Evidence for Multiple Signaling Pathways in the Regulation of Gene Expression by Amino Acids in Human Cell Lines

Céline Jousse, Alain Bruhat, Marc Ferrara and Pierre Fafournoux

ABSTRACT In mammals, plasma concentrations of amino acids (AA) are affected by nutritional or pathologic conditions. Alterations in AA profiles have been reported as a result of a deficiency of any one of the essential AA, a dietary imbalance of AA or an insufficient intake of protein. In recent years, evidence has accumulated that AA availability regulates the expression of several genes involved in the regulation of a number of cellular functions or AA metabolism. Nevertheless, the molecular mechanisms involved in the AA regulation of mammalian gene expression are limited, particularly the signaling pathways mediating the AA response. This work provides a better understanding of the signaling pathways involved in the AA control of gene expression. We studied the expression of C/EBP homologous protein (CHOP) and asparagine synthetase (AS) in response to deprivation of a single AA and investigated the possible link between protein synthesis inhibition due to amino acid limitation and gene expression. We have shown the following: 1) several mechanisms are involved in the AA control of gene expression. When omitted from the culture medium, each AA can activate one (or several) specific signaling pathways leading to the regulation of one specific pattern of genes. 2) AA limitation by itself can induce gene expression independently of a cellular stress due to protein synthesis inhibition. Together, these results suggest that AA control of gene expression involves several specific mechanisms by which one AA (or one group of AA) can activate one signaling pathway and thus alter one specific pattern of gene expression.

KEY WORDS: amino acid • CHOP • asparagine synthetase • gene expression • human cultured cells

In addition to their role as substrates for protein synthesis, amino acids (AA) have multiple functions. They can act as gluconeogenic substrates, regulators of protein turnover, neurotransmitters or precursors of signal transducers (Young et al. 1994). Because important dispensable AA stores do not exist as they do for lipids or glucose, AA metabolism can be altered in response to various forms of malnutrition or trauma (e.g., sepsis, fevers or thermal burns). A dramatic diminution of the plasma concentration of certain AA occurs after a dietary imbalance, a deficiency of any one of the essential AA or a deficient intake of protein. Moreover, multicellular organisms are unable to synthesize all of the AA; as a result, they have to adjust several of their physiologic functions involved in the adaptation to AA limitation by regulating numerous genes. For example, an AA limitation, as occurs in animals subjected to a low protein diet, increases the level of insulin-like growth factor binding protein-1 (IGFBP-1) mRNA, thereby participating in the down-regulation of growth (Jousse et al. 1998). Other genes have been shown to be regulated by AA availability. Marten et al. (1994) showed that the abundance of several different mRNAs [e.g., C/EBP homologous protein (CHOP), C/EBPβ or ubiquitin] is affected by AA deprivation. The regulation of asparagine synthetase (AS) and CHOP expression by AA availability has been studied at the molecular level (Bruhat et al. 1997, Guerrini et al. 1993). AS is the gene encoding the enzyme responsible for the biosynthesis of asparagine from aspartate and glutamate. CHOP encodes a transcription factor that regulates certain aspects of the response of cellular stress. CHOP induction is generally linked to a perturbation of the endoplasmic reticulum (ER) unfolded protein response (UPR). However, it was shown recently that AA deprivation induces CHOP expression through a pathway distinct from the ER stress-signaling cascade (Jousse et al. 1999b).

The mechanisms involved in the AA control of gene expression are poorly understood in mammalian cells. However, both transcription and translation can be regulated by AA availability. It has been shown that the induction of CHOP and AS genes by AA deprivation involves transcriptional mechanisms. A promoter fragment, when linked to a reporter gene, is sufficient to reproduce the regulation of CHOP or AS expression by leucine deprivation in cultured cells (Bruhat et al. 1997, Guerrini et al. 1993). Moreover, it has been shown that AA availability can regulate mRNA expression by Amino Acids in Human Cell Lines1

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2 To whom correspondence should be addressed.

3 Abbreviations used: AA, amino acid; AS, asparagine synthetase; β-Gal, β-galactosidase; CHOP, C/EBP homologous protein; CHX, cycloheximide; DMEM, Dulbecco’s modified Eagle’s medium; eIF-2α, eucaryotic initiation factor 2α; eIF-4E, eucaryotic initiation factor 4E; ER, endoplasmic reticulum; IGFBP-1, insulin-like growth factor binding protein-1; LUC, luciferase; UPR, unfolded protein response.
translation. AA (particularly leucine) can modulate the protein synthesis rate through changes in the phosphorylation of translation factors and regulatory proteins such as eukaryotic initiation factor 2a (eIF-2a), eukaryotic initiation factor 4E (eIF4E) and protein S6 (Hara et al. 1998, Kimball et al. 1999, Wang et al. 1998, Xu et al. 1998). In yeast, the regulation of gene transcription in response to AA deprivation has been studied extensively. Two types of regulation have been characterized: 1) a specific control whereby numerous operons are regulated by the specific AA end products of the corresponding enzymes (Marczak and Brandris, 1991, Se et al., 1992); and 2) a general control process whereby a subset of genes is coordinately induced by depriving the cell of any single AA (Hinnebusch, 1988). In the general control process, uncharged tRNAs accumulate under conditions of deprivation, thus stimulating the activity of the protein kinase GCN2, which turns on a signaling pathway leading to the translational up-regulation of GCN4. GCN4 is the transcription factor that is responsible for the pleiotropic increase in gene expression. Regulation of genes involved in AA metabolism can contain elements of both general and specific controls.

In this study, experiments were conducted to understand the signaling pathways that link AA limitation to regulated gene expression in mammalian cells. In particular, we examined whether one or several control processes are activated in response to AA availability and the role of protein synthesis inhibition due to AA limitation in the induction of gene expression.

MATERIALS AND METHODS

Cell culture and treatment conditions. Human cervical carcinoma cells (HeLa) and human hepatoma cells (HepG2) were cultured at 37°C under 95% air/5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Sigma Chemical, St. Louis, MO), containing 10% decompemental fetal bovine serum and 10% fetal bovine serum, respectively.

When indicated, DMEM/F12 lacking leucine, methionine, glutamine or lysine was used. For phenylalanine, histidine, tryptophan, asparagine, arginine, cysteine, alanine or several nonessential AA (NEAA; aspartate + glutamate + glycine + proline + serine) deprivation experiments, MEM medium (Life Technologies, Frederick, MD) was used. For AA deprivation experiments, 10% dialysed calf serum was used. The amino acid content was measured at the end of the experiments and was found not to change during the incubation time. Cycloheximide (CHX) was purchased from Sigma Chemical.

RNA isolation and Northern blot analysis. Total RNA was prepared as described previously (Chomczynski and Sacchi 1987). Northern blots were performed according to the procedure of Sambrook et al. (1989); the RNA was cross-linked to the membrane by UV irradiation. The human CHOP cDNA (BH1), generously provided by Dr. N. J. Holbrook (Park et al. 1992) was used as a probe. BH1 plasmid was linearized by PstI and 17P-riboprobes were synthesized (Sambrook et al. 1989) using T7 RNA polymerase (Promega, Madison, WI). Prehybridization was carried out for 2 h at 55°C in 50% formamide, 6X SSC, 5X Denhardt’s reagent, 0.5% SDS, plus 0.5% SDS. Hybridization was carried out for 16 h at 42°C, washed twice in PBS and then incubated with DMEM/F12 containing 10% calf serum. Twenty-four hours after transfection, cells were starved for 16 h. After starvation, cells were harvested in 150 µL of lysis buffer (Promega) and centrifuged at 13,000 × g for 2 min. The supernatant (20 µL) was assayed for LUC activity (PRODEMAT, Anduze, France). β-Gal activity was measured as described by Hall et al. (1983). Relative LUC activity was given as the ratio of relative light unit/relative β-gal unit. All values are the means calculated from the results of at least three independent experiments.

RESULTS

CHOP and AS genes are regulated differentially according to the limiting AA. Among the AA-regulated genes, only the regulation of AS and CHOP has been studied at the molecular level. To understand the signaling pathway activated by AA limitation and leading to gene regulation, we studied the regulation of CHOP and AS expression in response to deprivation of an individual AA. The expression of CHOP and AS genes varied according to the AA that was omitted from the culture medium (Fig. 1). Among the AA tested, 16 h of deprivation in alanine or NEAA (aspartate, glutamate, glycine, proline, serine) did not affect CHOP and AS expression. On the other hand, deprivation of leucine, methionine, lysine, phenylalanine or tryptophan induced the expression of both CHOP and AS genes in the two cell lines tested. A discrepancy between CHOP and AS expression was observed in response to deprivation of histidine, asparagine, cysteine and methionine. AS expression was induced in response to deprivation of histidine, asparagine or cysteine, whereas CHOP expression was little affected. On the contrary, methionine deprivation induced CHOP mRNA content to a greater extent than that of AS mRNA. Similar regulation of CHOP and AS genes was observed in HeLa and HepG2 cells, suggesting that the same regulatory mechanisms are involved in both cell line.
It was shown previously that induction of AS and CHOP expression in response to AA limitation involves a transcriptional component. We sought to determine whether the transcriptional activities of the CHOP and AS promoters are differentially regulated in response to deprivation of leucine, methionine, cysteine, asparagine, and histidine. LUC activity expressed under the control of the CHOP and AS promoter was induced differentially according to the omitted AA (Fig. 2). The transcriptional activity of the AS promoter fragment was induced in response to leucine, methionine, cysteine, asparagine, or histidine deprivation. The activity of the CHOP promoter fragment was poorly increased by cysteine, asparagine or histidine limitation, whereas it was strongly induced in response to methionine deprivation. Taken together, these results show that CHOP and AS genes are regulated differentially, according to the AA that is omitted from the culture medium.

**Cellular stress due to protein synthesis inhibition is not responsible for AS and CHOP induction.** Deprivation of an individual AA can lead to protein synthesis inhibition and therefore induce cellular stress. In this set of experiments, we investigated the possible link between protein synthesis inhibition and CHOP and AS expression.

Deprivation of essential AA plus cysteine inhibited L-^{35}S-methionine incorporation and induces CHOP and AS expression, whereas a limitation in alanine and NEAA did not affect either protein synthesis rate or gene expression (Fig. 3A). Asparagine deprivation induced AS expression (Fig. 1) and did not affect L-^{35}S-methionine incorporation into the cells (Fig. 3).

Cells incubated in a leucine-free medium showed a 60% reduction of L-^{35}S-methionine incorporation into total protein together with a drastic increase in CHOP and AS mRNA level (Fig. 3A and B). However, a moderate limitation in leucine (30 μmol/L) did not reduce global protein synthesis, whereas CHOP and AS mRNA levels were increased significantly. Therefore, a moderate AA restriction, which does not inhibit protein synthesis, can induce CHOP and AS expression.

Because a slight inhibition of protein synthesis, which is not detectable using methionine incorporation, could be responsible for gene induction, we inhibited methionine incorporation by treatment of the cells with CHX, a protein synthesis inhibitor. As shown in Figure 4A, CHX strongly inhibited methionine incorporation into cell proteins. A CHX concentration (0.2 mg/L) inhibiting ~50% of L-^{35}S-methionine incorporation into cell proteins did not induce CHOP or AS expression. Taken together, these results show that there is no association between CHOP and AS expression and protein synthesis inhibition due to AA limitation. Therefore, protein synthesis inhibition is not a prerequisite for gene induction in response to AA deprivation.

**DISCUSSION**

In recent years, evidence has accumulated that AA play an important role in controlling gene expression (Jouss et al., 1999). Nevertheless, the knowledge of the molecular mechanisms involved in the AA regulation of mammalian gene expression is limited. In particular, the signaling pathways mediating the AA response are not known. The objective this paper was to gain a better understanding of the signaling pathways involved in the AA control of gene expression. We investigated whether one or several pathway(s) are involved in gene regulation by AA limitation and whether a cellular stress due to protein synthesis inhibition subsequent to AA limitation is responsible for gene regulation.

Because no general accumulation of mRNAs in AA-deprived cells has been observed (Marten et al., 1994), cells must have a specific mechanism(s) that enables them to alter one specific pattern of gene expression in response to AA deprivation. In a previous study (Jouss et al., 1999b), we showed
that a limitation in one AA does not activate the UPR and therefore regulates CHOP expression through a pathway that is distinct from the ER stress signaling cascade. Consequently, the pathways linking the AA limitation to gene regulation remain unknown. However, in mammalian cells, several of the following observations suggest that depletion in any one AA activates a signaling pathway that could be related to the general control of yeast:

1) CHOP and AS mRNAs are regulated by the level of many different AA (Bruhat et al., 1997, Hutson and Kilberg, 1994, Thissen et al., 1994).

2) Andrulis et al. (1979) showed a correlation among asparagine starvation, amino-acylation of tRNA_{asn} and AS activity.

3) Inhibition of the leucyl-tRNA synthetase induces CHOP expression (Jousse et al. 1999b).

In addition, we hypothesize that different pathways will be activated, depending on the AA that is omitted from the culture medium. Our results effectively demonstrate that, in addition to this type of general control of yeast: 1) CHOP and AS mRNAs are regulated by the level of many different AA (Bruhat et al., 1997, Hutson and Kilberg, 1994, Thissen et al., 1994). 2) Andrulis et al. (1979) showed a correlation among asparagine starvation, amino-acylation of tRNA^{asn} and AS activity. 3) Inhibition of the leucyl-tRNA synthetase induces CHOP expression (Jousse et al. 1999b).

In addition, we hypothesize that different pathways will be activated, depending on the AA that is omitted from the culture medium. Our results effectively demonstrate that, in addition to this type of general control, starvation in certain AA can activate other pathway(s) that lead to up-regulation of a distinct pattern of genes. This conclusion can be drawn from the study of the regulation of AS and CHOP expression in response to methionine, histidine, asparagine or cysteine deprivation. CHOP expression was strongly induced in response to methionine deprivation but was affected only slightly by histidine, asparagine or cysteine deprivation. Under the same experimental condition, AS expression was induced equally in response to a limitation in any one of these AA. The discrep-
The main effect of AA limitation on cellular functions is a general control process described in yeast. This mechanism will lead to the up-regulation of several genes, including AS and CHOP. In addition, methionine deprivation can also turn on a more specific control process that activates CHOP expression. Both the general and specific control processes could regulate certain target genes such as CHOP. Similar examples have been found in yeast. LEU4, which encodes the first enzyme of the pathway of leucine biosynthesis, is subject to regulation by the following: 1) the general control system in response to starvation in any AA and 2) the Leu3p transcriptional activator in response to the levels of the metabolic intermediate α-isopropyl malate, which serves as a sensor of leucine availability (Friden and Schimmel 1988).

This conclusion is strengthened by the study of the regulation of the CHOP- and AS-promoter activities in response to AA deprivation. It was shown previously that these two promoter fragments are regulated by AA availability (Bruhat et al. 1997, Guerrini et al. 1993). The transcriptional activity of the CHOP promoter fragment is strongly induced in response to methionine deprivation, whereas it is only slightly affected by cysteine, asparagine or histidine deprivation. The activity of the AS promoter is increased by a limitation in any one of the AA tested. These results show that, like mRNA content, transcription of the CHOP and AS genes is regulated by different mechanisms according to the AA that is omitted from the culture medium. However, we cannot exclude the possibility that post-transcriptional events are involved in the regulation of the mRNA content of the endogenous gene. Indeed, in response to histidine deprivation, AS mRNA content was strongly enhanced, whereas the transcriptional activity of the AS promoter fragment was only slightly affected. This result also could be explained by the lack of regulatory elements in the AS promoter fragment.

The main effect of AA limitation on cellular functions is the inhibition of protein synthesis. Several lines of evidence show that CHOP or AS induction is dissociated from the inhibition of the protein synthesis. First, a low leucine concentration can induce CHOP and AS expression but does not significantly inhibit total protein synthesis. Second, deprivation of asparagine induces AS expression but has a limited effect on L-35S-methionine incorporation into the cell proteins. Third, CHX treatment, reproducing protein synthesis inhibition resulting from leucine deprivation, does not induce CHOP or AS expression. Taken together, these results show that although a limitation in AA affects L-35S-methionine incorporation, protein synthesis inhibition by itself is not responsible for CHOP and AS induction. However, we cannot exclude the possibility that AA limitation activates a mechanism involved in both protein synthesis inhibition and induction of gene expression. For example, free transfer RNAs could accumulate in response to AA deprivation and rapidly induce the up-regulation of CHOP or AS expression. The accumulation of free tRNA and the lack of charged tRNA could also inhibit protein synthesis.

In humans, amino acids requirements have been very well studied (Millward 1994, Rose 1957, Young 1998). In healthy adult humans, nine amino acids (valine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and histidine) were shown to be indispensable. Therefore, these amino acids must be supplied in the diet. Amino acid and protein homeostasis are finely maintained by the integrated action of all of the tissues and organs. However, in certain situations, nitrogen metabolism can become deranged, and a loss of body protein and/or amino acids will be at the expense of essential elements. The main factors affecting nitrogen metabolism and aminoacidemia are nutritional status and various forms of stress. For example, amino acid patterns have been shown to be altered in humans and animals when there is a deficient intake of protein, a dietary imbalance of amino acids or a deficiency in any one of the essential amino acids. Moreover, in response to various forms of stress, changes in the patterns of free amino acids are observed in plasma and urine. It follows that mammals must adjust several of their physiologic functions involved in the defense/adaptation to amino acid limitation by regulating the expression of numerous genes.

The idea that amino acids can regulate gene expression has just begun to emerge. It is now clear that amino acids by themselves can play (in concert with hormones) an important role in the control of gene expression; however, the underlying processes have only begun to be discovered. Among the genes regulated by amino acid availability, AS encode a gene responsible for the biosynthesis of one amino acid (asparagine), and CHOP encode a transcription factor. The physiologic importance of CHOP regulation in response to amino acid limitation is not yet understood. However, through its association with the C/EBP transcription factor, CHOP could be involved in the regulation of several mechanisms (McKnight et al. 1989) involved in the response to nutrient limitation. Further work will be required to understand the molecular steps by which the cellular concentration of an individual AA can regulate gene expression. These studies will provide an understanding of the role of AA in the regulation of cellular functions such as cell division, protein synthesis or proteolysis. The molecular basis for gene regulation by dietary protein intake is important in the regulation of physiologic functions of individuals living under conditions of restricted or excessive food intake.

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