Induction of *gadd153* mRNA by nutrient deprivation is overcome by glutamine

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The growth arrest and DNA damage-inducible (*gadd*) genes are co-ordinately activated by a variety of genotoxic agents and/or growth-cessation signals. The regulation of *gadd153* mRNA was investigated in renal proximal tubular epithelial cells (LLC-PK1) cultured in a nutrient- and serum-deprived medium. The addition of glutamine alone to LLC-PK1 cells cultured in Earl's balanced salt solution (EBSS) is sufficient to suppress *gadd153* mRNA expression, and the removal of only glutamine from Dulbecco's modified Eagle's medium (DMEM) is also sufficient to induce *gadd153* mRNA expression. Consistent with these findings, the inhibition of glutamine utilization with acivicin and 6-diazo-5-oxo-L-norleucine (DON) in cells grown in a glutamine-containing medium effectively induces *gadd153* expression. Glutamine can be used as an energy source in cultured mammalian cells. However, it is unlikely that deficits in cellular energy stores (ATP) are coupled to *gadd153* mRNA expression, because concentrations of ATP, UTP and GTP are all elevated in EBSS-

exposed cells, and the addition of α -oxoglutarate to cells grown in EBSS has no effect on *gadd153* mRNA expression. In contrast, concentrations of CTP decline substantially in EBSS and glutamine-deprived DMEM-cultured cells. Glutamine also serves as a precursor for the synthesis of protein and DNA. The addition of glutamine to cells grown in EBSS partly restores CTP concentrations. The addition of pyrimidine ribonucleosides (cytidine and uridine) to LLC-PK1 cells also restores CTP concentrations, in a manner commensurate with their relative abilities to overcome *gadd153* expression. Finally, glutamine does not completely suppress DNA damage-induced *gadd153* expression, suggesting that multiple signalling pathways lead to the expression of *gadd153* mRNA under conditions of nutrient deprivation and DNA damage.

Key words: CTP, glutamine, growth arrest, LLC-PK1 cells, nucleotide depletion.

INTRODUCTION

The growth arrest and DNA damage-inducible (*gadd*) genes encode a series of low-abundance transcripts originally isolated from UV-irradiated hamster cells by hybridization subtraction [1]. These genes are of interest because they are co-ordinately induced by DNA-damaging agents and growth cessation signals such as serum removal, medium depletion and contact inhibition [2]. The nature of *gadd153* expression in response to chemically induced DNA damage is comparable to its induction after physiologically induced growth arrest, because one common cellular response to DNA damage is the transient inhibition of DNA synthesis and cell growth. The transfection of *gadd* into human cells markedly inhibits cell growth, and the growth-inhibitory effects of different *gadd* family members are synergistic [3]. The *gadd153* protein product, CHOP-10, heterodimerizes with the CCAAT-enhancer-binding protein (C/EBP) family of transcription factors, blocks their ability to bind to C/EBP sites, and thereby inhibits DNA transcription [4]. However, the growth-inhibitory effect of *gadd153* might be mediated by the binding of CHOP-C/EBP dimers to alternative DNA sequences [5].

The absence of growth factors (serum deprivation) does not induce *gadd153* mRNA expression in renal proximal tubular epithelial cells (LLC-PK1) grown in an otherwise nutrient-rich culture medium [6]. In contrast, *gadd153* is readily induced in subconfluent LLC-PK1 cells cultured in Earl's balanced salt solution [EBSS; 0.8 mM MgSO₄·7H₂O/116.3 mM NaCl/

10.0 mM NaHCO₃/1.0 mM NaH₂PO₄·H₂O/5.4 mM KCl/5.5 mM D-glucose/20 mM Hepes/1.8 mM CaCl₂·2H₂O (pH 7.4)] [6,7] and the addition of glucose fails to suppress *gadd153* induction under these conditions [6]. Glucose is not essential for energy production in cultured cells [8]; glutamine, not glucose, is the major energy source in HeLa cells [8]. Glutamine also serves as a precursor for the synthesis of purine and pyrimidine nucleotides, via amidophosphoribosylamine transferase and carbamoyl phosphate synthetase II respectively, and cells grow readily in sugar-free medium supplemented with uridine or cytidine [9,10]. The mitochondrial pathway from glutamine to α -oxoglutarate is also the major site of ammoniogenesis in LLC-PK1 cells [9]. We therefore examined the possibility that the absence of glutamine might initiate *gadd153* mRNA expression in nutrient-depleted LLC-PK1 cells and whether the ability of glutamine to supply energy or nucleotides has the major role in the growth-supporting effect of glutamine. Our results indicate that glutamine and pyrimidine ribonucleotides restore intracellular concentrations of CTP and concomitantly overcome *gadd153* mRNA induction in nutrient-deprived LLC-PK1 cells.

EXPERIMENTAL

Chemicals

2-Bromo-bis(glutathion-S-yl)hydroquinone (BBGHQ) was synthesized as described [11]. [³H]Thymidine (specific radioactivity 65 Ci/mmol) was purchased from ICN Biochemicals (Cleveland,

Abbreviations used: BBGHQ, 2-bromo-bis(glutathion-S-yl)hydroquinone; C/EBP, CCAAT-enhancer-binding protein; DMEM, Dulbecco's modified Eagle's medium; DON, 6-diazo-5-oxo-L-norleucine; EBSS, Earl's balanced salt solution; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ppGpp, guanosine tetraphosphate; TCA, trichloroacetic acid.

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OH, U.S.A.). Nucleotide precursors (L-glutamine, uridine, cytidine, aspartic acid, α -oxoglutarate, ribose and ribose 5-phosphate), the nucleotide standards for the capillary electrophoresis assay and acivicin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 6-Diazo-5-oxo-L-norleucine (DON) is a product from Fluka Chemical Co. (Milwaukee, WI, U.S.A.). All other reagents were of the highest grade commercially available.

Cell culture conditions

LLC-PK1 cells, a pig renal proximal-tubule epithelial cell line (used between passage numbers 211 and 240), were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (high glucose without pyruvate) containing 10% (v/v) fetal bovine serum (Atlanta Biologicals, Norcross, GA, U.S.A.) at 37 °C under air/CO₂ (19:1) in a humidified incubator.

Determination of DNA synthesis

Cells were seeded at a density of 2×10^5 cells per well in six-well plates and used after culture overnight. Cells were washed in EBSS and incubated with 5 μ Ci/ml [³H]thymidine in either EBSS or EBSS with nutrient supplementation for 0, 0.5, 1, 2, 3 or 4 h. Cultures were then washed three times with PBS, pH 7.4, and 5% (w/v) trichloroacetic acid (TCA) was added. Cells were harvested by scraping, then washed with TCA three times to remove unincorporated [³H]thymidine. A final washing with ethanol was performed to remove residual TCA; the pellet was then left to dry in air. The pellet was dissolved in 1 ml of 0.1 M NaOH and the resulting solution was used for the determination of radioactivity by liquid-scintillation spectroscopy, and for the determination of protein concentration by the Bradford method [12].

Northern-blot analysis

Cells were seeded at a density of 2×10^6 cells per 100 mm dish and left to grow overnight. Cells were washed and treated with EBSS in the presence or absence of glutamine, aspartate, cytidine or uridine. Total RNA was isolated with Ultraspect (Cinna/Biotech Laboratory International, Friendswood, TX, U.S.A.) by following the 'single-step' method [13]. RNA concentrations were measured and equal amounts of RNA were subjected to electrophoresis in 1.0% (w/v) agarose gel containing formaldehyde, then transferred to Zeta Probe membranes (Bio-Rad, Richmond, CA, U.S.A.). The gadd153 cDNA probe was labelled with [³²P]dCTP (ICN, Costa Mesa, CA, U.S.A.) by the random primer method [14] with a random labelling kit (Boehringer Mannheim, Mannheim, Germany). The resulting probes were incubated with RNA blots in hybridization solution [0.25 M Na₂HPO₄ (pH 7.2)/7% (w/v) SDS] overnight at 65 °C. All blots were washed in 25 mM Na₂PO₄ (pH 7.2) containing 5% (v/v) SDS and then 1% SDS. All blots were rehybridized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to correct for potential variations in RNA loading. Autoradiography was performed with Hyperfilm (Amersham Life Science, Little Chalfont, Bucks., U.K.) for several days at -80 °C, depending on the intensity of the signal. All blots were quantified with a Packard Instant ImagerTM Electronic Autoradiography System (Packard, Meriden, CT, U.S.A.) and gene expression relative to respective controls expressed as c.p.m. of gadd153/c.p.m. of GAPDH. The plasmid pBluescript containing a 600 bp fragment of cDNA of gadd153 was kindly provided by

Dr. Nikki J. Holbrook (National Institute on Aging, National Institutes of Health, Gerontology Research Center, Baltimore, MD, U.S.A.).

Extraction of cellular nucleotide pools

Cells were cultured and treated under the same conditions as those described above. Nucleotide pools were extracted with 0.2 M HClO₄ for 30 min with the use of a modified procedure of Smeets et al. [15]. Extracts were centrifuged at 6000 g for 5 min at 4 °C. The resulting supernatant, containing the nucleotides, was collected and neutralized with 2 M KOH (usually 0.1 vol.). KClO₄ precipitates were cleared by centrifugation and the supernatant was stored at -20 °C if not analysed immediately. Protein pellets were dissolved in 0.1 M NaOH; protein concentrations were determined by the Bradford method [12].

Capillary electrophoresis

Nucleotide concentrations were determined by capillary electrophoresis with a P/ACE 5010 system (Beckman, Fullerton, CA, U.S.A.). Samples were introduced by electrokinetic injection at 5 kV and separated at 12.5 kV in uncoated silica capillaries (Beckman) 57 cm in length and 75 μ m in internal diameter at 25 °C. The running buffer contained 50 mM Na₂HPO₄, 100 mM dodecyltrimethylammonium bromide and 1 mM EDTA, pH 4.0. Peak identification, as well as quantification of the various ribonucleotides and deoxyribonucleotides, was based on their characteristic UV absorption spectra at 260 nm and their migration times compared with those of a mixture of 18 authentic standards.

Statistical analysis

All data are expressed as means $\pm$ S.E.M. Mean values were compared by analysis of variance with a *post hoc* Student Newman Kuels test. *P* < 0.05 was accepted as significant.

RESULTS

Effects of glutamine on gadd153 mRNA expression and DNA synthesis in nutrient-depleted renal tubular epithelial cells

Depriving LLC-PK1 cells of serum and nutrients by culturing them in EBSS induces *gadd153* expression; the addition of 20 mM glucose to EBSS fails to overcome *gadd153* induction [7]. Thus the induction of *gadd153* in physiologically arrested LLC-PK1 cells is a response to the absence of nutrients other than glucose. Glutamine is an important energy source and essential precursor for nucleotide synthesis *de novo* in mammalian cells. The addition of glutamine to EBSS suppresses *gadd153* mRNA expression in a time-dependent (Figure 1) and concentration-dependent (Figure 2) manner. Maximal suppression (62%) of *gadd153* mRNA by glutamine (Figure 2) occurs at concentrations (4 mM) usually included in commercially prepared DMEM. The suppression of *gadd153* mRNA by glutamine required a lag phase of 2–5 h (Figure 1), which is presumably a consequence of a requirement for its metabolism to key intermediates required for DNA synthesis. Consistently with this view, glutamine stimulates the incorporation of [³H]thymidine into newly synthesized DNA (Figure 3); this stimulation also occurs after a lag phase of 2–3 h. In addition, the removal of glutamine alone from cells grown in DMEM strongly induces (approx. 13-fold) *gadd153* mRNA (Figure 4, lane 4). *gadd153* mRNA is also induced in cells grown in a complete medium in which glutamine utilization is inhibited by treatment with either acivicin or DON

Figure 1 Time-dependent induction of *gadd153* mRNA by nutrient deprivation is suppressed by glutamine in renal tubular epithelial cells

Subconfluent LLC-PK1 cells were cultured in either EBSS (●) or EBSS plus 4 mM L-glutamine (○) for 0, 0.5, 1, 2 and 5 h. Total RNA was extracted and separated, then examined by Northern blot analysis (upper panel). The level of *GAPDH* was used as the internal control. The upper panel is a representative Northern blot from one of three experiments, and the lower panel represents the relative expression of *gadd153* mRNA following quantification of *gadd153* and *GAPDH* mRNA using a Packard Instant ImagerTM electronic autoradiography system. Results are means $\pm$ S.E.M. of three separate experiments, calculated as c.p.m. *gadd153*/c.p.m. *GAPDH* with the ratio at zero time randomly assigned the value 1.0.

Figure 2 Concentration-dependent suppression of *gadd153* mRNA expression by L-glutamine in nutrient-depleted renal tubular epithelial cells

Subconfluent LLC-PK1 cells were cultured in EBSS supplemented with increasing concentrations of L-glutamine for 5 h. Total RNA was extracted and separated, then examined by Northern blot analysis (upper panel). The level of *GAPDH* was used as the internal control. The upper panel is a representative Northern blot from one of three experiments, and the lower panel represents the relative expression of *gadd153* mRNA following quantification of *gadd153* and *GAPDH* mRNA using a Packard Instant ImagerTM electronic autoradiography system. Results are means $\pm$ S.E.M. for three separate experiments calculated as c.p.m. *gadd153*/c.p.m. *GAPDH*, with the ratio at zero time randomly assigned the value 1.0.

(Figure 5). DON and acivicin are both anti-metabolites of glutamine and their cellular effects are therefore relatively specific. Indeed, decreases in UTP and CTP concentrations induced by *N*-(phosphonacetyl)-L-aspartate do not render L1210 cells more sensitive to acivicin [16], indicating that the effects of acivicin are unlikely to encompass glutamine-independent events.

Effect of ribonucleotide precursors on *gadd153* mRNA expression: suppressive effect of cytidine and uridine on *gadd153* mRNA induced by nutrient depletion

Uridine and cytidine can supply precursors for growth in many cell types, primarily through the formation of ribose 1-phosphate, a key precursor for nucleotide biosynthesis *de novo* [10]. The ribonucleosides can also be phosphorylated and enter the salvage pathway for the synthesis of nucleotide pools [17,18]. We therefore studied the effects of uridine and cytidine on *gadd153* induction in cells cultured in EBSS. Both uridine and cytidine (25 mM) overcome *gadd153* expression induced by nutrient deprivation (Figures 6 and 7), although cytidine was more effective (more than 80% inhibition at 5 h) than either uridine (65%) or glutamine (Figures 1 and 2). Aspartic acid provides nitrogen for the synthesis of both purines and pyrimidines *de novo* and when added to EBSS it also partly overcomes *gadd153* mRNA expression (30% and 40% at 2 and 5 mM respectively) (Figure 8). A major glutamine metabolite, α -oxoglutarate, provides cells with energy via the citric acid cycle; it also participates in pyrimidine synthesis through aspartic acid. However, α -oxoglutarate fails to suppress *gadd153* mRNA expression induced by nutrient deprivation (Figure 8). Krebs cycle inter-

Figure 3 Effect of glutamine on DNA synthesis in renal tubular epithelial cells

Subconfluent cells were cultured in either EBSS (●) or EBSS plus 4 mM L-glutamine (○) in the presence of [³H]thymidine for 0, 0.25, 0.5, 1, 2, 3, 4 and 5 h. The incorporation of [³H]thymidine into newly synthesized DNA was determined by liquid-scintillation spectroscopy. Results are means $\pm$ S.E.M. (*n* = 3). *Significantly different from EBSS cultured cells at *P* < 0.05. Experiments were performed at least twice and produced similar results.

mediates are actively transported into renal epithelial cells [19,20] and α -oxoglutarate is a substrate for the rat Na⁺-dicarboxylic acid transporter [19–21], which has been localized to the S3

Figure 4 Effect of glutamine and BBGHQ on *gadd153* mRNA expression in renal tubular epithelial cells

Subconfluent LLC-PK1 cells were cultured in DMEM or DMEM without glutamine and exposed to BBGHQ (200 μ M) for 5 h. Total RNA was extracted and separated, then examined by Northern blot analysis (upper panel). The level of GAPDH was used as the internal control. The upper panel is a representative Northern blot from one of three experiments, and the lower panel represents the relative expression of *gadd153* mRNA following quantification of *gadd153* and GAPDH mRNA using a Packard Instant ImagerTM electronic autoradiography system. Results are means $\pm$ S.E.M. for three separate experiments calculated as c.p.m. *gadd153*/c.p.m. GAPDH, with the ratio of the control (— BBGHQ/ + glutamine) randomly assigned the value 1.0.

Figure 5 Effects of glutamine-utilizing enzyme inhibitors on *gadd153* mRNA expression in renal tubular epithelial cells

Subconfluent LLC-PK1 cells were cultured in DMEM and exposed to acivicin (1 mM) or DON (10 mM) for 3 h. Total RNA was extracted and separated, then examined by Northern blot analysis (upper panel). The level of GAPDH was used as the internal control. The upper panel is a representative Northern blot from one of three experiments, and the lower panel represents the relative expression of *gadd153* mRNA following quantification of *gadd153* and GAPDH mRNA using a Packard Instant ImagerTM electronic autoradiography system. Results are means $\pm$ S.E.M. of three separate experiments calculated as c.p.m. *gadd153*/c.p.m. GAPDH, with the ratio of the control (— inhibitors) randomly assigned the value 1.0.

segment of proximal tubules [21]. It is therefore unlikely that the inability of α -oxoglutarate to suppress *gadd153* mRNA expression is a consequence of its inability to be transported into LLC-PK1 cells. Ribose and ribose 5-phosphate, precursors of phosphoribosyl pyrophosphate, which are essential for nucleotide synthesis *de novo*, also fail to overcome *gadd153* induction in response to nutrient depletion (Figure 6), even though ribose can

Figure 6 Effects of nucleotide precursors on *gadd153* mRNA expression in renal tubular epithelial cells

Subconfluent LLC-PK1 cells were cultured in either EBSS or EBSS plus different nucleotide precursors (25 mM) for 2 or 5 h. Total RNA was extracted and separated, then examined by Northern blot analysis (upper panel). The level of GAPDH was used as the internal control. The upper panel is a representative Northern blot from one of three experiments, and the lower panel represents the relative expression of *gadd153* mRNA following quantification of *gadd153* and GAPDH mRNA using a Packard Instant ImagerTM electronic autoradiography system. Results are means $\pm$ S.E.M. for three separate experiments calculated as c.p.m. *gadd153*/c.p.m. GAPDH, with the ratio of the control (DMEM at 2 h) randomly assigned the value 1.0. Each bar of the histogram relates to the corresponding lane in the Northern blot above.

Figure 7 Concentration-dependent suppression of *gadd153* mRNA by cytidine in nutrient depleted renal tubular epithelial cells

Subconfluent LLC-PK1 cells were cultured in EBSS supplemented with increasing concentrations of cytidine for 5 h. Total RNA was extracted and separated, then examined by Northern blot analysis. The level of GAPDH was used as an internal control. The upper panel is a representative Northern blot from one of three experiments, and the lower panel represents the relative expression of *gadd153* mRNA following quantification of *gadd153* and GAPDH mRNA using a Packard Instant ImagerTM electronic autoradiography system. Results are means $\pm$ S.E.M. for three separate experiments calculated as c.p.m. *gadd153*/c.p.m. GAPDH, with the ratio of the control (without cytidine) randomly assigned the value 1.0.

Figure 8 Effect of α -oxoglutarate and aspartic acid on *gadd153* mRNA expression in nutrient-depleted renal tubular epithelial cells

Subconfluent LLC-PK1 cells were cultured in EBSS plus α -oxoglutarate (●) or aspartic acid (○) for 5 h. Total RNA was extracted and separated, then examined by Northern blot analysis (upper panel). The level of GAPDH was used as the internal control. The upper panel is a representative Northern blot from one of three experiments, and the lower panel represents the relative expression of *gadd153* mRNA following quantification of *gadd153* and GAPDH mRNA using a Packard Instant Imager™ electronic autoradiography system. Results are means $\pm$ S.E.M. for three separate experiments calculated as c.p.m. *gadd153*/c.p.m. GAPDH, with the ratio of the control (without α -oxoglutarate/aspartate) randomly assigned the value 1.0. α -Ketoglutarate, α -oxoglutarate.

effectively overcome glucose-responsive protein (GRP 78) induction caused by glucose deprivation in LLC-PK1 cells [22]. It is not known whether ribose 5-phosphate can be transported into LLC-PK1 cells.

Effects of nutrient depletion (EBSS) on cellular nucleotide pools

In nutrient-depleted cells, no significant depletion in the overall nucleotide triphosphate pool is seen. However, an imbalance in the nucleotide pool is observed. Concentrations of ATP and GTP are all significantly elevated in nutrient-depleted LLC-PK1 cells, and although UTP levels also increase, the increases are not statistically significant (Table 1). In contrast, concentrations of CTP decrease to approx. 50% in cells cultured in EBSS (Table 1). The decrease is statistically significant when the data are analysed by a paired Student's *t* test. However, the extremely large increases in CTP concentrations after the addition of cytidine renders the data statistically insignificant when analysed by an analysis of variance. In addition, concentrations of UDP-glucose are also significantly increased (approx. 40%; results not shown) in nutrient-depleted cells. The addition of glutamine to cells grown in EBSS suppresses UTP and UDP-glucose to concentrations found in cells grown in DMEM; it also increases CTP concentrations (Table 1). In contrast, concentrations of ATP and GTP are unaffected by the addition of glutamine to EBSS (Table 1). The addition of cytidine to LLC-PK1 cells cultured in EBSS substantially decreases the levels of ATP and GTP (Table 1) but significantly increases concentrations of UTP, CTP (Table 1), UDP-glucose and UMP (results not shown). The addition of uridine to EBSS also decreases cellular levels of ATP

Table 1 Effects of nutrient deprivation and nucleotide precursors on intracellular NTP pools in LLC-PK1 cells

Subconfluent LLC-PK1 cells were cultured in DMEM, EBSS or EBSS supplemented with glutamine (4 mM), cytidine (25 mM) or uridine (25 mM) for 5 h. Intracellular nucleotides were extracted and quantified by capillary electrophoresis. The results are means $\pm$ S.E.M. (*n* = 3). *Significantly different from EBSS-cultured cells at *P* < 0.05; †significantly different from DMEM-cultured cells at *P* < 0.05. Experiments were performed at least twice and produced similar results.

Culture conditions	NTP (pmol/mg)			
	ATP	UTP	GTP	CTP
DMEM	91.3 $\pm$ 2.9*	75.9 $\pm$ 0.6	22.4 $\pm$ 1.0*	6.1 $\pm$ 0.5
EBSS	112.8 $\pm$ 3.8†	96.9 $\pm$ 1.9	43.7 $\pm$ 1.2†	3.5 $\pm$ 0.1
EBSS + G	129.4 $\pm$ 12.1†	75.7 $\pm$ 6.8	40.0 $\pm$ 3.9†	4.4 $\pm$ 0.4
EBSS + C	32.2 $\pm$ 2.5*†	247.8 $\pm$ 8.5*†	22.2 $\pm$ 1.6*	137.1 $\pm$ 10.6*†
EBSS + U	47.6 $\pm$ 2.9*†	426.2 $\pm$ 12.9*†	25.6 $\pm$ 1.2*	5.4 $\pm$ 0.5

and GTP (Table 1) and significantly increases levels of UTP, CTP (Table 1), UDP-glucose and UMP (results not shown). Culturing LLC-PK1 cells in glutamine-depleted DMEM produces similar effects to those seen by culturing cells in EBSS. Thus intracellular CTP concentrations (4.16 ± 0.32 pmol/mg in DMEM and 1.77 ± 0.40 pmol/mg in DMEM lacking glutamine; approx. 60% decrease) and UDP-glucose concentrations (approx. 40% increase; results not shown) are altered in a manner similar to that caused by culturing cells in EBSS. The results implicate decreases in CTP concentration as an important factor in signalling *gadd153* mRNA expression in response to glutamine withdrawal.

Relative effects of DNA damage and glutamine deprivation on *gadd153* mRNA expression

BBGHQ is genotoxic: it causes extensive karyorrhexis and karyolysis *in vivo* [23] and produces single-strand DNA breaks in LLC-PK1 cells [24,25]. BBGHQ also induces *gadd153* (5-fold) in subconfluent LLC-PK1 cells grown in a complete medium (Figure 4). However, the removal of glutamine from DMEM provides a far more potent (13-fold) signal than BBGHQ for *gadd153* mRNA expression (Figure 4). Thus *gadd153* expression is more sensitive to glutamine deprivation than to DNA damage. Moreover, BBGHQ-induced DNA damage slightly suppresses (approx. 10%) the *gadd153* response to glutamine deprivation (Figure 4), indicating that the combined effects of nutrient deprivation and DNA damage are not additive.

DISCUSSION

Nutrient deprivation of LLC-PK1 cells stimulates the expression of *gadd153* [6,26]. Because glutamine serves as both an energy source and as an essential nutrient for both purine and pyrimidine biosynthesis in cultured mammalian cells [8], we hypothesized that cellular glutamine concentrations might be important in regulating *gadd153* expression. Consistent with this view, (1) the addition of glutamine to EBSS suppresses *gadd153* expression in nutrient-depleted LLC-PK1 cells (Figure 1), (2) the removal of glutamine alone from DMEM induces *gadd153* expression (Figure 4), and (3) the inhibition of glutamine utilization in a glutamine-containing medium also induces *gadd153* expression (Figure 5).

Although glucose regulates *gadd153* in HeLa cells [26], glutamine, not sugar, is the major energy source in these cells [8].

Moreover, although the addition of pyruvate to HeLa cells restores cellular ATP concentrations, *gadd153* expression is only partly suppressed [26]. Glutamine provides energy via the citric acid cycle; deamidation and subsequent deamination result in the formation of α -oxoglutarate. However, it seems unlikely that the ability of glutamine to suppress *gadd153* expression is coupled to cellular energy supplies. Thus the addition of α -oxoglutarate to EBSS has no effect on *gadd153* mRNA expression (Figure 8). Consistent with this finding, but in contrast with findings in HeLa cells [26], glucose does not overcome *gadd153* expression in nutrient-deprived LLC-PK1 cells [6], suggesting that compromised energy supplies in LLC-PK1 cells do not serve as a signal for growth arrest under these conditions. Indeed, ATP (and UTP and GTP) concentrations are slightly elevated in nutrient-deprived cells (Table 1), perhaps because decreases in DNA synthesis lower the utilization of ATP. The only essential function of sugar in HeLa cells is to provide ribose phosphate(s), the primary precursor for nucleotides [9]. The finding that both uridine and cytidine suppress *gadd153* mRNA expression, while simultaneously decreasing cellular ATP concentrations (Table 1), suggests that *gadd153* mRNA induction is linked more closely to imbalances in the nucleotide pool than to energy stores.

The importance of glutamine for sustaining the growth of LLC-PK1 cells was confirmed by examining the effects of acivicin and DON on *gadd153* mRNA expression in cells cultured in a glutamine-containing medium (Figure 4). DON, a glutamine anti-metabolite, inhibits various mitochondrial glutaminases [27,28] and glutamine-dependent amidotransferase [29,30]. Acivicin, another anti-metabolite, irreversibly inactivates several glutamine-utilizing enzymes including amidophosphoribosyl transferase, carbamoyl phosphate synthetase II and CTP synthetase [31–34] but not glutaminase. Inhibition of these enzymes, which are essential for the synthesis of nucleotides *de novo*, results in a rapid depletion of CTP and GTP pools [32–34], whereas ATP and UTP pools remain unchanged [32]. The finding that DON and acivicin induce *gadd153* mRNA (Figure 5) and are known to deplete CTP pools [32–34] is consistent with nutrient-deprivation-mediated decreases in CTP (Table 1) being coupled to *gadd153* induction (Figure 1). Thus although DON and acivicin inhibit different glutamine-utilizing enzymes, they both induce *gadd153* mRNA expression. The removal of only glutamine from DMEM also strongly induces *gadd153* mRNA (Figure 4) and causes a concomitant selective decrease in intracellular CTP concentrations; the addition of glutamine to LLC-PK1 cells cultured in EBSS partly replenishes intracellular CTP (Table 1), increases DNA synthesis (Figure 3) and suppresses *gadd153* expression (Figure 1). In HeLa cells, ribonucleosides (cytidine and uridine) support cell growth, in the absence of sugar, by providing a source of ribose 1-phosphate [35]. However, the suppression of *gadd153* expression by cytidine and uridine in LLC-PK1 cells does not seem to be related to ribose 1-phosphate pools, because the addition of either ribose or ribose 5-phosphate has little effect on *gadd153* mRNA expression in nutrient-deprived cells (Figure 6). Hence glutamine is the key nutrient regulating both *gadd153* expression and CTP concentrations in LLC-PK1 cells.

The phosphorylation of cytidine and uridine by uridine-cytidine kinase [17,18] might be the major site of the nucleotide salvage pathway in LLC-PK1 cells. Extracellular nucleosides are efficiently transported into mammalian cells, where they are efficiently phosphorylated; the rate of nucleotide synthesis is therefore highly dependent on the concentration of the precursors [36–38]. The synthesis of UMP requires both glutamine and aspartate, for carbamyl aspartate generation, and aspartic acid

partly suppresses *gadd153* mRNA induction in nutrient-deprived LLC-PK1 cells (Figure 8). In contrast, α -oxoglutarate, a metabolite of glutamine, has no effect on *gadd153* mRNA induction (Figure 8) despite its ability to support nucleotide synthesis via metabolism to aspartic acid. Thus the suppression of *gadd153* mRNA expression by glutamine does not occur through the mitochondrial pathway from glutamine to α -oxoglutarate. These results provide additional evidence that glutamine exerts its growth promoting effects by donating nitrogen for nucleotide biosynthesis *de novo*.

Of the four nucleotide triphosphates, CTP is present at the lowest concentration in cells [39–41]; depletion of intracellular CTP or UTP is sufficient to induce growth arrest, even when the GTP and ATP levels are increased [42]. Our own results are consistent with these findings (Table 1). CTP pools might contribute to signalling *gadd153* mRNA induction in LLC-PK1 cells; however, the mechanism of this effect is not known. The relative ability of cytidine (80%), uridine (65%), glutamine (62%) and aspartic acid (40%) to suppress *gadd153* expression correlates with their roles in nucleotide biosynthesis. Cytidine and uridine can readily be incorporated into nucleotide pools, via the uridine-cytidine kinase salvage pathway. In contrast, glutamine provides essential nitrogen and carbon atoms for the *de novo* ribonucleotide biosynthetic pathway, which also requires other precursors, including phosphoribosyl pyrophosphate, glycine and aspartic acid. Extracellular cytidine is converted into both uracil and cytosine nucleotides in rat cardiomyocytes, whereas uridine serves only as a precursor for uracil nucleotides [43]. This result is similar to our own findings (Table 1) and might explain why cytidine is more efficient than uridine in suppressing *gadd153* induction. Furthermore, the relative abilities of cytidine, uridine and glutamine to restore cellular CTP pools also correlates with their abilities to suppress *gadd153* mRNA induction (Figures 1 and 4).

The mechanism by which cellular CTP concentrations are coupled to *gadd153* expression is not yet known. Bacterial growth in response to starvation is under 'stringent' control [44]. Nutrient deprivation induces the synthesis and accumulation of guanosine tetraphosphate (ppGpp) in *Escherichia coli* [45,46]. ppGpp influences several key cellular processes. It inhibits RNA transcription, DNA replication and subsequently cell growth [44,47,48]. ppGpp inhibits DNA replication by repressing the expression of *dna A* [49], a replication initiation gene, and halting replication forks post-initiationally [50]. Although similar factors have not been identified in mammalian cells, a similar mechanism might exist. CHOP-10, the *gadd153* protein product, shows obvious growth-inhibitory properties [5]. The accumulation of ppGpp and the inhibition of RNA synthesis during glucose starvation are not as rapid as during amino acid deprivation [51], which is consistent with our finding that *gadd153* is more responsive to deprivation of amino acids (especially glutamine).

Finally, the induction of *gadd153* mRNA by nutrient deprivation is far more marked than that of DNA damage (Figure 4) [6]. *gadd153* is inducible in the presence of glutamine when LLC-PK1 cells are treated with an agent that induces DNA damage (Figure 4). However, glutamine does not completely suppress DNA-damage-induced *gadd153* expression, which is consistent with reports that multiple signalling pathways can lead to the expression of GADD153 [52]. *gadd153* is more responsive to nutritional status than to DNA damage.

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REFERENCES

- 1 Fornace, Jr., A. J., Alamo, I. J. and Hollander, M. C. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8800–8804
- 2 Fornace, Jr., A. J., Nebert, D. W., Hollander, M. C., Luethy, J. D., Papathanasiou, M., Fargnoli, J. and Holbrook, N. J. (1989) *Mol. Cell. Biol.* **9**, 4196–4230
- 3 Zhan, Q., Lord, K. A., Alamo, Jr., I., Hollander, C. M., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A. and Fornace, Jr., A. J. (1994) *Mol. Cell. Biol.* **14**, 2361–2371
- 4 Ron, D. and Habener, J. F. (1992) *Genes Dev.* **6**, 439–453
- 5 Ubeda, M., Wang, X. Z., Zinszner, H., Wu, I., Habener, J. F. and Ron, D. (1996) *Mol. Cell. Biol.* **16**, 1479–1489
- 6 Jeong, J. K., Huang, Q., Lau, S. S. and Monks, T. J. (1997) *J. Biol. Chem.* **272**, 7511–7518
- 7 Chen, Q., Yu, K., Holbrook, N. J. and Stevens, J. L. (1992) *J. Biol. Chem.* **267**, 8207–8212
- 8 Reitzer, L. J., Wice, B. M. and Kennell, D. (1979) *J. Biol. Chem.* **254**, 2669–2676
- 9 Wice, B. M., Reitzer, L. J. and Kennell, D. (1981) *J. Biol. Chem.* **256**, 7812–7819
- 10 Wice, B. M. and Kennell, D. E. (1982) *J. Biol. Chem.* **258**, 13134–13140
- 11 Monks, T. J., Highet, R. J. and Lau, S. S. (1988) *Mol. Pharmacol.* **34**, 492–500
- 12 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 13 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 14 Feineberg, A. P. and Vogelstein, B. (1985) *Anal. Biochem.* **137**, 266–267
- 15 Smee, D. F., Bome, R., Chernow, M., Binko, B. P. and Matthews, T. R. (1984) *Biochem. Pharmacol.* **34**, 1049–1056
- 16 Cory, A. H. and Cory, J. G. (1998) *Biochem. Biophys. Res. Commun.* **249**, 687–691
- 17 Liacouras, A. S., Garvey, III, T. Q., Millar, F. K. and Anderson, E. P. (1975) *Arch. Biochem. Biophys.* **168**, 74–80
- 18 Krawitt, E. L., Betel, I. and Potter, V. R. (1969) *Biochim. Biophys. Acta* **174**, 763–765
- 19 Chen, X. Z., Shayakul, C., Berger, U. V., Tian, W. and Hediger, M. A. (1998) *J. Biol. Chem.* **273**, 20972–20981
- 20 Wright, E. M. (1995) *Annu. Rev. Physiol.* **47**, 127–141
- 21 Welborn, J. R., Shpun, S., Dantzer, W. H. and Wright, S. H. (1998) *Am. J. Physiol.* **274**, F165–F174
- 22 Gstraunthaler, G., Harris, Jr., W. and Handler, J. S. (1987) *Am. J. Physiol.* **252**, C239–C242
- 23 Rivera, M. I., Jones, T. W., Lau, S. S. and Monks, T. J. (1994) *Toxicol. Applied Pharmacol.* **128**, 239–250
- 24 Mertens, M. J., Gibson, N. W., Lau, S. S. and Monks, T. J. (1995) *Arch. Biochem. Biophys.* **320**, 51–58
- 25 Jeong, J. K., Stevens, J. L., Lau, S. S. and Monks, T. J. (1996) *Mol. Pharmacol.* **50**, 592–598
- 26 Carlson, S. G., Fawcett, J. D., Bartlett, J. D., Bernier, M. and Holbrook, N. J. (1993) *Mol. Cell. Biol.* **13**, 4736–4744
- 27 Shapiro, R. A., Clark, V. M. and Curthoys, N. P. (1979) *J. Biol. Chem.* **254**, 2835–2838
- 28 Lebedeva, Z. L., Kabanova, E. A. and Berezov, T. T. (1986) *Biochem. Int.* **12**, 413–420
- 29 Vollmer, S. J., Switzer, R. L., Hermodson, M. A., Bower, S. G. and Zalkin, H. (1983) *J. Biol. Chem.* **258**, 10582–10585
- 30 Boss, G. R., Idriss, S. D., Willis, R. C. and Seegmiller, J. E. (1983) *Anal. Biochem.* **130**, 283–286
- 31 Aoki, T., Sebolt, J. and Weber, G. (1982) *Biochem. Pharmacol.* **31**, 927–932
- 32 Denton, J. E., Lui, M. S., Aoki, T., Sebolt, J. and Weber, G. (1982) *Life Sci.* **30**, 1073–1080
- 33 Achleitner, E., Lui, M. S. and Weber, G. (1985) *Adv. Enzyme Regul.* **24**, 225–232
- 34 Pausch, J., Rasenack, J., Haussinger, D. and Gerok, W. (1985) *Eur. J. Biochem.* **150**, 189–194
- 35 Wice, B. M. and Kennell, D. (1981) *J. Biol. Chem.* **257**, 2578–2583
- 36 Loret, S., Aussedat, J. and Rossi, A. (1986) *Basic Res. Cardiol.* **81**, 303–310
- 37 Loret, S., Aussedat, J. and Rossi, A. (1987) *Arch. Int. Physiol. Biochem.* **95**, 289–298
- 38 Zimmer, H. G. and Gerlach, E. (1978) *Pflügers Arch.* **376**, 223–227
- 39 Perrett, D. (1987) in *CRC Handbook of Chromatography – Nucleic Acid and Related Compounds*, vol. 1, part B., (Krstulovic, A. M., Zweig, G. and Sherm, J., eds.), pp. 3–29, CRC Press, Boca Raton
- 40 Huang, M., Liu, S., Murray, B. K. and Lee, M. L. (1992) *Anal. Biochem.* **207**, 231–235
- 41 Shao, X., O'Neill, K., Zhao, Z., Anderson, S., Malik, A. and Lee, M. L. (1994) *J. Chromatogr. A* **680**, 463–468
- 42 Linke, S. P., Clarkin, K. C., Leonardo, A. D., Tsou, A. and Wahl, G. M. (1996) *Genes Dev.* **10**, 934–947
- 43 Olivares, J., Dubus, I., Barrieux, A., Samuel, J., Rappaport, L. and Ross, A. (1992) *J. Mol. Cell Cardiol.* **24**, 1349–1359
- 44 Cashel, M. and Rudd, K. E. (1987) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M. and Umberger, H. E., eds.), pp. 1410–1438, American Society for Microbiology, Washington, DC
- 45 Cashel, M. and Gallant, J. (1969) *Nature (London)* **221**, 838–841
- 46 Cashel, M. (1969) *J. Biol. Chem.* **244**, 3133–3141
- 47 Gallant, J. A. (1979) *Annu. Rev. Genet.* **13**, 393–415
- 48 Baracchini, E. and Bremer, H. (1998) *J. Biol. Chem.* **263**, 2597–2602
- 49 Chiamello, A. E. and Zyshind, J. W. (1990) *J. Bacteriol.* **172**, 2013–2019
- 50 Levine, A., Vannier, F., Dehbi, M., Henckes, G. and Séror, S. J. (1991) *J. Mol. Biol.* **219**, 605–613
- 51 Molin, S., Meyenburg, K. V., Maaløe, O., Hansen, M. T. and Pato, M. L. (1977) *J. Bacteriol.* **131**, 7–17
- 52 Gately, D. P., Sharma, A., Christen, R. D. and Howell, S. B. (1996) *Br. J. Cancer* **73**, 18–23

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