of leucine starvation was measured on both TRB3 mRNA content and TRB3 promoter-dependent transcription in GCN2-deficient MEFs and in the corresponding wild-type cells. MEF cells were transfected with four amino acid responsive luciferase constructs (see rows 1 and 2 of Figure 3A and rows 2 and 3 of Figure 3C) and then incubated either in control or leucine-starved medium. Lack of GCN2 abolished the response of TRB3 transcription to leucine depletion: the amino acid inducibility of both TRB3 mRNA (Figure 4A) and TRB3 AARE (Figure 4B) were completely lost. Protein analysis confirms that the induction of eIF2α phosphorylation was completely lost in MEFs deficient in GCN2 (Figure 4C). These results demonstrate that GCN2 plays a critical role in the activation of TRB3 promoter in response to amino acid starvation in MEF cells.

TRB3 was previously described as an ER stress-inducible gene [17,29]. To address directly the role of PERK, the ER stress-activated eIF2α kinase, in the transcriptional response of TRB3 to leucine starvation, PERK-deficient MEFs were used. In PERK+/− MEFs (Figure 4D), TRB3 exhibited a normal response to leucine starvation and to an agent (tunicamycin) that induces ER stress. Lack of PERK resulted in a complete loss in the TRB3 mRNA inducibility by tunicamycin but did not affect the mRNA induction level by leucine starvation. Protein analysis confirms that inductions of eIF2α phosphorylation and TRB3, ATF4 expression were still observed in leucine-starved PERK−/− cells (Figure 4E). On the other hand, eIF2α remained unphosphorylated and TRB3 and ATF4 levels did not increase when these cells were treated with tunicamycin. Thus, PERK is not required for the TRB3 induction following leucine starvation.

To explore in vivo the role of GCN2 in the induction of TRB3 expression, we examined the kinetic of the increase in TRB3

Figure 1. Induction of TRB3 mRNA in the liver of mice fed a diet lacking leucine vs control diet. Mice were fed for 2 h either control (Ctr) or leucine-deficient (−leu) diets or refed for 2 h on the control diet after a 2 h-consumption of the leucine-deficient diet (−leu/+leu) prior to liver mRNA and protein or plasma leucine levels measurement. (A) Total hepatic RNA from mice fed the indicated chow was subjected to qRT-PCR analysis for mRNA content as indicated. (B) Plasma leucine levels (μM) from mice fed as indicated above. (C) Liver protein extracts from mice fed the indicated chow were prepared as described under "Materials and Methods" and immunoblots against eIF2α phosphorylated on serine 51, ATF4 and TRB3 were performed.
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mRNA in the liver of both knockout (GCN2 \(-/-\)) and wild-type (GCN2 +/+ ) mice fed a control or leucine-deficient diet (Figure 5A). In GCN2 +/+ mice, TRB3 mRNA was increased 2.5-fold, 1 h after the beginning of the meal and reached a maximum level (6-fold) after 4 h. In contrast, lack of GCN2 resulted in a complete loss of TRB3 mRNA inducibility. Protein analysis confirms that the lack of GCN2 affected the phosphorylation of eIF2\(\alpha\) as well as the level of TRB3 and ATF4 expression (Figure 5B). Serum leucine levels were decreased to similar levels by the leucine-deficient diet in both genotypes thereby indicating an altered response to amino acid deficiency in mice lacking GCN2 activity (Figure 5C). Thus, in the mice liver, GCN2 is essential for induction of the TRB3 gene in response to a leucine-deficient diet.

**Identification of factors involved in TRB3 AARE regulation in liver tissues**

It has been previously demonstrated that in amino acid-starved cells, a multiprotein complex is bound to the AARE sequences including a number of regulatory proteins like ATF4 [7,12], ATF2 [10] and C/EBP\(\beta\) [14]. To determine whether these factors bind the AARE sequences within the TRB3 promoter, HepG2 cells were incubated in control or leucine-free medium for 4 h and ChIP assays were performed with primer sets covering either the 5’ region (amplicon A), the AARE (amplicon B) or the 3’ region (amplicon C) of the TRB3 gene (Figure 6A). The results show an increase in ATF4 binding to the AARE following 4 h of leucine deprivation whereas the binding of ATF2 and C/EBP\(\beta\) remained constitutive (Figure 6B). Furthermore, binding of ATF4, ATF2 and C/EBP\(\beta\) was not detected in the 5’ and 3’ regions of TRB3 confirming that these factors bind specifically to the AARE. Protein analysis confirms that ATF4 and C/EBP\(\beta\) expression increased in leucine-starved HepG2 cells as described previously [37] while ATF2 level remained unchanged (Figure 6C). To test the influence of leucine deprivation on the modification of chromatin structure, acetylation of histones was analyzed. The abundance of acetylated histones H3, H4 and H2B significantly increased in leucine-deprived HepG2 cells compared to the control (Figure 6D). This increase was observed in the AARE as well as in the 5’ and 3’ regions of TRB3 indicating that the change in histone acetylation is propagated in the vicinity of the AARE (data not shown).

To determine whether ATF4, ATF2 or C/EBP\(\beta\) are required to mediate the TRB3 mRNA induction following leucine starvation, MEFs deficient in ATF4, ATF2 or C/EBP\(\beta\) and their corresponding wild-type cells were used. TRB3 mRNA level was enhanced in wild-type MEF cells deprived of leucine. Lack of ATF4 abolished (Figure 7A) whereas lack of C/EBP\(\beta\) only reduced the TRB3 mRNA inducibility by leucine depletion (Figure 7B). In contrast, the increased expression level of TRB3 mRNA resulting from amino acid deprivation was not affected in ATF2\(-/-\) cells (Figure 7C). Protein analysis shows that lack of ATF4 resulted in a complete loss of the induction of TRB3 expression (Figure 7D) confirming that ATF4 is required for the transcription activation of TRB3 following leucine starvation. By contrast, leucine deprivation induced phosphorylation of cI\(\alpha\)\(\alpha\), ATF4 and TRB3 expression in C/EBP\(\beta\)\(-/-\) (Figure 7E) and ATF2\(-/-\) MEFs (Figure 7F) providing evidence that ATF2 and C/EBP\(\beta\) are not involved in amino acid regulation of TRB3.

The results described above highlight the key role of ATF4 in the transcriptional activation of TRB3 following amino acid starvation in HepG2 cells. To examine whether the binding of ATF4 to TRB3 AARE is also increased in the liver of mice fed either the leucine-deficient or the control diet for 2 h, ChIP experiments were performed (Figure 8). The results show an increase in ATF4 binding to the AARE in the liver of wild type mice (GCN2 +/+ ) in response to leucine starvation and are consistent with those described in HepG2 cells. A marked decrease in ATF4 binding was observed when mice were refed 2 h with a control diet after leucine deprivation. As expected the increase in ATF4 binding to the TRB3 AARE was lost in the liver of knockout (GCN2 \(-/-\)) mice fed a leucine-deficient diet. Taken together, these results demonstrate that ATF4 binding to the AARE, is a key event in the transcriptional induction of TRB3 in the liver of mice fed with a leucine-deficient diet.
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A

Relative Luciferase Activity

(1) -7857 -6940
AARE LUC
(3.9)

(2) -7857 -7033
AARE LUC
(6.6)

(3) -7857 -7132
LUC
(0.4)

(4) -7032 -6940
LUC
(0.7)

(5) -7131 -7033
LUC
(17.5)

B

TRB3
(-7131/-7099) (-7098/-7066) (-7066/-7034)
CHOP (-313/-295)
SNAT2 (+709/-724)
ASNS (-72/-57)
ATF3 (-12/-27)

AARE core
CGGTTGATACCCCG
AACATGGATACCC
GATATGGATACGTAT
GAAGTTTCAATGCGC
AGCGTTGATACCCCG

consensus
\[ R^T G \]

C

Relative Luciferase Activity

(1) TK-LUC
TK LUC
(1.2)

(2) 1XAARE TRB3
TK LUC
(15)

(3) 2XAARE TRB3
TK LUC
(21)

(4) 2XAARE CHOP
TK LUC
(14)

(5) 2XAARE ATF3
TK LUC
(6)

D

Relative Luciferase Activity (fold induction)

Leucine (μM)
420 140 70 30 15 7.5 0

2XAARE TRB3
Discussion

In order to adapt to an amino acid imbalanced diet, mammalian cells regulate expression of specific genes involved in transport, intermediary metabolism, oxidative stress and energy metabolism. In cultured cell lines, hundreds of amino acid-responsive genes have been identified by microarray analysis [36,38,39], although few of them have been extensively investigated with regard to transcriptional mechanisms. In the last decade, significant progress has been achieved in the understand-

![Figure 3. Identification of the AARE regulating activation of transcription of TRB3 in response to amino acid starvation in HepG2 cells.](image)

(A) HepG2 cells were transiently transfected with LUC constructs containing internal deletions of the TRB3 promoter as described under "Materials and Methods". Twenty-four hours after transfection, cells were incubated for 24 h in control DMEM F12 (+leu) or in DMEM F12 lacking leucine (−leu) and assayed for LUC activity. The relative fold induction, defined as the ratio of the relative LUC activity of leucine-starved cells to unstarved cells, is indicated in parentheses to the right of the bars. (B) Sequence comparison of the TRB3 33-bp repeat (−7313/−7099, −7098/−7066, −7066/−7034) with the CHOP AARE (−313/−295), the SNAT2 AARE (+709/+724), the ASNS NSRE-1 (−577/−72) and the ATF3 AARE (−12/−27). The position of the minimum AARE core sequence is boxed in grey. The resulting minimum consensus sequence is shown at the bottom. (C) HepG2 cells were transiently transfected with LUC constructs containing one or two copies of the TRB3 AARE (−7313/−7033) or two copies of the CHOP AARE or ATF3 AARE inserted 5' to the TK promoter. Twenty-four hours after transfection, cells were incubated for 24 h in DMEM F12 (+leu) or in DMEM F12 lacking leucine (−leu) and assayed for LUC activity. (D) HepG2 cells were transiently transfected with a 2XTRB3AARE-LUC construct. Twenty-four hours after transfection, cells were incubated for 24 h in DMEM F12 (420 μM) or in DMEM F12 containing the indicated leucine concentration and assayed for LUC activity.

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![Figure 4. Role of GCN2 and PERK in the transcriptional regulation of TRB3 following amino acid starvation.](image)

(A) GCN2 +/- or GCN2 +/- MEFs were incubated either in control (+leu) or leucine-free medium (−leu) and harvested after 4 hours and total RNA was analyzed for TRB3 mRNA content as described under "Materials and Methods". The graphs show means ± S.E.M. of three independent experiments. (B) GCN2 +/- or GCN2 +/- MEFs were transiently transfected with four amino acid responsive luciferase constructs as described under "Materials and Methods". Twenty-four hours after transfection, cells were incubated for 24 h in control DMEM F12 (+leu) or in DMEM F12 lacking leucine (−leu) and assayed for LUC activity. (C) HepG2 cells were transiently transfected with a 2XTRB3AARE-LUC construct. Twenty-four hours after transfection, cells were incubated for 24 h in DMEM F12 containing the indicated leucine concentration and assayed for LUC activity.

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ing of molecular mechanisms involved in the control of gene transcription following amino acid limitation in cultured cells [1,5]. However, so far no results concerning transcriptional mechanisms was obtained directly in mammalian tissues.

In a mammalian organism, the control of gene transcription differs in many aspects from that operating in cultured cells, and involves complex interactions between hormonal, neuronal and nutritional factors. The regulation of TRB3 gene expression represents a new mechanistic model to investigate how the consumption of a leucine-deficient diet can activate gene transcription in mice liver. The experiments included in this report have led to the following novel observations. 1) TRB3 is up-regulated in the liver of mice fed a leucine-deficient diet and this induction is quickly reversible. 2) Using transient transfection and chromatin immunoprecipitation approaches in hepatoma cells, we report the characterization of an AARE in the TRB3 promoter and the binding of ATF4, ATF2 and C/EBPβ to this AARE sequence. 3) We provide also evidence that only ATF4 bound to the AARE plays a crucial role in the amino acid-regulated transcription of TRB3. 4) Using GCN2−/− mice and a chromatin immunoprecipitation approach, we demonstrate that the GCN2/eIF2α/ATF4 pathway is essential for induction of the TRB3 gene transcription in response to a leucine-deficient diet.

The 5′-upstream region of the human TRB3 gene contains three identical tandemly arranged repeats each consisting of 33-bp. This promoter region was reported to contain an endoplasmic reticulum (ER) stress response element [17,29]. The present results provide evidence that these 33-bp repeats are also responsible for the amino acid control of TRB3 transcription. These DNA sequence can be called an AARE for the following reasons: (i) the 33-bp repeats can regulate a basal promoter in response to leucine (see Figure 3C) or some other essential amino acids starvation (data not shown); (ii) the sequence of the middle part of the 33-bp repeat (5′-TTTGCATCA-3′) differs from the AARE core sequence of the CHOP (5′-ATTGCATCA-3′), SNAT2 (5′-ATTGCATCA-3′) and ATF3 (5′-ATTGCATCA-3′) genes by only one nucleotide and from the AARE core sequence (5′-GTTTCATCA-3′) of the ASNS gene by 2 nucleotides (see Figure 3B); (iii) mutations affecting the core sequence of the 33-bp repeat result in a sharp decrease of amino acid responsiveness (Figure S1). In addition, the TRB3 AARE confers a higher amino acid responsiveness than CHOP or ATF3 AAREs.

Figure 5. Role of GCN2 in the induction of TRB3 expression in the liver. GCN2+/+ and GCN2−/− mice were fed either control (Ctr) or leucine-deficient diets (~leu) prior to liver mRNA levels measurement. (A) Total hepatic RNA from GCN2+/+ and GCN2−/− mice fed for the indicated time was analyzed for TRB3 mRNA content. The TRB3 mRNA induction level is defined as the ratio of the relative mRNA level of leucine-starved mice to that of control mice. (B) Liver protein extracts from GCN2+/+ or GCN2−/− mice fed the indicated chow for 2 h were prepared and immunoblots against eIF2α phosphorylated on serine 51, ATF4, TRB3 and eIF2α were performed. (C) Plasma leucine levels (μM) of GCN2+/+ or GCN2−/− mice fed the indicated chow for 0–9 h. Each animal experiment was repeated three times with four animals in each group in order to confirm the reproducibility of the results.

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As for the other AAREs described previously, the sequence of the TRB3 AARE show some homology with the specific binding sites of the C/EBP and ATF/cAMP response element-binding protein transcription factors. Du et al [33] reported that C/EBPβ is involved in the induction of TRB3 by insulin. In the context of gene regulation by amino acid starvation, C/EBPβ like ATF2, do not seem to be involved in the amino acid-regulated transcription although both factors bind to the TRB3 AARE sequence. Using EMSA (electrophoresis mobility shift analysis), Ord et al. [29] demonstrated that ATF4 binds in vitro the TRB3 AARE in response to ER stress. The present observations provide in vivo evidence for ATF4 binding to TRB3 AARE in both amino acid-starved hepatoma cells and in the liver of mice fed a leucine-deficient diet. The effects of ATF4 on TRB3 transcription in mice liver appears to be quickly reversible since marked decreases in both ATF4 expression and binding to AARE were observed when mice are refed 2 h a control diet after leucine deprivation. Therefore, TRB3 can be quickly switched on or switched off in the liver of mammals according to the amino acid content of the diet. It is possible that other ATF4 interacting factors or co-factors should be involved in this regulatory process of transcription in the liver. These proteins remain to be identified.

At a physiological level, TRB3 is involved in the regulation of many biological functions in several tissues. By inhibiting Akt activation by insulin in liver [19] and acetyl-coenzyme A carboxylase (ACC) in adipose tissue [21], TRB3 has been found to modulate lipid and glucose metabolisms. Currently, the mechanisms involved in the regulation of TRB3 expression in...
liver and adipose tissue are not well understood. An increasing number of data provides arguments suggesting that TRB3 could be also involved in the control of amino acid and protein metabolism. It was shown that (i) TRB3 is a potent regulator of P70S6 kinase (S6K1) activation by insulin [40]; (ii) TRB3 is involved in the induction of autophagy by inhibiting the Akt/mTORC1 axis [41]; (iii) TRB3 inhibits ATF4-dependent transcription in response to amino acid starvation [16,24]; (iv) We show in this work that GCN2 activation and ATF4 binding to AARE, are key events in the up-regulation of TRB3 transcription in the liver of mice fed a leucine-deficient diet. Thus, it is likely that TRB3 could participate in the control of amino acid homeostasis. However its precise role in the control of amino acid metabolism remains to be investigated.

It is now clear that when animals are presented with a diet devoid of a single essential amino acid, the GCN2/eIF2α pathway is activated in several tissues. Following the consumption of such diet, the decline in the amino acid blood levels is correlated with the GCN2-dependent phosphorylation of eIF2α and a sharp food intake inhibition [42,43]. Previous studies have involved the anterior piriform cortex in sensing blood amino acid levels and in initiating the food intake response [44]. GCN2 kinase-deficient mice illustrated that recognition of an amino acid imbalanced-diet by the brain requires uncharged tRNA sensing by GCN2 [42,43]. However, the role of ATF4 in this process remains to be demonstrated. It seems likely that in most tissues, following activation of the GCN2/eIF2α/ATF4 pathway, a highly coordinated time-dependent program of molecular events should take place, leading to the fine transcriptional regulation of specific target genes. Our results show for the first time that, in the liver, amino acid control TRB3 transcription through GCN2 activation and subsequent ATF4 binding to AARE sequences. The TRB3 transcription is also up-regulated in other tissues following the consumption of a leucine-deficient diet, such as the intestine (data not shown). Nevertheless, other ATF4-target genes remain to be identified in these tissues.

The idea that amino acids can regulate gene transcription in mammalian tissues through the GCN2/eIF2α/ATF4 pathway is now established. The molecular basis of gene regulation by dietary protein intake is an important field of research for studying regulation of physiological functions of individuals living under condition of restricted, imbalanced, or excessive food intake. Beyond gaining a basic understanding of the amino acid control of biological mechanisms, the characterization of how these processes contribute to the occurrence of various diseases represents an important field of investigation in molecular nutrition.

Materials and Methods

Ethics Statement

Maintenance of the mice and all experiments were conducted according to the guidelines formulated by the European Community for the use of experimental animals (L558-86/609/
Figure 8. ATF4 binding to TRB3 AARE in the liver of leucine-deprived mice. GCN2 +/+ and GCN2 −/− mice were fed for 2 h either the control (Ct) or leucine-deficient diets (−leu) or refed for 2 h a control diet after a 2 h-consumption of the leucine-deficient diet (−leu/−leu) and then ATF4 binding associated with the TRB3 AARE was monitored in liver extracts by ChIP assays. PCR products amplifying the AARE-containing promoter of the TRB3 gene were assayed by qPCR as described under “Materials and Methods”. Data were plotted as the percentage of antibody binding versus the amount of PCR product obtained using a standardized aliquot of input chromatin. Each point represents the mean value of three independent experiments, and the error bars represent the standard error of the means. * Statistical significance (p<0.05) of the leucine-deprived values compared with the values in the control medium-incubated cells.
system in the TRB3 promoter has been established according to the identification of the transcription start sites [49]. 2XAARE-ChOP-TK-LUC and 2XAARE-ATF3-TK-LUC plasmids were generated as previously described [10,50]. 2XAARE-TRB3-TK-LUC construct was generated by inserting MsiI-XhoI doubled stranded oligonucleotides containing two iterations of the TRB3 AARE sequence (~7131 to ~7033) into the MsiI-XhoI sites of TATA-TK-LUC [10].

Transient transfection and luciferase assay
Cells were plated in 12 well-dishes and transfected by the calcium phosphate co precipitation method as described previously [10]. One µg of luciferase plasmid was transfected into the cells along with 0.05 µg of pCMV-βGal, a plasmid carrying the bacterial β-galactosidase gene fused to the human cytoemalgovirus immediate-early enhancer/promoter region, as an internal control. Cells were then exposed to the precipitate for 16 h, washed twice in phosphate buffered saline (PBS), and then incubated with DMEM F12 containing 10% fetal bovine serum. Two days after transfection, cells were harvested in 100 µl of lysis buffer (Promega) and centrifuged at 13,000 g for 2 min. Twenty µl of the supernatant were assayed for luciferase activity (YELEN, Enseur La Redonne, France). For all the transfection experiments presented, a plasmid pCMV-βGal was used as an internal control. β-Galactosidase activity was measured as described previously [10]. Relative luciferase activity was given as the ratio of relative luciferase unit/relative β-Gal unit. All values are the means calculated from the results of at least three independent experiments performed in triplicate.

Antibodies
The following antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA): ATF2, sc-187; ATF4, sc-200; C/EBPβ, sc-150; β-actin, sc-7210. The TRB3 antibody (ST 1032) was obtained from Calbiochem and the P-eIF2α antibody (catalog no. 1090-1) was from Epitomics (Burlingame, CA). Acetylated histone H3, 06-599 (recognizes acetylated H3 at Lys-9 and Lys-14) and acetylated histone H4, 06-866 (recognizes acetylated H4 at Lys-5, -8, -12 and -16) antibodies were purchased from Upstate Biotechnology (Charlottesville, VA). Acetylated histone H2B (recognizes acetylated H2B at Lys-12 and Lys-15) antibody was from Abcam (Cambridge, UK).

Nuclear extracts
Nuclear extracts from mouse liver cells were prepared as described by Sierra [51].

Immunoblot analysis
To detect eIF2α and its phosphorylated form, liver cells were lyed in radiomunmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 2 mM Na3VO4, 100 mM sodium fluoride, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonil fluoride, protease inhibitor cocktail from Sigma). To determine ATF4 and TRB3 proteins in liver samples, nuclear extracts were prepared. Total or nuclear proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a Hybond-P PVDF membrane (Amersham Biosciences). Membranes were blocked for 1 h at room temperature with a solution of 5% nonfat milk powder in TN (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20). The blots were then incubated with primary antibody in blocking solution overnight at 4°C. Antibodies were diluted according to the manufacturer’s instructions. The blots were washed three times in TN and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) (Santa Cruz, CA) in blocking buffer for 1 h at room temperature. After three washes, the blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

Chromatin immunoprecipitation analysis (ChiP)
ChiP analysis was performed according to the protocol of Upstate Biotechnology, Inc. (Charlottesville, VA) with minor modifications. HePG2 cells were seeded at 1 x 10^6/100-mm dish with DMEM F12 and grown for 24 h. Cells were transferred to fresh DMEM F12 12 h before transfer to either complete DMEM F12 or DMEM F12 lacking leucine for the time period indicated in each figure. For liver, fresh samples (900 mg) were chopped in small pieces and rinsed with fresh ice-cold phosphate buffered saline, pH 7.5 (PBS). Protein-DNA was cross-linked by adding formaldehyde directly to the culture medium or to liver samples to a final concentration of 1% and then stopped 15 min later by the addition of glycine to a final concentration of 0.125 M. For mice liver samples, nuclear extracts were then prepared. Cross-linked chromatin was sonicated using a Vibra cell sonicator (Biorbic Scientific Technology) for ten bursts of 1 min at power 2 with 1 min cooling on ice between each burst to obtain DNA fragments of an average of 400 bp. Liver or cells extracts were incubated with 5 µg of antibody, A rabbit anti-chicken IgG was used as the nonspecific antibody control. The antibody-bound complex was precipitated by protein A-Agarose beads (Upstate Biotechnology).

The DNA fragments in the immunoprecipitated complex were released by reversing the cross-linking overnight at 65°C and purified using a phenol/chloroform extraction and ethanol precipitation. Real-time quantitative PCR was performed by using a LightCycler (Roche) and a SYBR-Green-I-containing PCR mix (Qiagen), following the recommendations of the manufacturer. The immunoprecipitated material was quantified relative to a standard curve of genomic DNA. Primers used for the human TRB3 promoter: hTRB3 amplicon A, 5’-AAGAGA AAAAG-CAGCCTTCTTG-3’ and 5’-AGCGAGA AAGAATTGGTG- A-3’; hTRB3 amplicon B (AARE), 5’-GC GGAGTGCAGAGGA GAGA-3’ and 5’-CACC CTTCCTGGCAGGATCT-3’; hTRB3 amplicon C, 5’-CCCATGTCCCAGGAAGAAG-3’ and 5’-AG TCCTGGAGGGTATGGG-3’. The set of primers used for the analysis of the mouse TRB3 promoter were 5’-GGGC GGTTTCAGATGGTG-3’ and 5’-GACCGC CAGAGCC TAGCTG-3’. The reactions were incubated at 95°C for 15 min to activate the polymerase, followed by amplification at 95°C for 15 s, 59°C for 20 s (human primers), 68°C for 20 s (mouse primers) and 72°C for 20 sec for 45 cycles. After PCR, melting curves were acquired by stepwise increases in the temperature from 65 to 95°C to ensure that a single product was amplified in the reaction. The results are expressed as the percentage of antibody binding versus the amount of PCR product obtained using a standardized aliquot of input chromatin. Samples are the means from at least three independent immunoprecipitations.

Supporting Information
Figure S1 Responsiveness of the wild-type and mutant 33-bp repeats to leucine starvation. HepG2 cells were transfected with luciferase reporter constructs [29] driven by the wild-type (wt) 33-bp repeat or mutant 33-bp repeats (mut-5’, mut1-AARE core, mut2-AARE core, mut-3’). The AARE core sequence within the nucleotide sequence of the 33-bp repeat is boxed and mutated nucleotides are in italic lower-case letters. Twenty-four hours after transfection, cells were incubated for 24 h in DMEM F12 (+leu) or
in DMEM F12 lacking leucine (−leu) and then were harvested for preparation of cell extracts and determination of luciferase (LUC) activity. Relative LUC activities were determined as described in “Materials and Methods”. The relative fold induction, defined as the ratio of the relative LUC activity of leucine-starved cells to unstarved cells, is indicated in parentheses to the right of the bars. Each data represents the mean of at least three independent experiments performed in triplicate.

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References

1. Bruhat A, Cherasse Y, Chaverrous C, Maurin AC, Jousse C, et al. (2009) Amino acids as regulators of gene expression in mammals: molecular mechanisms. Biofactors 35: 249–257.

2. Kimball SR, Jefferson LS (2004) Amino acids as regulators of gene expression. Nutr Metab (Lond) 1: 3.

3. Wec RC, Jiang HY, Anthony TG (2006) Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans 34: 7–11.

4. He TP, Zhang Y, Zeng H, Novoa I, Lu PD, et al. (2005) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 11: 619–633.

5. Kilberg MS, Shan J, Su N (2009) ATF4-dependent transcription mediates signaling of amino acid limitation. Trends Endocrinol Metab 20: 436–441.

6. Barbosa-Tessmann IP, Chen C, Zhong C, Suo F, Schuster SM, et al. (2000) Activation of the human asparagine synthetase gene by the amino acid response and the endoplasmic reticulum stress response pathways occurs by common genomic elements. J Biol Chem 275: 26076–26085.

7. Suo F, Bain PJ, LeBlanc-Chaffin R, Chen H, Kilberg MS (2002) ATF4 is a mediator of the nutrient-replication response pathway that activates the human asparagine synthetase gene. J Biol Chem 277: 24120–24127.

8. Chen H, Pan VX, Dudenhausen EE, Kilberg MS (2004) Amino acid deprivation induces the transcription rate of the human asparagine synthetase gene through a timed program of expression and promoter binding of nutrient-responsive basic region/leucine zipper transcription factors as well as localized histone acetylation. J Biol Chem 279: 50829–50839.

9. Bruhat A, Jousse C, Wang XZ, Ron D, Ferrara M, et al. (1997) Amino acid limitation induces expression of CHOP, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels. J Biol Chem 272: 17292–17297.

10. Bruhat A, Jousse C, Barraro V, Reimold AM, Ferrara M, et al. (2000) Amino acids control mammalian gene transcription: activating transcription factor 2 is essential for the amino acid responsiveness of the CHOP promoter. Mol Cell 5: 1023–1031.

11. Bruhat A, Averous J, Barraro V, Zhong C, Reimold AM, et al. (2002) Differences in the molecular mechanisms involved in the transcriptional activation of the CHOP and asparagine synthetase genes in response to amino acid deprivation or activation of the unfolded protein response. J Biol Chem 277: 41007–41114.

12. Averous J, Bruhat A, Jousse C, Barraro V, Thiaville MM, et al. (2009) Activation of the ATF3 gene through a co-ordinated amino acid-sensing response programme that controls transcriptional regulation of responsive genes following amino acid limitation. Biochem J 401: 299–307.

13. Pan VX, Chen H, Thiaville MM, Kilberg MS (2007) Activation of the ATF3 gene through a co-ordinated amino acid-sensing response programme that controls transcriptional regulation of responsive genes following amino acid limitation. Biochem J 401: 299–307.

14. Ord D, Dexe P, Brd AB. Conceived and designed the experiments: TO PF AB. Performed the experiments: VO PF AB. Analyzed the data: VC A-CM SL-J, JAC CC CJ AB. Contributed reagents/materials/analysis tools: DO TO. Wrote the paper: AB.

Author Contributions

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43. Hao S, Sharp JW, Ross-Inta CM, McDaniel BJ, Anthony TG, et al. (2005) Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. Science 307: 1776–1778.
44. Gietzen DW, Ross CM, Hao S, Sharp JW (2004) Phosphorylation of eIF2alpha is involved in the signaling of indispensable amino acid deficiency in the anterior piriform cortex of the brain in rats. J Nutr 134: 717–723.
45. Harding HP, Novoa II, Zhang Y, Zeng H, Wek R, et al. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 6: 1099–1108.
46. Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D (2000) Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol Cell 5: 897–904.
47. Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, et al. (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 12: 982–995.

48. Breitwieser W, Lyons S, Flenniken AM, Ashton G, Beuler G, et al. (2007) Feedback regulation of p38 activity via ATF2 is essential for survival of embryonic liver cells. Genes Dev 21: 2069–2082.
49. Ord T, Ord D, Koivomagi M, Juhkam K, Ord T (2009) Human TRB3 is upregulated in stressed cells by the induction of translationally efficient mRNA containing a truncated 5’-UTR. Gene 444: 24–32.
50. Chaveroux C, Jousse C, Cherasse Y, Maurin AG, Parry L, et al. (2009) Identification of a novel amino acid response pathway triggering ATF2 phosphorylation in mammals. Mol Cell Biol 29: 6315–6326.
51. Sierra F, Tamone F, Muelder CR, Schuhler U (1990) Differential in vitro transcription from the promoter of a rat alpha 2u globulin gene in liver and spleen nuclear extracts. Mol Biol Med 7: 131–146.