Amino Acid Limitation Induces Expression of CHOP, a CCAAT/Enhancer Binding Protein-related Gene, at Both Transcriptional and Post-transcriptional Levels*

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In mammals, plasma concentrations of amino acids are affected by nutritional or pathological conditions. Here we examined the role of amino acid limitation in regulating the expression of CHOP, a CCAAT/enhancer binding protein (C/EBP)-related gene. CHOP protein is capable of interacting with other C/EBPs to modify their DNA binding activities and may function as a negative regulator of these transcription factors. Our data show that leucine limitation in human cell lines leads to induction of CHOP mRNA and protein in a dose-dependent manner. CHOP mRNA induction is rapidly reversed by leucine replenishment. Elevated mRNA levels result from both an increase in the rate of CHOP transcription and an increase in the CHOP mRNA stability. Using a transient expression assay, we show that a promoter fragment, when linked to a reporter gene, is sufficient to mediate the regulation of CHOP expression by leucine starvation in HeLa cells. In addition, we found that decreasing amino acid concentration by itself can induce CHOP expression independently of a cellular stress due to protein synthesis inhibition. Moreover, CHOP expression is induced at leucine concentrations in the range of those observed in blood of protein-restricted animals suggesting that amino acids can participate, in concert with hormones, in the regulation of gene expression.

Cells regulate gene expression in response to changes in the external environment. Metabolite control of gene expression has been well documented in prokaryotes and lower eukaryotes. Specific mechanisms have evolved to allow these organisms to quickly metabolize various molecules based on their availability in the external medium (1, 2).

However, much less is known about the response of multicellular organisms to nutrient variations. The control of gene expression differs in many aspects from those operating in single cell organisms and involves complex interactions of hormonal, neuronal, and nutritional factors. It has been shown that major (carbohydrates, fatty acids, sterols) or minor (minerals, vitamins) dietary constituents participate, in concert with many hormones, in the regulation of gene expression in response to nutritional changes (3–7). There is considerably less information available concerning the control of mammalian gene expression by amino acids. However, it has been shown that starvation of one essential amino acid causes a specific increase in mRNA abundance of certain genes including c-myc, c-jun, ornithine decarboxylase (8), asparagine synthetase (9), the mammalian equivalent of ribosomal protein L17 (10), the insulin-like growth factor binding protein gene (11). Moreover, Marten et al. (12) have shown that the abundance of several different mRNAs is affected by amino acid starvation. In this study the greatest induction in response to amino acids starvation was exhibited by the CHOP gene. However, little is known about the molecular mechanisms involved in gene regulation by amino acids. It has only been shown that the induction of asparagine synthetase gene by amino acid starvation involves both transcriptional and post-transcriptional mechanisms (9). These authors have characterized cis-acting elements involved in transcriptional regulation of that gene in response to amino acid starvation.

CHOP (also called gadd153) is a mammalian gene whose expression is also induced in all tested cells by a wide variety of stresses and agents (13–16). CHOP encodes a small nuclear protein related to the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors. Members of the C/EBP family have been implicated in the regulation of processes relevant to energy metabolism (17), cellular proliferation, differentiation, and expression of cell type-specific genes (18–20). By forming heterodimers with the members of the C/EBP family, CHOP protein can influence gene expression as both a dominant negative regulator of C/EBP binding to one class of DNA targets and by directing CHOP-C/EBP heterodimers to other sequences (21–26).

In mammals, plasma concentrations of glucose and free amino acids are markedly affected by nutritional or pathological conditions (27, 28). Carlson et al. (15) have shown that CHOP mRNA expression is induced by glucose deprivation in mammalian cell lines, suggesting a close relationship between nutrient variation and CHOP expression. In the present study we have examined the role of amino acids in the regulation of CHOP expression. We demonstrate that amino acid limitation, in conditions which do not inhibit protein synthesis, can induce

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1 The abbreviations used are: C/EBP, CCAAT/enhancer binding protein; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco’s modified Eagle’s medium; MEM, minimum Eagle’s medium; CHOP, C/EBP homologous protein.
CHOP expression. Particularly, we show that leucine starvation induces CHOP expression through both transcriptional and post-transcriptional mechanisms. The implication of these findings are discussed in a general context of the control of mammalian gene expression by amino acids in various nutritional conditions.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions—Cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium F12 (DMEM/F12) (Sigma) containing 10% (HeLa, HepG2) or 20% (Caco-2) fetal bovine serum. Where indicated, DMEM/F12 lacking leucine was used. For other amino acid or glucose starvation experiments, MEM medium (Life Technologies, Inc.) was used. For amino acid starvation experiments 10% diazylated calf serum was used.

RNA Isolation and Northern Blot Analysis—Total RNA was prepared as described previously (29). Northern blots were performed according to the procedure of Sambrook et al. (30). The membranes were UV cross-linked and then prehybridized was carried out for 2 h at 55 °C in 50% formamide, 5 × SSC, 5 × Denhardt’s reagent, 0.5% SDS, 0.1 mg/ml sonicated salmon sperm DNA, and 10 μg/ml yeast tRNA. The human CHOP cDNA (BH1), generously provided by Dr. N. J. Holbrook (31), was used as a probe. BH1 plasmid was linearized by PstI, and 32P-end-labeled CHOP probes were synthesized (30) using T7 RNA polymerase (Promega). Hybridization was carried out for 16 h at 55 °C. The membranes were washed for 15 min at 55 °C successively in 2 × SSC containing 0.1% SDS, 0.5 × SSC containing 0.1% SDS, 0.1 × SSC containing 0.1% SDS. Labeled bands were detected by autoradiography. Autoradiogram signals were quantified by using a densitometric scanner (Applegate) and NIH image software. To control for variation in either the amount of RNA in different samples or loading errors, all blots were rehybridized with an oligonucleotide probe corresponding to 18 S RNA. All densitometric values for CHOP mRNA were normalized to 18 S RNA values obtained on the same blot. Relative CHOP mRNA was determined as the ratio of CHOP mRNA to 18 S RNA.

DNA Transfection and CAT Assay—HeLa cells (5 × 10^5) were plated in 60-mm diameter dishes and transfected by the calcium phosphate coprecipitation method as described previously (32). Ten micrograms of CAT plasmid were transfected into the cells along with 2 μg of pCMV-βGal, a plasmid carrying the bacterial β-galactosidase gene fused to the human cytomegalovirus immediate-early enhancer/promoter region, as an internal control. Cells were exposed to the precipitate for 16 h, washed twice in phosphate-buffered saline, and then incubated with DMEM/F12 containing 10% fetal calf serum. Twenty-four hours after transfection, cells were amino acid-starved for the desired time and then collected for CAT assay (33). The protein concentration of the cell extracts was determined using the BCA method (34). β-Galactosidase activity was measured as described by Hall et al. (35) and used to calibrate transfection efficiency. Relative CAT activity was given as a percentage of pSV2CAT activity. All values are the means calculated from the results of at least three independent experiments.

Primer Extension—Total cellular RNA from transfected cells was isolated as described above. A 20-base pair oligonucleotide (5′-CAAGGGTGTATATCAGTG-3′), complementary to the DNA sequence located 11–30 base pairs downstream from the transcription initiation site of the cat gene, was end-labeled with T4 polynucleotide kinase (Eurorgenet) and then used for primer extension as described previously (36).

Nuclear Run-on Transcription Assays—In vitro transcription experiments in isolated HeLa cell nuclei were carried out essentially as described by Liu et al. (37). RNA was labeled with [32P]UTP and then hybridized to filter-bound cDNAs of CHOP (31), ribosomal S26 protein (38), and pBluescript DNA (Stratagene). Hybridization with labeled RNA was performed at 45 °C for 24 h. The filters were washed twice for 15 min in 5 × SSC plus 0.2% SDS at 45 °C, followed by three washes in 2 × SSC plus 0.2% SDS at 45 °C. Radioactive dots were visualized and quantified by using a PhosphorImager (Bio-Rad) and the MOLECULAR ANALYST software.

Protein Synthesis Measurements—HeLa cells were incubated for 16 h in DMEM/F12 containing 420, 140, 70, 35, or 0 μM leucine. During the last 3 h of incubation, 0.5 μCi/ml [35S]methionine were added. The medium was then removed, and the cells were incubated for 30 min in cold 5% trichloroacetic acid. The wells were washed once with trichloroacetic acid and three times with water. The radioactivity incorporation into trichloroacetic acid-precipitable material was measured by liquid scintillation counting after protein solubilization in 0.1 M NaOH plus 0.5% SDS. Results are given as a percentage of methionine incorporation in cells incubated in DMEM/F12 control medium.

RESULTS

Induction of CHOP mRNA Expression by Leucine Limitation—To understand the regulation of gene expression by amino acids at a molecular level, we have studied the regulation of CHOP expression in response to leucine limitation because (i) leucine is an essential amino acid that is poorly utilized by cells during a 16-h incubation period (data not shown), (ii) leucine, which is transported by system L, is rapidly equilibrated through the cell membrane (41, 42), and (iii) Marten et al. (12) have shown that leucine depletion strongly induces CHOP expression. To test the possibility that leucine concentration can influence CHOP expression, HeLa, HepG2, or Caco-2 cells were incubated for 16 h in medium containing different concentrations of leucine. As shown in Fig. 1A, CHOP mRNA levels were very low in each cell type in control medium containing 420 μM leucine and were inversely proportional to the leucine concentration in the medium, ranging from 15- to 30-fold over the control value. These results indicate that the expression of CHOP mRNA in human cells is regulated in response to changes in leucine concentration.
that the increase in CHOP mRNA levels results in the increase in the CHOP protein. Kinetic analysis of CHOP mRNA level in HeLa cells exposed to medium lacking leucine indicated that mRNA was detectable 2 h after starvation, and a maximum level was reached after 10–12 h (Fig. 2A). To determine whether the induction of CHOP expression by leucine starvation is reversible by leucine replenishment, 420 μM leucine was added to the culture medium of HeLa cells, and the RNA was harvested at the times indicated. The error bars represent standard deviation from the mean of two independent experiments in duplicate.

**Inhibition of Protein Synthesis Is Not Responsible for Induction of CHOP mRNA Expression**—To determine whether leucine limitation affects protein synthesis, HeLa cells were incubated in medium containing different concentrations of leucine and then [35S]methionine incorporation in the acid-precipitable fraction was measured (Fig. 3A). Cells incubated in medium lacking leucine showed a 40% reduction of methionine incorporation into total protein together with a drastic increase in CHOP mRNA level (Fig. 3B, lane b). However, cells incubated in medium containing 35 or 70 μM leucine gave no significant reduction of the global protein synthesis, whereas CHOP mRNA expression was significantly increased (Fig. 3B, lanes c and d). These observations are consistent with the idea that inhibition of protein synthesis is not responsible for the induction of CHOP mRNA expression.

**The Induction of CHOP Expression by Leucine Starvation Involves Both Transcriptional and Post-transcriptional Mechanisms**—Leucine starvation could increase CHOP mRNA expression either by increasing the rate of transcription or by stabilizing existing transcripts, or through both mechanisms. Nuclear run-on experiments provided evidence that the rate of CHOP transcription was increased by leucine starvation (Fig. 4A). Four hours of leucine starvation increased dramatically the transcription of CHOP (21-fold), while the transcription of the S26 ribosomal gene remained unchanged. To determine whether leucine starvation can affect the half-life of CHOP mRNA, HeLa cells were first incubated for 16 h in medium lacking leucine and then incubated with actinomycin D (4 μg/ml) in the presence or absence of 420 μM leucine, and total mRNA was extracted from cells at various times. As shown in Fig. 4B, addition of leucine resulted in a rapid decline in CHOP mRNA levels. In starved cells, the CHOP mRNA half-life was increased about 3-fold compared with cells incubated in the control medium. These findings indicate that leucine starvation elevates CHOP mRNA levels both by increasing the rate of CHOP transcription and by enhancing the stability of CHOP mRNA. To assess the importance of protein synthesis for the increase of CHOP mRNA expression during leucine starvation, cells were leucine-starved and treated with cycloheximide for 4 h. As shown in Fig. 4C, cycloheximide present during leucine starvation prevented the accumulation of CHOP mRNA. This result indicates that the increase in CHOP mRNA during leucine starvation is dependent on de novo protein synthesis.

**Regulation of CHOP Promoter Activity by Leucine Starvation**—To analyze the role of CHOP promoter in transcription activation by leucine starvation, a chimeric gene (pCHOP-CAT) containing the 5′-flanking sequence from nucleotides −954 to +91 fused to the cat gene (31) was transiently transfected in HeLa cells. The data presented in Fig. 5A (summarized in the graph of Fig. 5B) show that CAT activity expressed under the control of the CHOP promoter was induced 7-fold by 16 h of leucine starvation, whereas CAT activity expressed from the pSV2CAT construct used as a control was not induced. These results gave direct evidence that regulation of CHOP transcription by leucine starvation is mediated through the promoter sequence situated between nucleotide position −954 and +91. Similar increased levels of CAT activity were also observed.
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FIG. 4. Transcriptional and post-transcriptional regulation of CHOP by leucine starvation. A, nuclear run-on analysis of CHOP transcription. HeLa cells were incubated for 4 h in DMEM/F12 control medium (420 μM) or in DMEM/F12 lacking leucine (0 μM). 32P-Labeled RNA isolated from HeLa cells was hybridized to filter-bound DNAs of ribosomal S26, CHOP, and bluescript vector. The fold induction was determined as the ratio of mRNA expressed in leucine-starved to non-starved media. The numbers are the average of two separate experiments. B, effect of leucine starvation on CHOP mRNA stability. HeLa cells were initially incubated for 16 h in DMEM/F12 lacking leucine. At this point (time 0), cells were incubated in the presence of 4 μg/ml actinomycin D (Act D), either in DMEM/F12 (+Leu + Act D) or in DMEM/F12 lacking leucine (−Leu + Act D). Total RNA was extracted from each group of cells after the indicated incubation times. Northern blot analysis was performed as described under "Materials and Methods." C, effect of cycloheximide on CHOP mRNA accumulation. HeLa cells were incubated for 4 h in DMEM/F12 (420 μM) or in DMEM/F12 lacking leucine (0 μM) with 0.1, 0.5, 2.5, or 5 μg/ml cycloheximide as indicated. Northern blot analysis was performed as described under "Materials and Methods."

with transfection of pCHOP-CAT into HepG2 and Caco-2 cells (data not shown). To correlate CAT activity and amounts of CAT mRNA transcribed under leucine-starved and non-starved conditions, primer extension experiments were performed. As shown in Fig. 6, under leucine starvation, the amounts of CAT mRNA initiating at the correct start site of the promoter were much higher (lane b) than those transcribed in normal conditions (lane a), and the levels of CAT mRNA derived from pSV2CAT remained unchanged (lanes c and d). These results show that the degree of induction of pCHOP-CAT mRNA expression (6–7-fold) is in agreement with the degree of induction determined in CAT assays and indicate that, under our experimental conditions, leucine starvation does not affect significantly translation of the CAT mRNA.

To determine whether the CHOP promoter-driven CAT induction is consistent with that described for the endogenous CHOP mRNA, we examined the characteristics of the CHOP promoter activity in response to leucine limitation. Fig. 7A shows that the transcriptional activity from CHOP promoter was enhanced by a decrease in leucine concentration in a dose-dependent manner. Furthermore, kinetic analysis of the cat gene expression revealed that maximal CAT activity induction was reached 16 h after starvation (Fig. 7B).

FIG. 5. Regulation of CAT activity under the control of the CHOP promoter in leucine-starved HeLa cells. The plasmid pCHOP-CAT corresponds to the human CHOP promoter region from nucleotide −954 to +91 fused to the bacterial chloramphenicol acetyltransferase (CAT) gene (31). HeLa cells were transiently transfected with pCHOP-CAT or with plasmid pSV2CAT along with plasmid pCMV-βGal carrying the β-galactosidase gene as described under “Materials and Methods”; 24 h after transfection, cells were incubated for 16 h in DMEM/F12 (420 μM) or in DMEM/F12 lacking leucine (0 μM) and harvested for preparation of cell extracts and CAT activity determination. A, autoradiogram corresponding to CAT assays from pCHOP-CAT and pSV2CAT. B, relative CAT activity of these constructs normalized with respect to the plasmid pCMV-βGal as described under “Materials and Methods.”

In mammals, plasma concentrations of amino acids are affected by nutritional or pathological conditions. The experiments reported in this paper were designed to investigate the role of amino acids in the control of gene expression. A study performed by Marten et al. (12) showed that in a rat hepatoma cell line, removal of one amino acid in the culture medium
induced an increase in the expression of several genes. Among these genes, CHOP expression exhibited the greatest induction in response to amino acid starvation. Nevertheless, molecular mechanisms involved in the regulation of CHOP mRNA expression have not been elucidated to date. To understand the regulation of gene expression by amino acids at a molecular level, we have studied the regulation of CHOP expression in response to leucine limitation.

The main effect of amino acid limitation on cellular function is the inhibition of protein synthesis. We show that low leucine concentrations (35 and 70 μM) can induce CHOP expression but do not significantly inhibit total protein synthesis. However, this does not preclude the possibility that low leucine concentrations could affect the synthesis of particular proteins. These findings demonstrate that the regulation of CHOP expression by amino acid limitation is not a consequence of a cellular stress due to protein synthesis inhibition.

Since no general accumulation of mRNAs in amino acid-starved cells has been observed, mammalian cells must have a specific mechanism(s) that enables them to alter one specific pattern of gene expression in response to amino acid deprivation. Accumulation of asparagine synthetase, c-myc, c-jun, and c-fos mRNA have been reported to be induced transcriptionally and/or post-transcriptionally by amino acid starvation (8, 43, 44). We show that regulation of CHOP expression by leucine limitation has both transcriptional and post-transcriptional components. Our results clearly establish that the stability of CHOP mRNA is very low in the presence of leucine and is markedly increased in the absence of leucine. However, the mechanisms affecting CHOP mRNA stability in leucine-starved cells remain to be characterized. Furthermore, the induction of CHOP mRNA expression is sensitive to cycloheximide treatment suggesting that signaling pathways activated by leucine starvation involve synthesis of essential regulatory protein(s). We also show that starvation of other amino acids like lysine, methionine, arginine, phenylalanine, or threonine increases strongly CHOP promoter activity. These results suggest that gene regulation by leucine may be an example of a more general regulatory mechanism by which CHOP expres-
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