Induction of p21 and p27 expression by amino acid deprivation of HepG2 human hepatoma cells involves mRNA stabilization

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mRNA abundance for a number of genes is increased by amino acid limitation. From an array screening study in HepG2 human hepatoma cells, it was established that one set of genes affected by amino acid availability is the set associated with cell-cycle control. The present study describes the increased expression of both mRNA and protein for the cyclin-dependent kinase inhibitors p21 and p27 in response to deprivation of HepG2 cells for a single essential amino acid, histidine. The increase in p21 and p27 mRNA content depended on *de novo* protein synthesis and involved a post-transcriptional mRNA stabilization component. For p21, increase in mRNA by histidine depletion appeared to be independent of p53 transactivation, and the absolute level of p53 protein was unaffected by this treatment. Histidine limitation

caused an increase in the phosphorylation of ERK1/ERK2 (extracellular-signal-regulated kinase), and inhibition of the ERK signal transduction pathway resulted in a reduction in the starvation-dependent increase in p21 mRNA. Blockade of the phosphoinositide 3-kinase and mTOR (mammalian target of rapamycin) pathways also blunted the increase in p21 mRNA content. These results document the amino acid-dependent control of the synthesis of specific cell-cycle regulators and help to explain the block at G₁ phase after amino acid limitation.

Key words: amino acid starvation, cell cycle, cyclins, cyclin-dependent kinase inhibitor, mRNA stability, nutrient control.

INTRODUCTION

Adequate amount of protein in the diet plays an important role in general nutrition as a source of essential amino acids. Furthermore, changes in amino acid availability for specific tissues are a crucial factor in the pathology, therapy and recovery of several diseases. A molecular understanding of metabolite control of gene expression in mammalian cells is an area of investigation that is still at its early stages, but nutrient control is clearly an important factor in regulating cellular response to the environment (reviewed in [1,2]). Amino acid availability is known to alter a number of cellular functions, including transcription [1] and translation [3]. One subset of genes that is probably affected by nutrient availability is the subset associated with cell division. In culture, cells can be forced to undergo growth arrest by limiting the supply of nutrients or by depriving the cell of essential growth factors [4]. Of particular interest is the observation that depriving the cell of a single essential amino acid causes a block at the G₁ phase of the cell cycle [5]. However, the exact mechanism by which the block occurs is not well understood. Progression through the cell cycle is controlled by three different groups of proteins: (1) cyclins, (2) CDKs (cyclin-dependent kinases) and (3) CDKIs (CDK inhibitors). CDK activation can be highly regulated by modulating the synthesis and degradation of cyclin proteins, and through inhibition of CDK activity by CDKIs. Cyclins and CDKs become active only when they associate with each other to form a complex, whereas CDKIs function by binding to and inhibiting the action of these complexes [6,7].

During the G₁- to S-phase progression, two key CDKI proteins, p21 and p27, can inhibit the activity of the cyclin D–CDK and cyclin E–CDK complexes and thereby control the G₁ to S transition checkpoint [6,7]. The p21 protein, also known as CIP1 (CDK-interacting protein 1) and WAF1 (wild-type p53-activated

fragment 1), was identified as a regulator of CDK2 [8–10], and as a target gene subject to transcriptional activation by wild-type p53 [11]. It is believed that p21 serves as a downstream effector for p53-mediated cell-cycle control [11], but p21 expression can also be regulated by p53-independent mechanisms in response to a number of stimuli [12]. Moreover, the p21 mRNA level can be regulated at both transcriptional and post-transcriptional steps [13,14]. Similar to the actions of p21, the CDKI, known as p27 or KIP1 (kinase inhibitory protein 1), also inhibits cyclin–CDK complexes; in fact, p27 shares approx. 44 % homology with p21 in the N-terminal 65 amino acids, the region which contributes to the inhibitory function of CDKIs [15,16]. Similar to p21, p27 expression can be regulated by either transcriptional or post-transcriptional mechanisms [17,18].

In the present study, we describe the up-regulation of p21 and p27 mRNA and protein expression in response to amino acid deprivation of HepG2 human hepatoma cells. Our results also show that the p21 response is independent of the changes in p53 protein expression and that it depends, at least in part, on increased mRNA stability. The ERK (extracellular-signal-regulated kinase), PI3K (phosphoinositide 3-kinase) and mTOR (mammalian target of rapamycin) signal transduction pathways all appear to contribute to this response.

MATERIALS AND METHODS

Inhibitors

ActD (actinomycin D), CHX (cycloheximide), wortmannin and rapamycin were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). The kinase inhibitors PD98059, SB203580, JNK (c-Jun N-terminal kinase) inhibitor II (SP600125) and LY294002 were obtained from Calbiochem (San Diego, CA, U.S.A.). Stock

Abbreviations used: ActD, actinomycin D; Cat-1, cationic amino acid transporter 1; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; CHX, cycloheximide; ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEM, minimal essential medium; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; RT, reverse transcriptase; UTR, untranslated region.

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solutions were prepared for the inhibitors in the following concentrations: ActD (300 μ M, dissolved in 100 % ethanol), CHX (10 mM, dissolved in water), PD98059 (10 mM, dissolved in DMSO), SB203580 (10 mM, dissolved in DMSO) and SP600125 (20 mM, dissolved in DMSO). DMSO was obtained from Sigma–Aldrich.

Cell culture

Human HepG2 hepatoma cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.), and maintained in MEM (minimal essential medium, pH 7.4; Mediatech, Herndon, VA, U.S.A.), supplemented with 25 mM NaHCO₃, 2 mM glutamine, 10 μ g/ml streptomycin sulphate, 100 μ g/ml penicillin G, 28.4 μ g/ml gentamicin, 0.023 μ g/ml *N*-butyl-*p*-hydroxybenzoate, 0.2 % (w/v) BSA and 10 % (v/v) foetal bovine serum. Cells were grown at 37 °C in a 5 % CO₂ and 95 % air incubator. Nutrient deprivation was performed by incubating the cells for 12 h in complete MEM or in MEM lacking histidine or glucose (Invitrogen, Carlsbad, CA, U.S.A.). When inhibitors were used, an aliquot of the stock solution (or the solvent for the control) was added directly to the cells in a fresh medium.

Genomic constructs

The reporter construct used to measure the activity of the p21 promoter was prepared in the following manner. The promoterless pGL3 vector containing firefly luciferase was used as the reporter plasmid (Promega, Madison, WI, U.S.A.). A p21 genomic fragment, provided generously by Dr B. Vogelstein (John Hopkins University, Baltimore, MD, U.S.A.), containing nt –4500 to +500 of the 5' upstream genomic sequence of the p21 gene, was cloned into the *Bgl*II site of the pGL3 reporter. The *Renilla* luciferase construct phRL-SV40 was used as a co-transfection control.

Transfections

For the p21 promoter-luciferase reporter assays, HepG2 cells were transfected on 24-well dishes using Superfect reagent (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. Cells were seeded at a density of 2×10^5 cells/well in a total volume of 1 ml/well complete MEM plus 10 % serum. Approx. 16 h after plating, the reporter construct p21 (–4500/+500) promoter-Luc (0.9 μ g/well) was mixed with the *Renilla* co-transfection control phRL-SV40 (0.1 μ g/well) and Superfect reagent (5 μ l/well), and transfection was initiated. After transfection (16 h), the cells were placed for an additional 12 h in either complete MEM or MEM lacking histidine, each containing 10 % dialysed foetal bovine serum. Cells were collected and luciferase activity was assayed using the Dual-Luciferase Reporter assay system (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions.

RNA isolation and Northern-blot analysis

Total cellular RNA was isolated from HepG2 cells using the Qiagen RNeasy kit (Qiagen). For each sample, 12 μ g of RNA was dried and resuspended in 20 μ l of 12.5 M formamide, 6.6 % (v/v) formaldehyde, 6 mM sodium acetate (pH 7.4), 0.5 mM EDTA (pH 8.0) and 20 mM Mops (pH 7.0). The resuspended RNA was incubated at 65 °C for 15 min and 4 μ l of loading dye [0.4 % xylene cyanole, 0.4 % Bromophenol Blue, 1 mM EDTA, (pH 8.0), 50 % (v/v) glycerol and 0.3 μ g/ μ l ethidium bromide] was added to each sample. RNA was size-fractionated as described previously [19]. The membrane was prehybridized for

30 min at 61 °C in hybridization solution [0.5 M sodium phosphate, pH 7.2/7 % (w/v) SDS/1 mM EDTA, pH 8.0/1 % (w/v) BSA] before adding the radiolabelled cDNA probe. Probes were prepared by labelling the respective cDNAs with [α -³²P] dATP using the Strip-EZ DNA kit (Ambion, Austin, TX, U.S.A.). A cDNA probe for p21 was obtained from HepG2 RNA by RT (reverse transcriptase)–PCR using the primers 5'-GCCGAAGTCAGTTCCTTGTGG-3' and 5'-AGCATTGTGGGAGGAGCTGTG-3'. A cDNA probe for p27 was obtained from HepG2 RNA by RT–PCR using the primers 5'-GTCAGCCTCCCTTCCACCGCCATATTG-3' and 5'-GAGGGGAAAACCTATTCTACCC-AACACAGC-3'. The p21 and p27 cDNA products were sequenced to confirm their identity and sequence fidelity. Probes for ribosomal protein L7a and glutamate dehydrogenase were prepared as described previously [20]. Unincorporated nucleotides were removed by filtering the labelling reaction mixture through a Sephadex G-50 column (Amersham Biosciences, Piscataway, NJ, U.S.A.). The eluate was boiled for 5 min, added to the membrane and incubated overnight at 61 °C. The membrane was washed for 4 $\times$ 10 min in high-stringency washing buffer [0.04 M sodium phosphate, pH 7.2/1 mM EDTA, pH 8.0/1 % (w/v) SDS] at 65 °C, exposed to Biomax MR film (Kodak, Rochester, NY, U.S.A.) and analysed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The results were quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Immunoblotting

Total cell extracts (30 μ g/sample) were separated on a 12 % Tris/HCl polyacrylamide gel (Bio-Rad Laboratories) and electrotransferred on to a Protran nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.). The membrane was stained with Fast-Green stain to check for equal loading and then incubated with 5 % blocking solution [5 % (w/v) Carnation non-fat dry milk/30 mM Tris, pH 7.5/0.1 % (v/v) Tween 20/200 mM NaCl] for 2 h at room temperature (21 °C) with mixing. Immunoblotting was performed using a rabbit polyclonal antibody against p21 (catalogue no. C-19), mouse monoclonal antibodies against p53 (catalogue no. DO-1) and p27 (catalogue no. F-8), a rabbit polyclonal antibody against total ERK1/ERK2 (catalogue no. K-23) and a mouse monoclonal antibody against phosphorylated-ERK1/ERK2 (catalogue no. E-4) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Each antibody was used at 0.2–0.4 μ g/ml in 1 % dry milk blocking solution and incubated overnight for 2 h at 4 °C. The blots were washed 5 $\times$ 5 min in 1 % blocking solution on a shaker and then incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody (Kirkegaard & Perry, Gaithersburg, MD, U.S.A.) at a 1:20 000 dilution for 45 min at room temperature. The blots were then washed for 5 $\times$ 5 min in 1 % dry milk blocking solution and 2 $\times$ 5 min in TBS/Tween (30 mM Tris/0.1 % Tween 20/200 mM NaCl, pH 7.5). The bound secondary antibody was detected by using an enhanced chemiluminescence kit (ECL[®]; Amersham Biosciences) and exposing the blot to a Biomax MR film.

Real-time quantitative RT–PCR

To measure the relative amount of the p21 mRNA, a quantitative real-time PCR was performed using a DNA Engine Opticon 2 system (MJ Research, Reno, NV, U.S.A.) and detection with SYBR Green I. The following primers were used for amplification: for p21, sense 5'-CCGCGACTGTGATGCGCTAATG-3' and antisense 5'-CTCGGTGACAAAGTCGAAGTTC-3'; for L7a, sense 5'-TTTGGCATTGGACAGGACATCC-3' and antisense

5'-AGCGGGGCCATTTCACAAAG-3'. In each reaction, 25 and 200 ng of HepG2 total RNA were used to measure the mRNA content for L7a and p21 respectively. The reaction mixture was incubated at 50 °C for 30 min followed by 95 °C for 15 min to activate the polymerase, and the amplification was of 35 cycles at 95 °C for 15 s followed by 60 °C for 60 s. After PCR, melting curves were acquired by a stepwise increase in the temperature from 55 to 95 °C to ensure that a single product was amplified in the reaction. Samples from at least three independent experiments were analysed.

RESULTS

mRNA and protein levels for both p21 and p27 are induced by depriving the cells of a single amino acid

To identify the target genes of the amino acid deprivation pathway, RNA was isolated from HepG2 cells that had been incubated in either amino acid-complete MEM or in MEM lacking histidine. These RNA samples were used to prepare probes for array screening, and from that screen, one of the genes chosen for further study was the cell-cycle regulatory protein p21. To confirm that p21 mRNA expression was induced by amino acid deprivation, HepG2 cells were incubated in either complete MEM or MEM lacking histidine; RNA was then isolated at specific time points and analysed by Northern blotting. An increase in p21 mRNA was first detected at 3 h after histidine limitation, and the level continued to increase until the highest level (seven times control) was reached at 12 h (Figures 1A and 1B). Given that other amino acid-regulated genes, such as asparagine synthetase, are also activated by glucose starvation [20], the response of p21 to glucose limitation was examined. The results showed that p21 mRNA content was not changed after glucose starvation (Figures 1A and 1B). An immunoblot analysis for p21 protein revealed a lag between the increases of mRNA and protein, with protein content increasing between 8 and 18 h of starvation and continuing up to 24 h (Figure 1C). Given that the p21 gene can be transcriptionally activated by p53 [11], the level of p53 protein was analysed by immunoblotting and observed not to change in response to histidine deprivation (Figure 1C).

Amino acid-dependent expression of other CDKIs that act at the G₁ to S checkpoint was also examined. Two members of the INK4 (Inhibitor of CDK4) family, p15 and p16 [7], were not detectable on Northern-blot analysis using RNA from HepG2 cells (results not shown). However, the mRNA content for p27, a CDKI that shares sequence similarity with p21 [15,16], increased after histidine deprivation (Figures 1D and 1E). Similar to p21, the p27 mRNA level did not increase significantly in response to glucose starvation. Despite this similarity in the specificity of metabolite control, the increase in p27 mRNA followed a different time course when compared with p21. The p27 mRNA level increased slightly after 3 h of histidine deprivation and continued upward until 8 h, after which there was no further increase. The p27 protein content in cells deprived of amino acid also increased and, similar to p21, it lagged behind the mRNA increase (Figure 1F).

The induction of p21 and p27 mRNA was also observed after limitation of other individual essential and non-essential amino acids, examples of which are shown in Figure 1(G). The absolute magnitude of induction differed for each amino acid, and there was no apparent rationale to explain the efficacy of one over another for either p21 or p27. Deprivation for all amino acids by using Earle's balanced salt solution yielded similar results to depletion of a single amino acid. As shown previously by our laboratory for HepG2 cells, histidine deprivation was routinely one of the more

effective treatments. Therefore the rest of the experiments used MEM lacking histidine as the activating condition.

Blockade of general transcription prevents the increase in mRNA by amino acid deprivation for both p21 and p27

To test if *de novo* transcription plays a role in the up-regulation of p21 and p27 mRNA levels, HepG2 cells were grown for 0–12 h in complete medium (MEM) or MEM lacking histidine, with or without 5 µM of the transcription inhibitor ActD. The resulting blockade of transcription prevented the induction of both p21 and p27 mRNA by histidine starvation (Figure 2). In fact, the mRNA levels of both CDKIs decreased compared with the basal state (MEM), consistent with previous reports of a short half-life for both of these mRNA species [13,18].

The p21 promoter does not respond to amino acid limitation

Inhibition of the p21 mRNA increase by ActD indicated that either transcriptional control of the p21 gene itself contributes to the amino acid-dependent regulation or the activation of an upstream regulatory gene is required. To distinguish between these two possibilities, the human p21 gene was tested for an amino acid response element. A construct containing 2.4 kb of the p21 5'-upstream genomic region, linked to the firefly luciferase reporter gene, was assayed for a response to amino acid deprivation. This 2.4 kb genomic fragment supported basal transcription rates, but did not exhibit increased transcription in the absence of amino acid (results not shown). To examine the possibility that an amino acid response element is located further upstream of the genomic region, a 4.5 kb upstream genomic fragment of the p21 gene (nt – 4500 to +500) was cloned in front of the luciferase reporter and tested for inducible transcription (results not shown). Once again, although the 4.5 kb promoter mediated basal transcription, no response was seen in amino acid deprivation (results not shown). The asparagine synthetase promoter (nt – 173 to +51) was used as a positive control for inducible transcription by histidine limitation [19]. Both the 2.4 and the 4.5 kb p21 genomic fragments contained known p53 activation sites [11], and yet, neither fragment responded to amino acid limitation. These results are consistent with the observation that histidine deprivation did not induce an increase in the p53 protein content (Figure 1).

Increased mRNA stability contributes to the induction of p21 and p27 mRNA

Although inhibition of the p21 mRNA increase by ActD is consistent with a transcriptional mechanism, the effect could also be due to inhibited mRNA synthesis of a required upstream activator. The delay of 3 h before a detectable increase in p21 mRNA (Figure 1) would allow for synthesis of such a regulator. Furthermore, the lack of promoter responsiveness is also consistent with a non-transcriptional mechanism for the p21 mRNA increase. If the induction of p21 is not caused entirely by increased transcription, an alternative explanation is amino acid-dependent control of mRNA stability. To investigate this possibility for both p21 and p27, cells were cultured for 12 h in medium lacking histidine to increase the mRNA levels and then transferred to either MEM or MEM lacking histidine, both media containing 5 µM ActD to eliminate further transcription. Northern-blot analysis revealed that the half-life of the p21 mRNA in the amino acid-fed condition was approx. 1 h, which was in clear contrast with the 8 h half-life observed in the histidine-deprived state (Figures 3A and 3B). Analysing the decay of p27 mRNA by the same procedure revealed that amino acid deprivation extended

Figure 1 Induction of p21 and p27 mRNA and protein content by histidine deprivation

HepG2 hepatoma cells were incubated for 0–12 h in complete MEM or MEM lacking histidine (– His) or MEM lacking glucose (– Glc). RNA (**A, D**) or total cellular protein (**C, F**) was isolated at the indicated times. Northern-blot analysis (12 µg of RNA/lane) was used to assay mRNA levels for p21, p27 or the ribosomal protein L7a used as a negative control. (**A, D**) A representative autoradiogram is shown, whereas (**B, E**) illustrate a compilation of three independent experiments for Northern-blot analysis and the results are shown as means ± S.D.; where not shown, the S.D. bars are within the symbol. To obtain the protein data of (**C, F**), total cell extracts were subjected to immunoblot analysis using antibodies against p21, p27 or p53. Each immunoblot analysis was performed with three different extracts with similar results. (**G**) A representative autoradiogram showing changes in p21 or p27 mRNA content in response to limitation of individual essential and non-essential amino acids, as well as total amino acid limitation (Earle's balanced salt solution).

the half-life of the p27 mRNA as well, from approx. 2.5 h in the amino acid-fed condition to 6 h in the absence of histidine (Figures 3C and 3D).

Blockade of *de novo* protein synthesis abolishes the induction of p21 mRNA

To determine if the induction of p21 and p27 mRNA was dependent on *de novo* protein synthesis, HepG2 cells were cultured for 0–12 h in MEM or MEM lacking histidine in the presence or absence of 0.1 mM CHX. Inhibition of protein synthesis in cells incubated in complete MEM caused a 2.5–3.0-fold increase in p21 mRNA relative to MEM alone (Figures 4A and 4B). This increase probably reflects the accumulation of mRNA due to inhibition of p21 mRNA turnover. Consistent with this inter-

pretation, the presence of CHX during amino acid refeeding of previously starved cells completely prevented p21 and p27 mRNA decay (results not shown). When CHX was included in the MEM lacking histidine, the increase in p21 mRNA by more than ten times the control value (MEM) was completely blocked (Figures 4A and 4B). These results imply that *de novo* protein synthesis is required for the induction of p21 mRNA. For p27, the presence of CHX in the complete MEM induced the accumulation of p27 mRNA to a level equal to that observed with histidine deprivation alone, suggesting inhibition of p27 mRNA turnover by CHX (Figures 4C and 4D). However, in contrast with the results for p21, when CHX was included in the MEM lacking histidine, the increase in p27 mRNA was not blocked at all, indicating that *de novo* protein synthesis is not required for the induction of p27 mRNA by amino acid limitation.

Figure 2 Effect of ActD on the induction of p21 and p27 mRNA by histidine deprivation

HepG2 cells were incubated in complete MEM or MEM lacking histidine with or without 5 μ M ActD. RNA was isolated at the indicated times and Northern-blot analysis was performed to measure p21, p27 or L7a mRNA content. (A, C) Representative autoradiograms are shown, whereas (B, D) display the quantification of three independent experiments representing means $\pm$ S.D.; where not shown, the S.D. bars are within the symbol.

Phosphorylation of ERK1/ERK2 increases after histidine depletion

Expression of p21 has been reported to be up-regulated by increased mRNA stability through a mechanism dependent on ERK1/ERK2, which are part of the mitogen-activated protein kinase signalling pathway [13,21]. Investigation of the effect of histidine limitation on ERK1/ERK2 activation in amino acid-deprived HepG2 cells revealed that the total amount of ERK1/ERK2 protein was unchanged, but the amount of the phosphorylated activated form increased after amino acid limitation (Figure 5). After approx. 4 h of amino acid deprivation, approx. 40 % of ERK1/ERK2 was phosphorylated and this level remained relatively high for 4–12 h. Analysis at 24 h indicated that the amount of phosphorylation decreased, but was still slightly increased when compared with the $t=0$ control. The time course of ERK activation by histidine deprivation is consistent with a potential role in p21 regulation, since the ERK activation occurs before the onset of p21 mRNA increase (Figure 1B).

Inhibition of ERK1/ERK2 activation blocks the increase in mRNA and protein for p21 after amino acid deprivation

To examine whether kinase signalling pathways are involved in the response of p21 to amino acid deprivation, selected known pathway inhibitors were tested (Figures 6A and 6B). HepG2 cells were incubated in either MEM or MEM lacking histidine in the presence or absence of the following inhibitors: for ERK1/ERK2, PD98059 (50 μ M); for p38 kinase, SB203580 (10 μ M); and for JNK inhibitor II, SP600125 (20 μ M). These concentrations have been shown by others to be effective in HepG2 cells [22,23]. Recent work has questioned the specificity of several kinase inhibitors [24], arguing that secondary targets are affected

at higher concentrations, thereby giving false results. However, the same report showed that the ERK inhibitor PD98059 did not affect other kinase targets. Blocking the ERK1/ERK2 pathway decreased the increase in p21 mRNA by histidine deprivation to approximately one-half of the control level (Figures 6A and 6B). In contrast, inhibition of either p38 kinase or JNK did not prevent the p21 mRNA increase. A dose-dependent curve for PD98059 action indicated that the induction of p21 mRNA content by histidine limitation was decreased with increasing concentrations of the ERK1/ERK2 inhibitor (Figure 6C), but concentrations above 50 μ M were not tested to avoid cytotoxicity as a possible complication.

When p21 protein content was examined in parallel experiments, inhibition of either p38 or JNK in MEM-incubated control cells caused an increase, whereas blocking the ERK1/ERK2 pathway had no effect (Figures 6D and 6E). In contrast, the increase in p21 protein after histidine deprivation was completely blocked by inhibiting the ERK1/ERK2 pathway, whereas inhibition of either p38 or JNK did not prevent the starvation-induced increase in p21 protein. Lower concentrations of PD98059 (5 and 10 μ M) partially inhibited the activation of the ERK1/ERK2 after histidine deprivation, whereas the increase in p21 protein content appeared to require a somewhat greater degree of inhibition. These results are consistent with those of Franchi-Gazzola et al. [25], who showed that amino acid deprivation caused an activation of the ERK1/ERK2 pathway, but also suggest that other pathways may contribute to the response.

Inhibition of PI3K and mTOR pathways

The expression of p21 can be regulated by PI3K and mTOR pathways after treatment with epidermal growth factor [26]. Given

Figure 3 Effect of histidine limitation on the turnover of p21 and p27 mRNA

HepG2 cells were incubated in MEM lacking histidine for 12 h and then transferred to fresh complete MEM or MEM lacking histidine, both containing 5 μ M ActD. At the indicated times, RNA was isolated and subjected to Northern-blot analysis for p21, p27 or L7a mRNA. (A, C) Representative autoradiograms of p21 and p27 mRNA content respectively. (B, p21) and (D, p27) represent the means $\pm$ S.D. for three independent experiments, the p21/L7a or p27/L7a ratio is expressed as a percentage of the value obtained for $t = 0$ (12 h histidine starvation) and then plotted as the semi-logarithm to estimate the decay rate of p21 or p27 mRNA.

Figure 4 Effect of CHX on the induction of p21 and p27 mRNA by histidine deprivation

HepG2 cells were incubated in either complete MEM (M) or MEM lacking histidine (– His) with or without 0.1 mM CHX. RNA was isolated at the indicated times and analysed by Northern-blot analysis for p21, p27 or L7a mRNA. (A, C) Representative Northern blots for p21 or p27 mRNA content respectively, whereas (B, p21) and (D, p27) are the summary of three independent experiments and the results are depicted as means $\pm$ S.D.; where not shown, the S.D. bars are within the symbol.

Figure 5 Abundance and activation state of ERK1/ERK2 after amino acid deprivation

A total cellular extract was collected from HepG2 cells at specific time points after incubation in complete MEM (M) or MEM lacking histidine (– His). Immunoblots were probed with an antibody against total ERK protein (upper box) or an antibody specific for phosphorylated ERK isoforms (lower box). (A) A representative autoradiogram is shown, whereas (B) is a summary of three independent experiments and the results are depicted as means $\pm$ S.D. * $P \leq 0.05$ compared with the control ($t = 0$). All blots were stained with Fast Green to confirm equal lane loading.

that 50 μ M PD98059 completely prevented ERK1 phosphorylation, but caused only a partial inhibition of p21 mRNA content, the possible involvement of additional signalling pathways was tested. HepG2 cells were incubated for 12 h in either complete MEM or MEM lacking histidine with or without wortmannin or LY294002 (PI3K inhibitors) or rapamycin (mTOR inhibitor) at concentrations shown to be effective by others [27]. The relative amount of the p21 mRNA was analysed by quantitative real-time RT–PCR. Results showed that blockade of either pathway partially inhibited the increase in p21 mRNA by histidine depletion (Figure 7). Simultaneous inhibition of both PI3K and mTOR pathways caused nearly a complete prevention of the starvation-dependent increase in p21 mRNA level. These results are consistent with the observations by others that changes in p21 expression, in response to a specific stimulus, can be mediated by multiple pathways [26].

DISCUSSION

Results in the present study report the up-regulation of two CDKIs, p21 and p27, in response to amino acid limitation of human HepG2 hepatoma cells. Increased expression of the mRNA and protein content was observed for p21 and p27, although the absolute magnitude of the increase and the time course of activation were different for the two proteins. The mRNA accumulation for p21 began earlier when compared with that for p27, but the time required to detect an increase in protein content was similar. The increase in CDKI expression was not a general nutrient stress response of the p21 and p27 genes, as starvation for glucose did not affect expression of either gene.

Interestingly, the total protein content for the tumour suppressor p53, a known activator of p21 gene transcription [11], did not

change after amino acid depletion. In addition, despite containing a known p53 *cis*-element [11], a 4.5 kb p21 genomic fragment did not exhibit increased promoter activity in response to amino acid deprivation. These results suggest that the p21 activation is independent of total p53 protein content, and they are consistent with an earlier study that the induction of asparagine synthetase by amino acid limitation is p53-independent also [28].

With regard to the mechanism for increased p21 and p27 mRNA expression, two possibilities existed: (1) increased transcription from the respective genes or (2) a decreased decay rate of the mRNA. Although ActD treatment completely prevented the increase in both p21 and p27 mRNA, when two different p21 genomic fragments, 2.4 and 4.5 kb in length, were tested for amino acid-dependent transcription, no regulated response was detected. Although not conclusive by themselves, these results indicate that either increased transcription does not contribute or that the amino acid response element is located elsewhere in the gene. Conversely, the sensitivity to ActD may also indicate that activation of an upstream regulatory gene is required. Additional experiments indicated that amino acid limitation slowed p21 mRNA turnover significantly, extending the apparent half-life from approx. 1 h to approx. 8 h. Similar results indicated that the half-life of p27 mRNA was also prolonged in cells cultured in histidine-deprived conditions, although the difference was less significant when compared with that for p21. Expression of the Cat-1 (cationic amino acid transporter 1) is also up-regulated by amino acid depletion, in part, through increased mRNA stability [29]. In this instance, an AU-rich *cis* element within the 3'-UTR (3'-untranslated region) of the Cat-1 mRNA was shown to be important and the involvement of the RNA-binding protein HuR was reported [29]. Interestingly, similar to the p21 mRNA stabilization shown in the present study, amino acid-dependent regulation of Cat-1 mRNA stability was inhibited by ActD [30]. p21 mRNA contains an AU-rich sequence in the 3'-UTR, which can mediate increased mRNA stability in response to either phenylephrine or UV treatment [13,31]. Furthermore, the p21 3'-UTR has also been reported to contain other *cis* elements that can influence its stability [32]. One or more of these elements may contribute to the amino acid-dependent control as described in this study.

For both p21 and p27, CHX treatment of the control cells maintained in complete MEM increased the steady-state mRNA abundance, probably the result of blocking the turnover of p21 and p27 mRNA. Inhibition of protein synthesis has been shown to cause the accumulation of mRNA species that contain an AU-rich element and that are rapidly degraded, a phenomenon often referred to as 'super-induction' presumed to result from inhibition of mRNA turnover. CHX treatment resulted in elimination of the starvation-dependent mRNA increase for p21, demonstrating a requirement for a newly synthesized protein factor(s) for the p21 response to histidine limitation. For p27, CHX did not block the increase in p27 mRNA caused by histidine deprivation, the level of mRNA in the histidine-deprived cells treated with CHX was the same as in the histidine-limited cells.

Screening of kinase inhibitors revealed that the increase in p21 mRNA after histidine deprivation was partially blocked when the ERK, PI3K or mTOR pathways were inhibited. In contrast, inhibition of p38 and JNK did not significantly decrease p21 mRNA induction. These results are consistent with a previous report showing that regulation of p21 protein expression by epidermal growth factor is dependent on ERK, PI3K and mTOR pathways [26]. Inhibition of the ERK pathway caused a decrease in the starvation-dependent activation of p21 mRNA expression that correlated with phosphorylation of ERK1/ERK2. Interestingly, the basal level of p21 mRNA expression was not affected by ERK inhibition, suggesting that the ERK pathway primarily

Figure 6 Role of mitogen-activated protein kinases in the induction of p21 mRNA and protein by histidine deprivation

HepG2 cells were transferred to complete MEM or MEM lacking histidine (– His) for 12 h with or without one of the following inhibitors: 50 μM PD98059, 10 μM SB203580 and 20 μM SP600125 (JNK Inhib. II). **(A)** RNA was isolated from the cells and subjected to Northern-blot analysis for p21 or GDH (glutamate dehydrogenase). **(B)** Quantified data are from three different experiments, represented as means ± S.D. * $P \leq 0.05$ compared with the control. To obtain the results shown in **(C)**, HepG2 cells were treated with specific concentrations of PD98059 for 12 h, as described in **(A)**. RNA was analysed by Northern blotting and results are the means ± S.D. for three independent experiments. * $P \leq 0.05$ compared with the histidine-deprived control (0 μM). The open (MEM) and black (– His) bars illustrate the relative amount of p21 mRNA at each inhibitor concentration. To examine the regulation of p21 protein content, a total cellular extract was collected from dishes incubated in parallel with those for RNA isolation. The levels of p21 protein, total ERK protein and phosphorylated ERK were analysed. **(D, F)** Representative immunoblots, whereas **(E)** and **(G, upper panel)** are means ± S.D. for three independent experiments. **(G, lower panel)** The percentage of phosphorylated ERK1/ERK2 at the indicated PD98059 concentration, with the untreated control (0 μM) set at 100%. In **(E, G)**, * $P \leq 0.05$ compared with the histidine-deprived control (no inhibitor); in **(E)**, ** $P \leq 0.05$ compared with the MEM control (no inhibitor).

mediates the regulated expression of p21 mRNA. A combinatorial inhibition of the PI3K and mTOR pathways decreased the p21 mRNA induction to almost the basal level, which agrees with other reports that wortmannin attenuates p21 action through PI3K activity [33,34]. Previous reports from two independent laboratories have also shown the involvement of ERK in the activation of system A amino acid transporter activity after amino acid limitation [25,35]. The steps between activation of these signal transduction pathways and the increase in p21 mRNA expression remain to be established, but the identification of their participation will aid in future investigations.

Interestingly, although inhibition of the p38 and JNK pathways did not significantly affect the starvation-dependent induction of p21 protein content, blockade of these two pathways actually caused an increase in the basal (MEM-incubated cells) level. The stress-activated protein kinases p38α and JNK1 have been reported to stabilize p21 protein by phosphorylation [36]. Hence, we speculate that during amino acid limitation, in addition to the increases in p21 mRNA content, the turnover of p21 protein might also be regulated by multiple pathways.

The findings presented in this study provide support for a broader explanation to the current view of how mammalian cells

Figure 7 Role of the PI3K and mTOR pathways in the induction of p21 by histidine limitation

HepG2 cells were incubated for 12 h in either complete MEM or MEM lacking histidine (– His) in the absence or presence of the following inhibitors: 50 μ M LY294002, 200 nM wortmannin or 200 nM rapamycin. RNA was isolated and then quantitative real-time PCR was performed to measure the relative amount of p21 and L7a mRNA, as described in the Materials and methods section. Results from three independent experiments are presented as means $\pm$ S.D. * $P \leq 0.05$ compared with the histidine-deprived control (no inhibitor).

control the cell cycle in response to amino acid limitation. Most of the current models argue that amino acid limitation leads to a general decrease in protein synthesis, including a decrease in the synthesis of cyclin D, which results in a block at the G₁ to S checkpoint [6]. This decrease in cyclin D production can affect the G₁ to S progression in two ways. First, it would cause a decrease in the formation of the cyclin D–CDK complex required to pass the G₁ to S restriction point. Secondly, it would increase the available CDK1 to bind and inhibit the required cyclin E–CDK complex [6]. A recent study also supports this model, in that Nelsen et al. [37] observed that starvation of rat hepatocyte primary culture for non-essential amino acids caused the down-regulation of cyclin D1 synthesis. Whereas it is probable that cyclin D biosynthesis is an important factor, the present results are consistent with an alternative mechanism of G₁ to S control involving the regulated synthesis of both p21 and p27. This specific regulation by amino acid availability would, in turn, lead to the inhibition of the cyclin D–CDK and cyclin E–CDK complexes, which are necessary for G₁ to S progression. In apparent contrast with the present results, Nelsen et al. [37] also reported that p21 was decreased when cells were starved for non-essential amino acids, but there was no effect after depriving the primary rat hepatocytes of essential amino acids. Although the reasons for these differences are unknown, three obvious differences in experimental design should be noted. First, Nelsen et al. [37] used primary hepatocytes, which are a non-dividing population of cells, whereas our studies involved rapidly growing hepatoma cells. Secondly, they saw no change in expression when the cells were starved for the essential amino acids, a situation very similar to the histidine deprivation used in the present study. Thirdly, in their experiments, the cells were deprived of amino acids for 3 days, whereas our studies were designed to observe the initial signalling events and, hence, were limited to 12–18 h. Which of these differences in experimental design are important and how different cell types respond to limitation of individual or groups of amino acids will need further investigation.

The observations presented here establish p21 and p27 mRNA turnover as a model for the control of mRNA stabilization by amino acid availability and they provide further insight into the mechanism by which amino acid limitation controls the cell cycle.

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