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ORIGINAL ARTICLE

Upregulation of capacity for glutathione synthesis in response to amino acid deprivation: regulation of glutamate–cysteine ligase subunits

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Abstract Using HepG2/C3A cells and MEFs, we investigated whether induction of GSH synthesis in response to sulfur amino acid deficiency is mediated by the decrease in cysteine levels or whether it requires a decrease in GSH levels per se. Both the glutamate–cysteine ligase catalytic (GCLC) and modifier (GCLM) subunit mRNA levels were upregulated in response to a lack of cysteine or other essential amino acids, independent of GSH levels. This upregulation did not occur in MEFs lacking GCN2 (general control non-derepressible 2, also known as eIF2α kinase 4) or in cells expressing mutant eIF2α lacking the eIF2α kinase Ser^{51} phosphorylation site, indicating that expression of both GCLC and GCLM was mediated by the GCN2/ATF4 stress response pathway. Only the increase in GCLM mRNA level, however, was accompanied by a parallel increase in protein expression, suggesting that the enhanced capacity for GSH synthesis depended largely on increased association of GCLC with its regulatory subunit. Upregulation of both GCLC and GLCM mRNA levels in response to cysteine deprivation was dependent on new protein synthesis, which is consistent with expression of GCLC and GCLM being mediated by proteins whose synthesis depends on activation of the GCN2/ATF4 pathway. Our data suggest that the regulation of GCLC expression may be mediated by changes in the abundance of transcriptional regulators, whereas the regulation of GCLM expression may be mediated by changes in the abundance of mRNA stabilizing or destabilizing proteins. Upregulation of GCLM levels in response to low cysteine levels may serve to protect the cell in the face of a future stress requiring GSH as an antioxidant or conjugating/detoxifying agent.

Keywords Glutamate–cysteine ligase catalytic subunit · Glutamate–cysteine ligase modifier subunit · mRNA stability · Sulfur amino acids · Cysteine

Abbreviations

AARE	Amino acid response element
ATF4	Activating transcription factor 4
CARE	CCAAT enhancer-binding protein–activating transcription factor response element
eIF2α	Eukaryotic initiation factor 2, subunit alpha
EpRE	Electrophile response element
GCN2	General control non-derepressible 2, also known as eIF2α kinase 4
GCL	Glutamate–cysteine ligase
GCLC	Glutamate–cysteine ligase catalytic subunit
GCLM	Glutamate–cysteine ligase modifier subunit
GSH	Glutathione
GSSG	Glutathione disulfide
MEF	Murine embryonic fibroblast
Nrf2	Nuclear factor erythroid 2-related factor 2

Introduction

Glutathione (γ-glutamylcysteinylglycine, GSH) is the most abundant non-protein thiol in mammalian tissues, being present at cellular concentrations of about 0.5–10 mM. The whole body turnover of GSH in human adults has been estimated to be ~40 mmol per day, which is slightly

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greater than estimates of the magnitude of cysteine turnover in the body protein pool (Lauterburg and Mitchell 1987; Storch et al. 1990, 1991; Fukagawa et al. 1996, 1998). GSH plays key roles in antioxidative and cellular redox processes and is present in the thiol/reduced (GSH) and disulfide/oxidized (GSSG) forms (Deponte 2013). GSH is the predominant cellular form, accounting for more than 98 % of total GSH (Kaplowitz et al. 1985; Ballatori et al. 2009; Forman et al. 2009). It serves as an essential coenzyme or cosubstrate for many reactions, including conjugation reactions involved in the detoxification of many endogenous and exogenous compounds and leukotriene synthesis. GSH also functions in the γ-glutamyl cycle by which amino acids may be transported into cells (Griffith et al. 1978; Taniguchi and Ikeda 1998) and in cell signaling through *S*-glutathionylation and *S*-nitrosylation of cysteine residues in proteins (Pastore and Piemonte 2012; Jacob et al. 2012; Janssen-Heininger et al. 2013; Hartmanová et al. 2013). In addition, GSH serves as a storage reservoir of the amino acid cysteine as evidenced by its depletion at sulfur amino acid intake levels that are marginal but still adequate for protein synthesis (Stipanuk et al. 1992; Lee et al. 2004).

GSH contains an unusual peptide linkage between the γ-carboxyl of glutamate and the α-amino group of cysteine. The formation of γ-glutamylcysteine is the first step of GSH synthesis. It is catalyzed by glutamate–cysteine ligase (GCL), which is a heterodimeric protein composed of catalytic (GCLC) and modifier (GCLM) subunits, which are encoded by separate genes in mammals (Franklin et al. 2009; Lu 2013). GCLC exhibits all of the catalytic activity, but its association with GCLM alters its kinetic properties to enhance GCL activity (Anderson 1998; Griffith 1999). Glycine is subsequently added to γ-glutamylcysteine in a second reaction that is catalyzed by glutathione synthetase to form GSH.

Glutathione synthesis is mainly regulated by the cellular concentration of cysteine and the activity of GCL (Stipanuk and Dominy 2006; Lu 2013). The GCL reaction is highly regulated by feedback inhibition by GSH and transcriptional regulation in response to oxidative or chemical stress (Lu 2013). Both GCLC and GCLM are transcriptionally upregulated through the redox-sensitive signaling pathways comprised of the nuclear factor erythroid 2-related factor 2 (Nrf2)/electrophile response element (EpRE) system and the activator protein 1 (AP-1)/TBP response element (TRE) system (Iles and Liu 2005; Zhang et al. 2007). GCL activity is also regulated by the association of GCLC with its modifier subunit; GCLM lowers the K_m of GCL for glutamate and increases the K_i of GCL for GSH (Huang et al. 1993; Lu 2013). Because the abundance of GCLM appears to be limiting for GCL holoenzyme formation in most cell types and tissues, changes in GCLM expression would be expected to affect cellular GCL activity (Tipnis et al. 1999; Krzywanski et al. 2004; Chen et al. 2005; Lee et al. 2006).

It has been long established that GCL activity increases when sulfur amino acids are deficient, and this response has usually been linked to a lack of sufficient glutathione, leading to oxidative stress and upregulation of GCLC gene expression mediated via Nrf2/EpRE (Iles and Liu 2005; Zhang et al. 2007). However, we have found that GCLC mRNA abundance, GCLC protein abundance, and GCL activity follow changes in cellular cysteine levels more closely than they follow changes in GSH levels, suggesting the regulation may not be related strictly to GSH concentration. For example, in primary rat hepatocytes, addition of cysteine to the culture medium dramatically decreased GCLC mRNA abundance, regardless of whether or not the cells were also treated with buthionine sulfoximine to inhibit GCL and thus GSH synthesis (Kwon and Stipanuk 2001). In HepG2/C3A cells, the mRNA abundances for both GCLC and GCLM were significantly increased in response to cysteine deprivation under conditions in which expression of most genes known to be regulated in response to oxidative stress (e.g., glutathione peroxidases, superoxide dismutase, thioredoxin, NAD(P)H dehydrogenase) was not increased (Lee et al. 2008). In studies with HepG2/C3A cells, the upregulation of GCLM expression was greater than that of GCLC, and formation of holoenzyme appeared to be a major factor in the regulation of GCL activity (Lee et al. 2008).

Thus, the evidence to date seems to point to the possibility that upregulation of GCLC and GCLM is one consequence of the cell's response to amino acid deprivation, which can be sensed by GCN2 (general control non-derepressible 2, also known as eIF2α kinase 4) (Palii et al. 2009; Sikalidis and Stipanuk 2010; Kilberg et al. 2009). Phosphorylation of the alpha subunit of eIF2 (eIF2α) by GCN2 or another eIF2α kinase blocks ternary complex formation, thereby blocking formation of the 43S preinitiation complex and suppressing global translation. The global attenuation of translation that results from a lack of 43S preinitiation complex paradoxically increases the translation of a subset of mRNAs, including that encoding activating transcription factor 4 (ATF4) (Harding et al. 2000). Upregulation of the translation of ATF4 and other target proteins can then lead to increased transcription of stress-related genes (such as *ATF3, ASNS, CEBPB* and *TRIB3*), allowing the cell to synthesize the subset of proteins needed to respond to the stress that initiated the response (Lee et al. 2008; Sikalidis et al. 2011; Shan et al. 2010).

In the work reported here, we performed a series of experiments designed to further test whether a decrease in cysteine or a decrease in GSH is needed to induce

expression of GCLC and GCLM, whether the effect can be replicated by other small sulfhydryl compounds, and whether the response to cysteine deficiency is a general response to amino acid deficiency. In addition, we evaluated the role of mRNA and protein synthesis in the response to cysteine deprivation.

Materials and methods

Rat study

Male Sprague–Dawley rats were obtained from Harlan-Sprague–Dawley and fed a diet adequate or deficient in sulfur-containing amino acids as described previously (Sikalidis and Stipanuk 2010). In brief, rats that weighed ~120 g were acclimated to a semi-purified diet that contained 100 g of soy protein isolate plus 3.4 g L-methionine per kg diet and then were placed on either the treatment diet (without supplemental methionine) or continued on the adequate control diet for 1 week. Rats were then anesthetized with CO_2, and liver was collected and frozen in liquid nitrogen. All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Soluble protein and total mRNA were prepared as previously described for western blotting and mRNA analysis (Lee et al. 2006; Sikalidis and Stipanuk 2010).

Studies with HepG2/C3A cells

For studies with cysteine-deficient medium, HepG2/C3A cells (clonal derivative of HepG2 human hepatocellular carcinoma cells; ATCC CRL-10741) were cultured in a humidified incubator at 37 °C and 5 % CO_2. Complete medium was prepared using sulfur amino acid-free high glucose Dulbecco's modified Eagle's medium (DMEM; custom prepared by Gibco/Invitrogen) supplemented with 0.1 mM L-methionine, 0.3 mM L-cysteine, 10 % (v/v) dialyzed fetal bovine serum (Hyclone), 4 mM L-glutamine, 1 mM sodium pyruvate, 1 × MEM non-essential amino acid solution and 0.05 mM bathocuproine disulfonate. All cells were plated in complete medium at a density of 1×10^6 cells per 100 mm diameter culture dish. After 24 h of culture in complete medium to allow the cells to reach 50–60 % confluence, the medium was replaced with experimental medium, which was either the same complete medium (+Cys) or medium prepared without cysteine/cystine (−Cys) but with all other supplements including 0.1 mM L-methionine. Other additions to the experimental medium included 0.3 mM 2-mercaptoethanol, 0.3 mM 3-mercaptopropionic acid, 0.3 mM penicillamine, 10 μg/ml actinomycin D in DMSO, or 10 μg/ml cycloheximide as indicated in results. In the case of actinomycin D, an equal volume of the vehicle (DMSO) was also added to control medium. For studies with leucine-, cysteine-, methionine-, and/or histidine-deficient media, a basal medium lacking sulfur amino acids, leucine, and histidine (custom prepared by Gibco/Invitrogen) was used to prepare medium deficient in individual amino acids by adding back cysteine, methionine, leucine and histidine to restore concentrations to 0.1 mM L-methionine, 0.3 mM L-cysteine, 0.2 mM L-leucine and 0.8 mM L-histidine as appropriate. All cell culture experiments were repeated three or more times to assure the repeatability of the results.

Cells were cultured and harvested at the indicated time-points. When cells were cultured in treatment medium for more than 20 h, medium was replaced with fresh medium at 18 h (i.e., for the cells harvested at the 24, 30 and 36 h time-points). For mRNA analysis, cultured cells were washed twice with ice-cold PBS and then directly lysed into denaturation solution. Total RNA was extracted from three separate plates of cells grown under each treatment with an RNeasy Micro Kit (Qiagen). Total RNA concentration was determined by absorbance at 260 nm, and RNA purity was assessed with the A_{260}/A_{280} ratio. For protein analysis by western blotting, cells were washed with ice-cold PBS containing 10 mM NaF. Monolayers were then harvested into lysis solution (50 mM Tris, pH 7.5, 1 % (v/v) Nonidet P-40, 2 mM EDTA, 150 mM NaCl, and 10 mM activated sodium orthovanadate) supplemented with 1 × PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science) and 1 × Complete Protease Inhibitor Cocktail (Roche). Cell lysates were centrifuged at 17,000×*g* for 30 min, and the protein concentration of the supernatants was determined using the bicinchonic acid assay (BCA, Pierce). For assay of thiol concentrations, cells were lysed in 2.5 % (w/v) perchloric acid, and the lysate was centrifuged to obtain the acid supernatant, which was used for thiol determinations. The protein pellets were resuspended in 5 N NaOH, and protein concentration was determined using the BCA assay.

Studies with GCN2 knockout and eIF2α(ala/ala) MEFs

For studies with murine embryonic fibroblasts (MEFs), cell culture and harvest were carried out as described for HepG2/C3A cells. Wild-type and *Gcn2*$^{-/-}$ MEFS, which had been immortalized with SV40 Large T antigen, were provided by Dr. David Ron (New York University School of Medicine, New York, NY, USA). The eIF2α(ala/ala) and isogenic wild-type MEFs were provided by Dr. Randal Kaufman (Sanford Burnham Medical Research Medical Institute, La Jolla, CA, USA).

Experiments with GCLC null MEFs

Gclc$^{-/-}$ MEFs were the gift of Dr. M.W. Lieberman (Methodist Hospital Research Institute, Houston, TX). Cells were grown in DMEM supplemented with 15 % (w/v) FBS, 2.5 mM glutathione (added just before use), 0.1 U/ml penicillin, and 0.1 μg/ml streptomycin at 5 % CO_2 and 37 °C until they reached approximately 60 % confluence. Medium was changed daily and 2 h before the start of each experiment. To start the experiment, cells were washed twice with PBS and treatment medium was added. Basal treatment medium was sulfur amino acid-free DMEM supplemented with 15 % dialyzed FBS, 0.1 U/ml penicillin, 0.1 μg/ml streptomycin, 2 mM glutamine, 0.05 mM bathocuproine disulfonate, and 0.1 mM L-methionine. Treatment medium included the basal medium and basal medium supplemented with 0.3 mM L-cysteine, 0.5 mM glutathione, or both cysteine and glutathione. Treatment medium was changed at 15 h, and cells were harvested at 22 h for mRNA and thiol analyses, as described for HepG23/C3A cells.

Quantitative PCR

Using the purified mRNA, cDNA was synthesized using an iScript cDNA Synthesis Kit (BioRad). PCR was done using an ABI Prism 7500 in a 96-well plate format. TaqMan Gene Expression Assays for human GCLM, human GCLC, and murine GCLM were obtained from Applied Biosystems. Target gene expression was normalized using the human rRNA 18S endogenous control or the murine beta actin endogenous control.

Northern blotting

Results for mRNA abundance in the experiments with actinomycin and cycloheximide were obtained by northern blotting as described previously (Lee et al. 2006). The GLCM mRNA and both GCLC mRNA transcripts were quantified.

Western blotting

For western blotting, 60 μg of total supernatant protein from each sample was separated by one-dimensional SDS-PAGE (12 % w/v acrylamide) and electroblotted overnight onto 0.45 μm (pore size) Immobilon-P PVDF membranes (Millipore). Membranes were immunoblotted for proteins of interest using the following antibodies: anti-pS51-eIF2α and anti-eIF2α (total) from Cell Signaling Technology; anti-GCLC from Neomarkers (Freemont, CA); and anti-GCLM (Lee et al. 2006). Bands were visualized using horseradish peroxidase-coupled secondary antibodies and chemiluminescent substrates (West Dura, Pierce) and autoradiography.

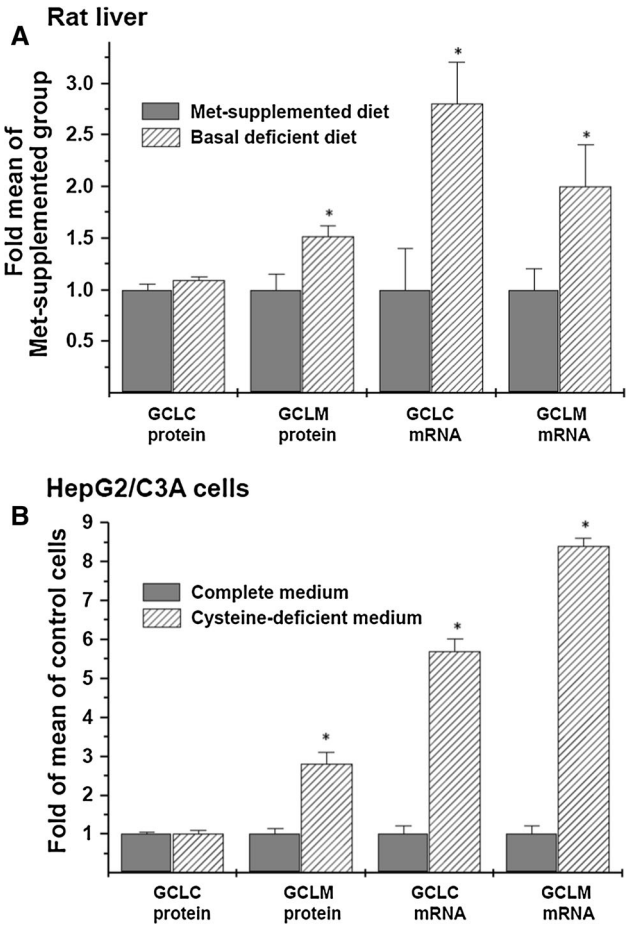

Fig. 1 Effect of sulfur amino acid deficiency on GCLC and GCLM expression in rat liver and in HepG2/C3A cells. **a** GCLC and GCLM protein and mRNA abundance in liver of rats fed a diet that contained 100 g soy protein isolate and no supplemental sulfur amino acids for 7 days. **b** GCLC and GCLM protein and mRNA abundance in cells cultured in medium with 0.1 mM L-methionine and no cyst(e)ine for 30 h. Values are expressed as fold of control values for rats fed a methionine-supplemented diet or for cells cultured in cysteine-supplemented medium. GCLC and GCLM protein levels were obtained by western blotting, and mRNA levels were obtained by quantitative PCR. *Bars* indicated by an *asterisk* represent values significantly greater than control values ($P \leq 0.05$)

Thiol analyses

Total cysteine concentration in cells was determined by the acid ninhydrin assay of Gaitonde (1967) as described previously (Dominy et al. 2007). Total glutathione concentration was determined by HPLC by the method of Cereser et al. (2001).

Statistical analyses

Data were statistically analyzed using Student's *t* tests or ANOVA followed by Dunnett's or Tukey's comparison tests, as indicated in figure legends. Statistical significance was accepted at $P \leq 0.05$.

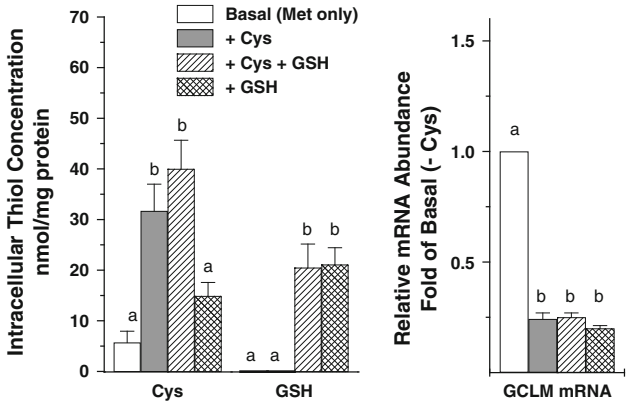

Fig. 2 Thiol and GCLM mRNA levels in cells null for GCLC. GCLC-null MEFs were cultured in cysteine-deficient medium (0.1 mM L-methionine) with or without addition of 0.3 mM L-cysteine and/or 0.5 mM glutathione. *Bars* not labeled with the *same letter* represent values that are significantly different at $P \leq 0.05$ by ANOVA and Tukey's comparison test

Results

Upregulation of GCLC and GCLM expression in liver of rats fed a low protein diet and in HepG2/C3A cells cultured in cysteine-deficient medium

In rats fed a diet that provided 100 g soy protein isolate supplemented with threonine and lysine but not with sulfur amino acids, hepatic GCLC mRNA and GCLM mRNA levels were significantly increased to 2.8 and 2.0 times, respectively, the levels present in liver of rats fed the same diet supplemented with 3.4 g L-methionine per kg diet (Fig. 1a). Hepatic GCLM protein abundance was increased by 50 %, whereas GCLC protein abundance was not significantly elevated ($P \geq 0.05$). In HepG2/C3A cells, culture in cysteine-deficient medium (with 0.1 mM Met) for 36 h led to GCLC mRNA abundance that was 5.7-times control and to GCLM mRNA abundance that was 8.4-times control (Fig. 1b). Cell GCLM protein abundance was increased to 2.8-times that of cells cultured in complete medium, but HepG2/C3A cell GCLC protein abundance was not affected by cysteine deprivation. Both the studies in rat liver and in HepG2/C3A cells show a strong response of GCLC and GCLM mRNA abundance to cysteine deprivation but an increase in protein abundance only for GCLM.

Effect of cysteine on GCLM expression in GCLC$^{-/-}$ MEFs

Using GCLC knockout MEFs, we tested the effect of cysteine supplementation on GCLM expression, without the accompanying increase in GSH levels. Addition of cysteine to GCLC null cells resulted in a marked decrease in GCLM mRNA levels despite the absence of GSH (Fig. 2). Addition of GSH similarly reduced GCLM mRNA abundance, but addition of GSH also led to an increase in cysteine levels, presumably due to hydrolysis of GSH. Addition of both cysteine and GSH had no additive effect, most likely because cysteine levels were sufficiently elevated by either one alone to yield a maximal response. This experiment demonstrates that GCLM expression can be regulated by cysteine in the absence of cellular GSH.

Effect of cysteine and its structural analogs on GCLC and GCLM expression in HepG2/C3A cells

The effects of addition of several thiol compounds to the culture medium on GCLC and GCLM mRNA levels in HepG2/C3A cells were compared to the effect of cysteine. Addition of cysteine to the medium resulted in a large increase in both cysteine and GSH levels (Fig. 3a) and the anticipated suppression of GCLC and GCLM mRNA abundance (Fig. 3b). In contrast to cysteine, however, the other thiol reagents that were tested (2-mercaptoethanol, 3-mercaptopropionic acid, and penicillamine) had no effect on either cysteine or GSH levels (Fig. 3a) and also had no significant effect on GCLC or GCLM mRNA abundance (Fig. 3b). These results indicate that suppression of GCLC and GCLM mRNA is not simply an effect of the presence of thiol/reducing compounds.

Effects of a deficiency of other essential amino acids and knockout of Gcn2 on Gclm and Gclc gene expression

In liver of rats fed sulfur amino acid-deficient diets, induction of ATF4 and phosphorylation of eIF2α were associated with higher levels of GCLC and GCLM mRNA (Sikalidis and Stipanuk 2010), and phosphorylation of eIF2α and upregulation of ATF4 mRNA is also observed in HepG2/C3A cells cultured in amino acid-deficient medium (Lee et al. 2008). Cysteine or sulfur amino acid deficiency could be upregulating *GCLC* and *GCLM* gene expression as a result of the general amino acid deprivation response mediated by activation of GCN2 (eIF2α kinase 4) when non-aminoacylated tRNAs accumulate in the cell. To explore this hypothesis, HepG2/C3A cells were cultured in medium deficient in cysteine or other essential amino acids. As shown in Fig. 4a, eIF2α was phosphorylated in response to culture of cells in medium deficient in leucine or cysteine, and to a lesser extent methionine, for 6–30 h, while levels of total eIF2α did not change. Consistent with the results shown in Fig. 1b, GCLC protein levels did not increase, whereas GCLM protein levels increased in the cells cultured in cysteine-deficient medium. However,

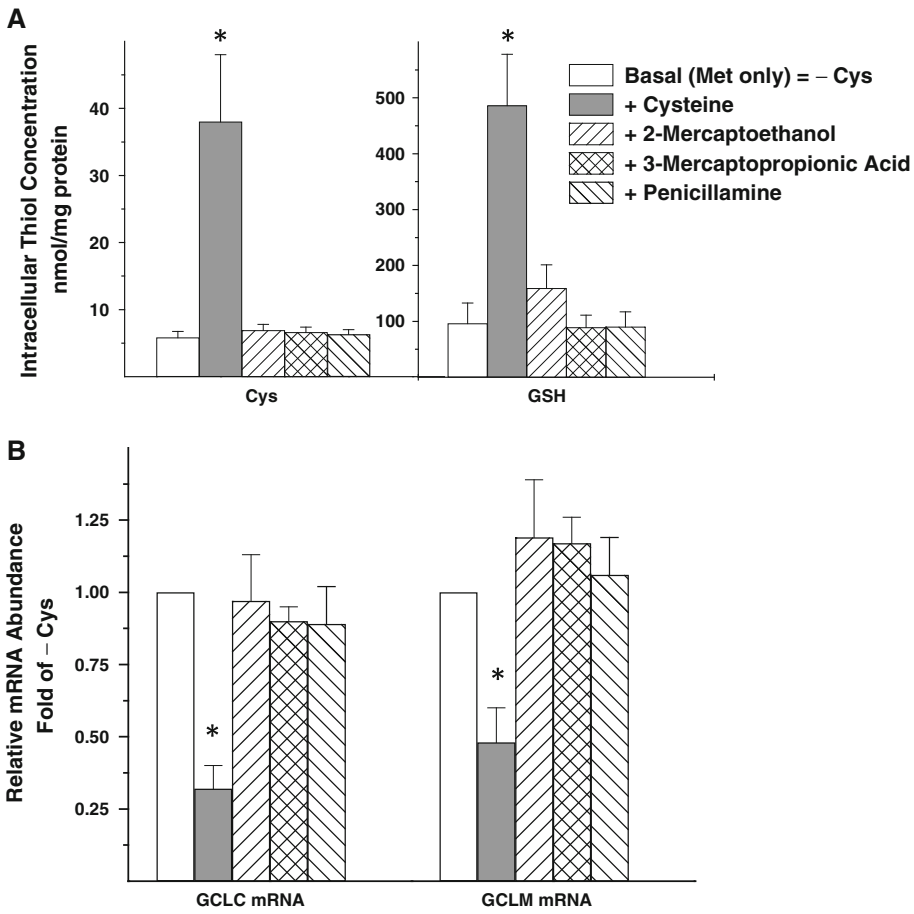

Fig. 3 Thiols (**a**) and GCLC and GCLM mRNA (**b**) in HepG2/C3A cells cultured for 20 h in cysteine-deficient medium (basal, containing 0.1 mM methionine) with/without addition of 0.3 mM cysteine or 0.3 mM other thiol compounds. *Bars* labeled with an *asterisk* are significantly different than values for the cysteine-deficient medium at $P \leq 0.05$ by ANOVA and Dunnett's comparison test

neither GCLC nor GCLM protein abundance was elevated in HepG2/C3A cells cultured in leucine- or methionine-deficient medium. Thus, based on protein expression, the response of GCLM seemed specific to cysteine deprivation. However, in studies described in Fig. 1, we similarly saw no change in GCLC protein levels despite marked increases in GCLC mRNA and much smaller fold changes in GCLM protein than in GCLM mRNA levels, indicating that changes in protein abundance may not accurately reflect changes in mRNA levels.

To further pursue the amino acid specificity of GCLC and GCLM gene expression, mRNA levels were determined in HepG2/C3A cells similarly cultured in medium deficient in cysteine, leucine, or histidine. As shown in Fig. 4b, a lack of cysteine, leucine, or histidine in the medium led to significantly higher levels of both GCLC and GCLM mRNA levels by 36 h of culture. The effects of cysteine or histidine deficiency appeared to be stronger than those of leucine deficiency, especially at 24 h when GCLC and GCLM mRNA abundances were not significantly elevated in cells cultured in leucine-deficient medium. This observation that both GCLC and GCLM mRNA levels were elevated by a deficiency of essential amino acids other than cysteine, together with the increased phosphorylation of eIF2α (Fig. 4a) suggests that activation of the GCN2 kinase may be responsible for increased expression of GCLC and GCLM mRNAs in response to amino acid deficiency, including cysteine deficiency.

To further evaluate the role of GCN2 and eIF2α Ser^{51} phosphorylation in the upregulation of GCLC and GCLM mRNA levels in response to amino acid deficiency, we tested the effect of amino acid deficiency in MEFs lacking GCN2 [$Gcn2^{-/-}$] and in MEFs in which Ser^{51} of eIF2α had been mutated to Ala [eIF2α(ala/ala)]. As shown in Fig. 5, removal of an essential amino acid from the medium resulted in the expected increases in GCLC and GCLM mRNA in wild-type control MEFs, whereas GCLC and GCLM mRNA levels were not significantly different from those of cells cultured in sufficient medium for the *Gcn2* knockout MEFs or the MEFs lacking the serine residue that is phosphorylated by GCN2 and other eIF2α kinases. This experiment clearly indicates that GCLC and GCLM mRNA levels are regulated via a GCN2/eIF2α-mediated pathway in MEFs. The similarity of results for both

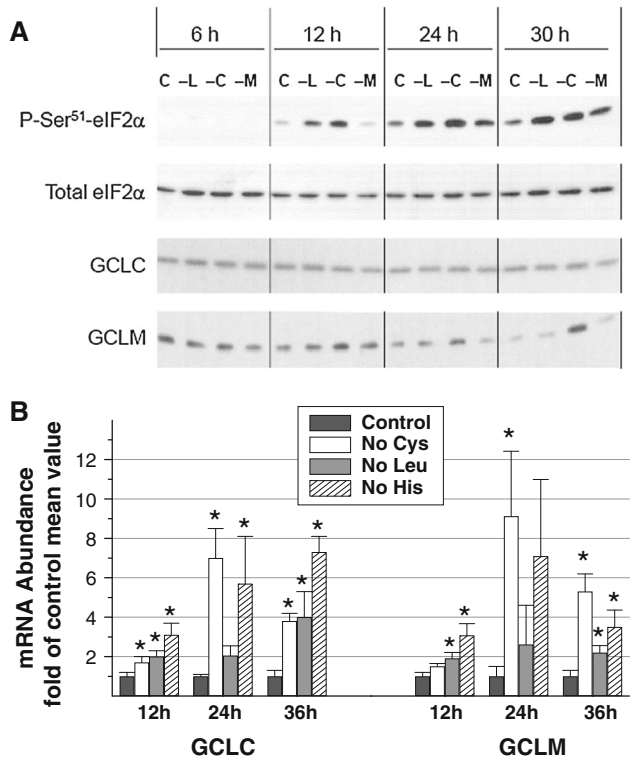

Fig. 4 Comparison of effects of various amino acid deficiencies on eIF2α phosphorylation and GCLC and GCLM expression in HepG2/C3A cells. **a** Western blots showing eIF2α phosphorylation in cells cultured in medium deficient in leucine, cysteine or methionine and an increase in GCLM protein abundance in cells cultured in cysteine-deficient medium. **b** GCLC and GCLM mRNA abundance in cells cultured for 12, 24, or 36 h in medium lacking cysteine, leucine or histidine. mRNA abundance was determined by quantitative PCR and is shown as fold of the control (complete medium) value. Values significantly different than the control value for a particular time-point are indicated by an *asterisk*

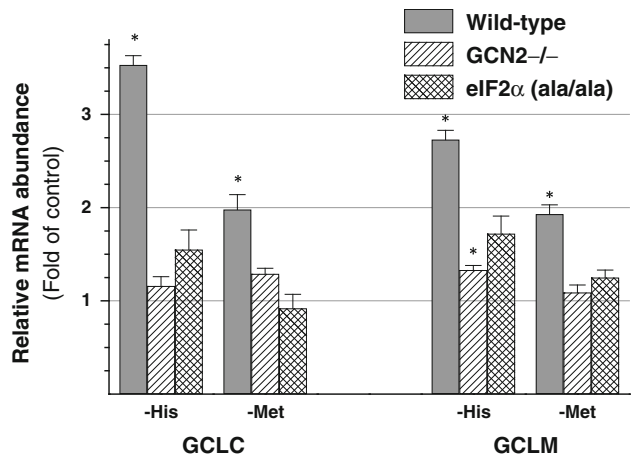

Fig. 5 Effect of deprivation of wild-type, *Gcn2* knockout, and eIF2α(ala/ala) mutant MEFs of histidine or methionine for 12 h on GCLC and GCLM mRNA levels. An *asterisk* above the *bar* indicates the value is different ($P \leq 0.05$) compared to the value (set as 1.0) for the same cells cultured in complete medium

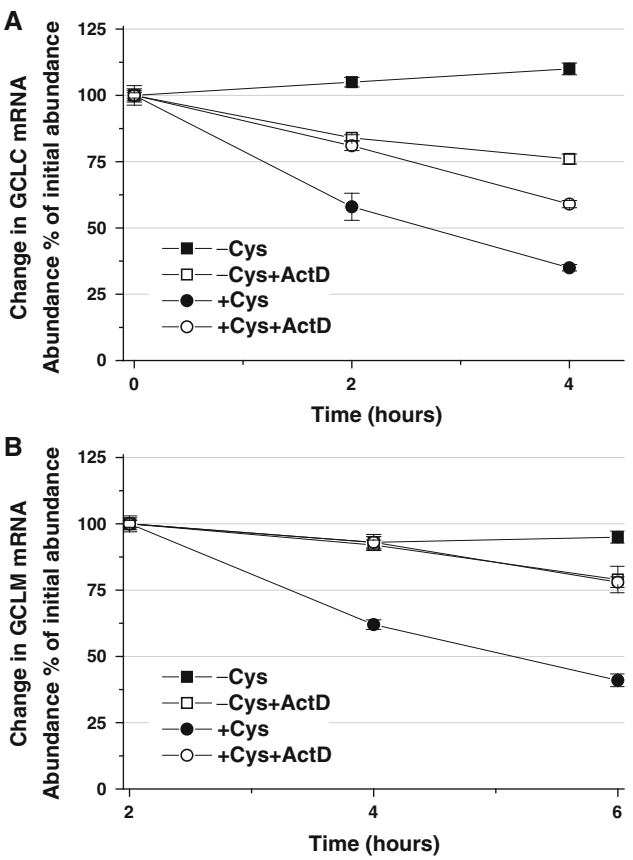

Fig. 6 Effect of actinomycin D on GCLC mRNA (**a**) and GCLM mRNA (**b**) abundance in HepG2/C3A cells. Actinomycin D was added with treatment media at time 0, and 2 h was allowed for actinomycin D to inhibit mRNA synthesis. All media contained 0.1 mM methionine. +Cys medium contained 0.3 mM cysteine. +ActD medium contained 10 μg/mL actinomycin D. Changes in mRNA levels were measured between 2 and 6 h

Gcn2$^{-/-}$ and eIF2α(ala/ala) MEFs also substantiates that the response is mediated by GCN2 and not by other eIF2α kinases.

Effects of actinomycin D and cycloheximide on GCLC and GCLM mRNA levels

The *GCLC* and *GCLM* genes have not been shown to contain functional amino acid response elements (AAREs) which are also known as CCAAT enhancer-binding protein-activating transcription factor response elements (CAREs) but may be downstream of genes that are regulated by ATF4 binding to AAREs, or CAREs. To further pursue the mechanism involved in the regulation of GCLC and GCLM mRNA abundance in response to cysteine deprivation, HepG2/C3A cells cultured in cysteine-deficient medium were transferred to fresh medium that was cysteine deficient or that had cysteine added back to restore

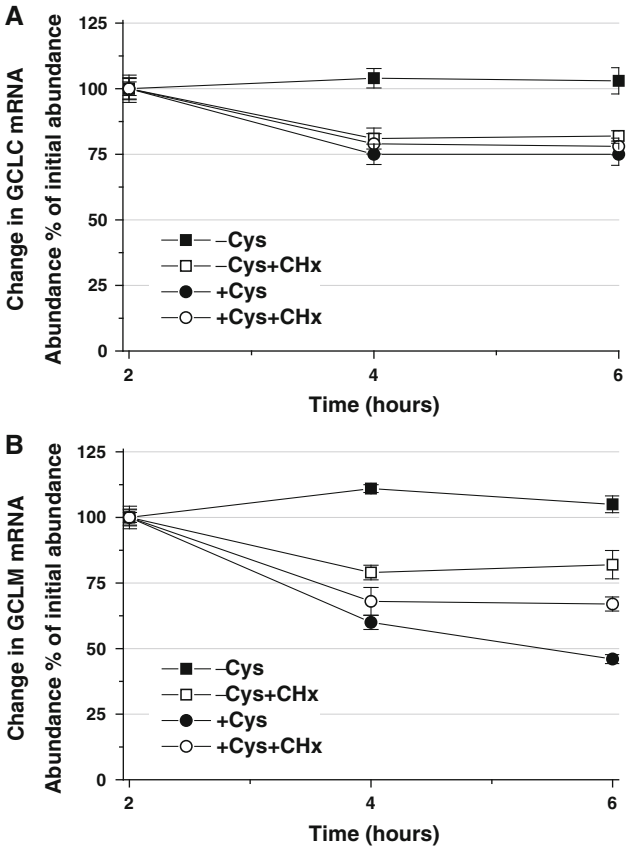

Fig. 7 Effect of cycloheximide on GCLC mRNA (**a**) and GCLM mRNA (**b**) abundance in HepG2/C3A cells. Cycloheximide was added with treatment media at time 0 and 2 h was allowed for cycloheximide to inhibit protein synthesis. All media contained 0.1 mM methionine. +Cys medium contained 0.3 mM cysteine. +CHx medium contained 10 μg/mL cycloheximide. Changes in mRNA levels were measured between 2 and 6 h

complete medium. In addition, actinomycin D or cycloheximide was added to some plates of cells to block mRNA synthesis or protein synthesis, respectively. As anticipated, when cells were switched to complete medium, GCLC mRNA abundance and GCLM mRNA abundance dramatically decreased over the subsequent 6 h, whereas GCLC and GCLM mRNA abundance in cells that remained in cysteine-deficient medium remained at the steady state level (Fig. 6a, b). Addition of actinomycin D to block new mRNA synthesis led to a similar rate of loss of GCLC mRNA in both cysteine-deficient and cysteine-supplemented cells, indicating that the GCLC mRNA degradation rate was similar under the two conditions. Thus, the differences in GCLC mRNA abundance in cells cultured in cysteine-deficient versus cysteine-supplemented medium appeared to be due to a higher rate of GCLC mRNA production (transcription and/or processing) in the cysteine-deficient cells.

Treatment of cysteine-deficient cells with actinomycin D had no effect (2 h) or a slight inhibitory effect (4 h) on the abundance of GCLM mRNA, suggesting the GLCM mRNA has a relatively long half-life and that mRNA levels might be regulated more by changes in the rate of GCLM mRNA degradation than by the rate of GCLM mRNA synthesis. On the other hand, treatment of cysteine-supplemented cells with actinomycin D dramatically eliminated the rapid GCLM mRNA degradation rate, restoring it to that observed in cysteine-deficient cells treated with actinomycin. The rapid loss of GCLM mRNA in cells cultured in cysteine-supplemented medium without actinomycin D, but not in those treated with actinomycin D, suggests that transcription of some gene other than *GCLM* is necessary for induction of the more rapid GCLM degradation rate in cysteine-supplemented cells.

To further assess whether upregulation of protein expression was involved in the regulation of GCLC mRNA and GCLM mRNA abundances, cells were treated with cycloheximide (Fig. 7a, b). Cycloheximide treatment resulted in cysteine-deficient and cysteine-supplemented cells having similar GCLC abundance and similar GCLM mRNA abundance. The similar mRNA abundances of cycloheximide-treated cells, regardless of cysteine level, suggest that upregulation of GCLC and GCLM mRNA levels in response to cysteine deprivation, whether mainly due to an increase in mRNA synthesis (i.e., GCLC mRNA) or a decrease in the rate of mRNA degradation (i.e., GCLM mRNA), requires protein synthesis. This would be consistent with the amino acid deprivation pathway inducing synthesis of ATF4 and other downstream proteins, some of which may be required to regulate GCLC mRNA synthesis and GCLM mRNA degradation.

The results for GCLM expression suggest that both transcription of some other gene and the translation of its mRNA into protein are necessary for induction of the more rapid GCLM mRNA degradation rate in cysteine-supplemented cells. This would be consistent with involvement of an mRNA-binding protein that destabilizes the GCLM mRNA. A requirement for new protein synthesis has been reported for upregulation of GCLM in L2 cells by 4-hydroxy-2-nonenal (Liu et al. 1998), which taken together with our findings, might suggest that GCLM mRNA levels are regulated by both stabilizing and destabilizing RNA-binding proteins under different circumstances. The results shown in Fig. 7b would be consistent with roles of both stabilizing (−Cys) and destabilizing (+Cys) proteins in regulation of GCLM mRNA turnover.

Discussion

Many studies over several decades have demonstrated a robust upregulation of the cell's capacity for glutathione synthesis in the face of oxidative or chemical stress.

Exposure of animal cells to low levels of electrophilic compounds (e.g., oxidizable phenols, isothiocyanates, quinones, Michael reaction acceptors, vicinal dimercaptans, heavy metals, trivalent arsenicals) results in induction of phase 2 detoxifying enzymes, such as glutathione *S*-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Lyakhovich et al. 2006; Purdom-Dickinson et al. 2007; Li et al. 2009; Mizuno et al. 2011; Emmert et al. 2012). The phase 2 detoxifying enzymes that are induced include GCLC and GCLM, and the increased expression of these GCL subunits results in the elevation of cellular GSH levels (Moinova and Mulcahy 1999; Wild et al. 1999; Chan and Kwong 2000; Dickinson et al. 2004). An essential feature of the stress response initiated by electrophilic species is the nuclear localization of transcription factor Nrf2 and the subsequent binding of an Nrf2:small Maf heterodimer to a DNA recognition sequence known as an antioxidant response element (ARE) or EpRE (Jaiswal 2004; Katsuoka et al. 2005; Nerland 2007; Hirotsu et al. 2012).

Because of the association of oxidative stress and upregulation of GCLC and GCLM and consequently cellular GSH levels, induction of GCLC or GCLM under conditions of GSH depletion has usually been attributed to the activation of Nrf2 and its binding to EpREs in the promoter regions of the *GCLC* and *GCLM* genes. Because GSH in animal tissues is generally limited by cysteine availability, such that low cellular cysteine levels tend to be paralleled by low cellular GSH levels, cellular responses to cysteine deficiency have also generally been attributed to a lack of GSH resulting in oxidative stress and activating the Nrf2/EpRE pathway. However, in our previous studies of cysteine deficiency in HepG2 cells, we found that *GCLC* and *GCLM* expression was upregulated in the absence of any signs of oxidative stress, as assessed by measures of ROS (dichlorodihydrofluorescein oxidation) and lipid peroxidation products (thiobarbituric acid-reactive substance formation) as well as by the failure to observe upregulation of the expression of most Nrf2/EpRE-regulated genes (such as those encoding NQO1, glutathione peroxidases, and superoxide dismutase 1) (Lee et al. 2006; Sikalidis et al. 2011). Furthermore, addition of cysteine to the culture medium dramatically decreased GCLC mRNA abundance in primary rat hepatocytes, regardless of whether or not the cells were also treated with buthionine sulfoximine to inhibit GCL and hence GSH synthesis (Kwon and Stipanuk 2001). The ability of hepatocytes to respond to changes in cellular cysteine levels in the absence of a change in GSH levels suggested that *GCLC* expression might be regulated in response to cysteine concentration rather than GSH concentration or oxidative stress caused by the lack of GSH. This possibility is supported by the gene expression studies in cysteine-deprived HepG2 cells (Lee et al. 2006, 2008), which demonstrated marked upregulation of a number of genes known to be upregulated by the eIF2α kinase/ATF4 pathway in response to amino acid deprivation.

Use of $Gclc^{-/-}$ MEFs allowed us to evaluate the effect of cysteine on *Gclm* gene expression in cells that could not synthesize GSH. GCLM mRNA levels were high in cells cultured in basal medium that contained only 0.1 mM methionine. Addition of cysteine reduced GCLM mRNA levels, demonstrating the responsiveness of *Gclm* gene expression to cysteine in the absence of GSH. Addition of GSH also reduced *Gclm* gene expression but, at the same time, increased cellular cysteine levels, presumably the result of GSH hydrolysis. Other thiols can sometimes replace GSH in preventing oxidative stress (Yan et al. 1995; Rabinkov et al. 2000; Takahashi et al. 2002), so we tested the effectiveness of several thiols, including 2-mercaptoethanol, and found that they had no effect on GCLC or GCLM expression in HepG2 cells cultured in cysteine-deficient medium. Thus, changes in cellular cysteine concentrations appeared to signal changes in GCLC and GCLM expression in a manner that was not dependent upon changes in cellular GSH levels or changes in oxidative stress.

Consistent with our previous observations that eIF2α kinase/ATF4 signaling was increased in cysteine-deficient cells (Lee et al. 2006, 2008), we found that *Gclc* and *Gclm* gene expression was upregulated in response to a deficiency of cysteine, histidine, methionine or leucine and that this upregulation was markedly suppressed in cells lacking the eIF2α kinase that senses amino acid deficiency (GCN2, or eIF2α kinase 4) by binding of uncharged tRNAs. These results clearly demonstrate that *Gclc* and *Gclm* are targets of the eIF2α kinase/ATF4 signaling pathways involved in stress responses and that a lack of any essential amino acid can activate the upregulation of *Gclc* and *Gclm* as has been shown for numerous other targets of this pathway (Harding et al. 2003; Lee et al. 2008; Kilberg et al. 2009, 2012; Sikalidis et al. 2011; Donnelly et al. 2013). Neither *Gclc* nor *Gclm* contain known CAREs that bind to ATF4-C/EBP heterodimers, suggesting that regulation of *Gclc* and *Gclm* expression by the eIF2α/ATF4 pathway must be downstream of ATF4 and, perhaps, dependent upon upregulation of other proteins or regulatory RNAs.

Indeed, our studies of GCLC mRNA and GCLM mRNA degradation rates demonstrated that upregulation of both GCLC and GLCM mRNA levels in response to cysteine deprivation was dependent on new protein synthesis, which is consistent with expression of GCLC and GCLM being

mediated by proteins whose synthesis depends on activation of the eIF2α/ATF4 pathway. The type and function of the regulatory proteins likely differ for GCLC and GCLM; however, based on our observation that the upregulation of GCLC mRNA in response to cysteine deprivation was due to an increase in mRNA synthesis, whereas the upregulation of GCLC mRNA in response to cysteine deprivation was due to a decrease in the rate of GCLM mRNA degradation. The regulation of GCLC expression is likely regulated by changes in abundance of transcriptional regulators, whereas the regulation of GCLM expression is likely regulated by changes in the abundance of mRNA stabilizing or destabilizing proteins or miRNAs.

Interestingly, the changes in GCLC mRNA were not translated into changes in GCLC protein levels in either rat liver or HepG2 cells, whereas changes in GCLM mRNA were associated with parallel changes in GCLM protein abundance in both rat liver and HepG2 cells. This suggests that the increase of GCL activity under conditions of amino acid limitation is largely dependent upon increased association of GCLC with its modifier subunit. This is consistent with our previously reported work showing increased association of GCLM with GCLC under conditions of cysteine deprivation (Lee et al. 2006).

Clearly, further work is needed to determine the details of the mechanisms and proteins involved in the regulation of GCLC and GCLM expression in response to amino acid availability. Nevertheless, this work, along with other work from our laboratory (Lee et al. 2004, 2006, 2008; Sikalidis et al. 2011), demonstrates that GCLC and GCLM mRNA levels are regulated in response to a deficiency of cysteine or other essential amino acids and that induction of an oxidative stress response is not necessary for upregulation of GCLC or GCLM mRNA levels. This can be seen as a protective mechanism because this upregulation serves to increase the capacity for GSH synthesis in the face of a possible deficiency of the precursors for synthesis of GSH and would serve to protect the cell in the face of a future oxidative or toxicant stress requiring GSH as an antioxidant or conjugating/detoxifying agent.

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Conflict of interest The authors declare that they have no conflict of interest.

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