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HepG2/C3A cells respond to cysteine deprivation by induction of the amino acid deprivation/integrated stress response pathway

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Lee J-I, Dominy JE Jr, Sikalidis AK, Hirschberger LL, Wang W, Stipanuk MH. HepG2/C3A cells respond to cysteine deprivation by induction of the amino acid deprivation/integrated stress response pathway. *Physiol Genomics* 33: 218–229, 2008. First published February 19, 2008; doi:10.1152/physiolgenomics.00263.2007.—To further define genes that are differentially expressed during cysteine deprivation and to evaluate the roles of amino acid deprivation vs. oxidative stress in the response to cysteine deprivation, we assessed gene expression in human hepatoma cells cultured in complete or cysteine-deficient medium. Overall, C3A cells responded to cysteine deprivation by activation of the eukaryotic initiation factor (eIF)2α kinase-mediated integrated stress response to inhibit global protein synthesis; increased expression of genes containing amino acid response elements (*ASNS*, *ATF3*, *CEBPB*, *SLC7A11*, and *TRIB3*); increased expression of genes for amino acid transporters (*SLC7A11*, *SLC1A4*, and *SLC3A2*), aminoacyl-tRNA synthetases (*CARS*), and, to a limited extent, amino acid metabolism (*ASNS* and *CTH*); increased expression of genes that act to suppress growth (*STC2*, *FOXO3A*, *GADD45A*, *LNK*, and *INHBE*); and increased expression of several enzymes that favor glutathione synthesis and maintenance of protein thiol groups (*GCLC*, *GCLM*, *SLC7A11*, and *TXNRD1*). Although *GCLC*, *GCLM*, *SLC7A11*, *HMOX*, and *TXNRD1* were upregulated, most genes known to be upregulated via oxidative stress were not affected by cysteine deprivation. Because most genes known to be upregulated in response to eIF2α phosphorylation and activating transcription factor 4 (ATF4) synthesis were differentially expressed in response to cysteine deprivation, it is likely that many responses to cysteine deprivation are mediated, at least in part, by the general control nondepressible 2 (GCN2)/ATF4-dependent integrated stress response. This conclusion was supported by the observation of similar differential expression of a subset of genes in response to leucine deprivation. A consequence of sulfur amino acid restriction appears to be the upregulation of the cellular capacity to cope with oxidative and chemical stresses via the integrated stress response.

general control nondepressible 2; eukaryotic initiation factor 2α; activating transcription factor 4; oxidative stress; sulfur amino acid

A FUNDAMENTAL QUESTION in biology is how cells sense and respond to changes in their nutrient supply. Our laboratory has had a long-term interest in the study of how cells and animals respond to changes in intake of sulfur-containing amino acids: methionine, which is absolutely indispensable, and cysteine, which is considered semi-indispensable for the whole animal because it can be synthesized from methionine sulfur and serine in the liver and some extrahepatic tissues and thus does not have to be obtained preformed from the diet. Nevertheless, most diets supply a mixture of methionine and cysteine, and the quality of many proteins low in sulfur-containing amino acids can be improved by addition of cyst(e)ine.

Despite wide ranges in sulfur amino acid intake, cysteine concentrations are maintained within a narrow range in most cells via regulation of amino acid transport, glutathione (GSH) synthesis, and cysteine catabolism (46, 47). Inside the cell, both cysteine and GSH are maintained in a highly reduced state, but cysteine levels are maintained quite low while GSH levels are much higher (~50–100 times those of cysteine). Cysteine is an essential substrate for synthesis of proteins, GSH, coenzyme A, hypotaurine, and taurine as well as an important precursor of sulfide and sulfate. In addition to being a storage reservoir of cysteine, GSH serves a myriad of protective functions, including the protection of cells from oxidative damage. GSH exerts its protective functions via its ability to participate in conjugation reactions catalyzed by glutathione *S*-transferases, to donate reducing equivalents via glutathione peroxidase- or thiotransferase-catalyzed reactions, to promote amino acid uptake via the γ-glutamyl cycle, and to nonenzymatically reduce free radicals or disulfides.

Whether a consequence of the primary cysteine deficiency or the secondary GSH deficiency, protective responses of cells to cysteine deprivation may underlie some of the observed effects of protein or sulfur amino acid restriction on longevity in rodent models. Lowering the content of sulfur amino acids in the diet by removing cysteine and restricting the concentration of methionine extended all parameters of survival in various rat strains, and pair-feeding demonstrated that the increase in survival was not due simply to caloric restriction (54). Although methionine restriction led to a decrease in hepatic GSH level, GSH levels in most extrahepatic tissues did not decline; in contrast, tissue cysteine concentrations were reduced to 28–75% of control levels in both liver and extrahepatic tissues (39). Methionine restriction was further shown to decrease mitochondrial oxygen radical production as well as oxidative damage to mitochondrial DNA and cellular proteins (41). These results with methionine restriction are somewhat in conflict with studies showing that an increase in cysteine supply or in GSH synthetic capacity, either of which would increase cellular GSH concentrations, is also associated with longevity. For example, long-living Ames dwarf mice exhibit enhanced flux of methionine to cysteine (51) as well as higher glutamate-cysteine ligase catalytic subunit (GCLC) levels and lower kidney γ-glutamyltranspeptidase levels (2). In contrast, short-lived senescence-accelerated mouse (SAM) P8 strain mice displayed lower levels of GSH and reactive protein sulfhydryls and higher levels of GSSG, consistent with a higher level of oxidative stress (38). In addition, studies in fruit flies (*Drosophila melanogaster)* demonstrated that overexpres-

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sion of either the catalytic or the modifier subunit of glutamate-cysteine ligase (GCL), the enzyme that catalyzes the first step in GSH synthesis, extended life span, and studies in mosquitoes (*Aedes aegypti*) showed that feeding a precursor of cysteine elevated both cysteine and GSH levels and extended life span (33, 40).

In studies with a human hepatoma cell line (HepG2/C3A) and with rats, we observed (6, 18, 19, 48, 49) that deprivation of cysteine, in the presence of adequate methionine to meet the cell's or whole animal's absolute requirement for methionine, led to a robust transcriptional induction of *GCLC* and *GCLM*, which encode the catalytic and modifier subunits, respectively, of GCL. In these models, addition of more methionine, under conditions in which transsulfuration was blocked, had no effect on *GCLC* or *GCLM* expression, whereas addition of cyst(e)ine readily reversed the induction. As would be anticipated, the increased expression of GCL subunit genes in response to cysteine deprivation was associated with a marked decrease in intracellular GSH, and the upregulation of *GCLC* and *GCLM* expression in response to cysteine deprivation is typically attributed to oxidative stress subsequent to GSH depletion. The promoters of the human *GCLC* and *GCLM* genes contain electrophile response elements (EpREs), which are the *cis*-acting sequences responsible for NF-E2-related factor 2 (Nrf2)-dependent regulation of gene expression, and expression of *GCLC* and *GCLM* has been shown to be upregulated in response to oxidative or electrophilic agents in numerous studies (4, 7, 27, 52). However, despite a marked depletion of intracellular GSH in C3A cells cultured in cysteine-deficient medium, we found no evidence for oxidative stress as assessed by dichlorodihydrofluorescein oxidation, a measure of reactive oxygen species (ROS) production, or thiobarbituric acid-reactive substance formation, a measure of lipid peroxidation (18). This led us to hypothesize that intracellular cysteine concentrations may be regulated via signaling pathways that are not induced by ROS and that changes in gene expression may occur in response to changes in cysteine concentrations under conditions that do not result in oxidative stress.

It seems possible that the amino acid deprivation pathway, which is initiated by general control nondepressible 2 (GCN2) or eukaryotic initiation factor (eIF)2α kinase 4 and is transcriptionally mediated by a consequent increase in activating transcription factor 4 (ATF4), is involved in the cellular response to cysteine deprivation. Harding et al. (10) found that murine embryonic ATF4−/− fibroblasts required much higher concentrations of cysteine to defend against GSH depletion than wild-type cells, suggesting that ATF4−/− cells may be defective in import and/or metabolism of cysteine and be predisposed to oxidative stress. However, because both GSH depletion and increased ROS production were observed in the ATF4−/− cells after withdrawal of supplemental cysteine from the medium (10), it was not possible to discern the relative contributions of amino acid deprivation and oxidative stress on induction of genes involved in cysteine uptake or metabolism. Furthermore, it was not clear whether the observed responses were mediated by amino acid deprivation directly, because, unlike yeast that have a single eIF2α kinase (GCN2), metazoans have a diversified group of eIF2α kinases that respond to various stresses to trigger common translational and transcriptional responses that have been termed the integrated stress response (ISR) (9, 10, 22, 23). Nevertheless, maintenance of reduced intracellular GSH levels appears to be a central component of the ISR.

Because our work with C3A cells (18, 19) indicated that this cell culture model was one in which we could explore the effect of cysteine deprivation on gene expression and cellular metabolism without the confounding influence of oxidative stress, we conducted microarray analyses of gene expression in C3A cells cultured in cysteine-sufficient or cysteine-deficient medium in order to obtain further information about the specific effects of cysteine deprivation on gene expression in mammalian cells. HepG2/C3A cells also have the advantage of being a model in which one can easily produce a cysteine deficiency in the presence of adequate methionine. C3A cells are a clonal derivative of the widely used human hepatocellular carcinoma HepG2 cell line that was selected for its better-differentiated hepatic phenotype (17). C3A cells, like HepG2 cells, have a very limited capacity to convert methionine to cysteine, due to a lack of expression of the high-K_m isoform of methionine adenosyltransferase (MAT1), and thus are unable to maintain intracellular cysteine and GSH levels without the provision of cyst(e)ine in the medium (18, 24). Our objectives were *1*) to further define the list of genes that are differentially expressed in response to cysteine deprivation and *2*) to evaluate the potential roles of amino acid deprivation vs. oxidative stress pathways in the regulation of gene expression in response to cysteine deprivation.

MATERIALS AND METHODS

Experimental treatment of C3A cells and RNA extraction. HepG2/C3A human hepatocellular carcinoma cells (American Type Culture Collection CRL-10741) were cultured in a humidified incubator at 37°C and 5% CO_2 in sulfur amino acid-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM L-methionine, 1× MEM nonessential amino acid solution, 0.05 mM bathocuproine disulfonate, and the indicated amount of cysteine. Cells were plated in cysteine-supplemented medium (0.1 mM L-cysteine + 0.1 mM L-methionine) at a density of a 1×10^6 cells per 100-mm-diameter culture dish. When the cells reached 50–60% confluence, the medium was replaced with experimental medium, which was either cysteine-sufficient medium (+Cys; + 0.1 mM cysteine + 0.1 mM methionine) or cysteine-deficient medium (−Cys; +0.1 mM methionine only), and cells were cultured in the experimental medium for 36 h with a medium change at 18 h. After 36 h of incubation the medium was changed to the opposite medium (Short treatments) or replaced with the same medium (Long treatments), and cells were incubated for an additional 6 h before cells were harvested. Thus there were four different experimental treatments, which were labeled as follows: *1*) cells cultured in +Cys medium for the entire 42 h (Long + Cys); *2*) cells cultured in −Cys medium for the entire 42 h (Long − Cys); *3*) cells cultured in +Cys medium for 36 h followed by −Cys medium for 6 h (Short − Cys); and *4*) cells cultured in −Cys medium for 36 h followed by +Cys for 6 h (Short + Cys). Three separate dishes of cells were analyzed by microarray for each of the treatments (i.e., a total of 12 RNA samples, 3 for each of 4 treatments, were analyzed). Other replicate dishes of cells were harvested separately for analysis of cysteine and GSH concentrations and for analysis of protein expression levels for a subset of genes.

In addition, in a separate experiment using a similar protocol, C3A cells were cultured for 42 h in control or leucine-devoid DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM L-methionine, and 1× MEM nonessential amino acid solution. Cells were harvested for RNA extraction and

analysis of a subset of genes by quantitative reverse transcriptase-PCR (qRT-PCR).

RNA extraction and microarray analysis. Cells cultured as described above were washed twice with ice-cold PBS, and cells were then directly lysed into denaturation solution in the culture dish. Total RNA was extracted from cells with an RNeasy Micro kit (Qiagen). These total RNA samples were labeled according to the standard one-cycle amplification and labeling protocol developed by Affymetrix (Santa Clara, CA). Labeled cRNAs were hybridized on Affymetrix GeneChip Human U133 plus 2.0 Array containing over 47,000 transcripts. Hybridized GeneChips were then stained, washed, and scanned by GeneChip Scanner 3000. The raw array data were processed by Affymetrix GCOS software to obtain detection calls and signal values. The signals were scaled to a target value of 500 with GCOS software. The scaled signal values were $\log_2$-transformed and centered by subtracting median signal from each gene across all arrays in the experiment. *t*-Test was applied on the normalized signal of each probe set to look for differential genes between any two conditions (i.e., Long − Cys vs. Long + Cys, Short − Cys vs. Long + Cys, and Short + Cys vs. Long − Cys). [Note that, in the results, all fold differences in expression are reported relative to the cysteine-deprived state for ease of comparison; thus the effect of the Short + Cys treatment is reported as Long − Cys/Short + Cys]. A significance level cutoff was empirically established at $P < 0.0001$, based on the number of genes passing the cutoff, the magnitude of fold changes, and the relationship between fold changes and P values, to select differential gene sets. Our complete microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO, accession no. GSE9517).

Data for groups of genes involved in sulfur amino acid metabolism or known to be regulated by the amino acid deprivation or oxidative stress pathways were specifically examined. Before looking at the array results, lists of genes were established for each category based on the literature, and then the Affymetrix probes for these genes were identified and the microarray results for these probes were evaluated. Genes with detection call of "present," determined by Affymetrix GCOS software, were considered to be reliably detected by the array. Informative genes were selected as having a "present" call in at least two of the nine arrays. A significance level of $P < 0.001$ was used as the cutoff for designating any of the listed genes as differentially expressed.

Microarray results were confirmed for selected genes with qRT-PCR. Isolated RNA was treated with DNase I, and first-strand cDNA was subsequently produced with the Invitrogen Superscript III kit and random primers. qRT-PCR was done with an Applied Biosystems 7500 Real Time PCR System, TaqMan Universal PCR Master Mix, and TaqMan gene expression assays for the specific genes (Applied Biosciences); 18S rRNA was used as the endogenous control. Northern blotting was also done for *GCLC* and *GCLM* as previously described, with actin as the endogenous control (18).

Measurement of cysteine and glutathione. Dishes of cells cultured as described above were washed twice with ice-cold PBS and harvested into 0.6 ml of PBS. One-half milliliter of cell solution was quickly transferred to a new tube including 0.2 ml of 35% (vol/vol) perchloric acid containing 3.75 mM bathocuproine disulfonate and 3.75 mM bathophenanthroline disulfonate, and the tube was placed on ice for 10 min. The mixture was then sonicated and centrifuged to obtain the acid supernatant. Acid supernatants were neutralized and treated with dithiothreitol (2.3 mM final concentration) to reduce disulfides. Total cysteine and total GSH were measured by formation of *S*-carboxymethyl derivatives followed by chromophore derivatization of primary amines with 1-fluoro-2,4-dinitrobenzene and separation of these derivatives by reverse-phase ion-exchange high-performance liquid chromatography with a 3-aminopropyl column (Brownlee Labs, Santa Clara, CA), as described previously (18, 49).

Analysis of protein expression levels in C3A cells. Dishes of C3A cells cultured as described above were washed twice with ice-cold PBS and harvested in TNES lysis buffer [50 mM Tris, pH 7.5, 1% (vol/vol) Nonidet P-40, 2 mM EDTA, 100 mM NaCl] with protease and phosphatase inhibitors. Samples were centrifuged for 4 min at 4°C and 13,000 *g* to obtain the soluble protein fraction. Soluble proteins were separated by SDS-PAGE and immunoblotted for proteins of interest with anti-GCLC (Neomarkers, Fremont, CA); anti-GCLM (generated in our laboratory; Ref. 19); anti-SLC7A11 (Abcam); anti-ATF3, anti-DDIT3/CHOP, and anti-HSP5A (all from Santa Cruz Biotechnology); anti-eIF2α (total) and anti-pS51-eIF2α (both from Cell Signaling Technology); anti-ASNS (12) and anti-ATF4 (both gifts of Dr. Michael Kilberg, Univ. of Florida, Gainesville, FL); and anti-NQO1 (44) (gift of Dr. David Ross, Univ. of Colorado Health Sciences Center, Denver, CO).

RESULTS

Removal of cysteine from cell culture medium resulted in decreased intracellular cysteine and GSH levels in C3A cells. The intracellular cysteine and GSH levels of HepG2/C3A cells used in this study were dramatically affected by the cysteine concentration of the medium. When measured after 36 h of culture (but only 18 h from the last medium change), total cysteine concentration was <0.04 μmol/g cells (wet wt) in cells cultured in cysteine-deficient medium and 0.41 ± 0.13 μmol/g in cells cultured in medium supplemented with 0.1 mM cysteine. Total GSH concentration was 0.75 ± 0.20 μmol/g for cells cultured in cysteine-deficient medium and 7.5 ± 3.4 μmol/g for cells cultured in cysteine-supplemented medium. Thus both cysteine and GSH concentrations in cells cultured in the cysteine-supplemented medium were ~10 times the concentrations found in cells cultured in cysteine-deficient medium.

Cysteine deprivation induced changes in gene expression in C3A cells. The results of the microarray analyses are summarized in Fig. 1 for the three different comparisons. First, all genes that were differentially expressed at a significance level of $P < 0.0001$ in any single treatment comparison were

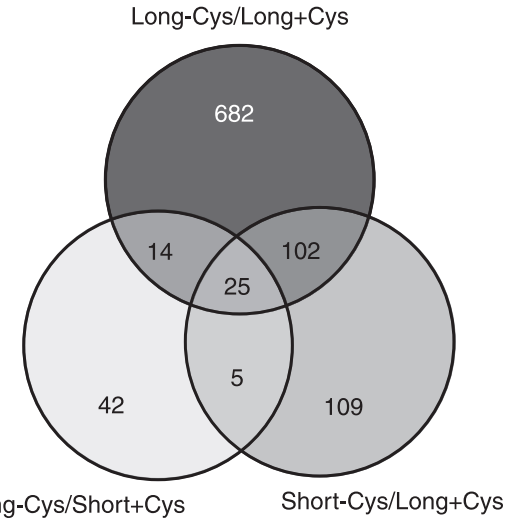

Fig. 1. Overlap of genes selected in the 3 different comparisons of gene expression in C3A cells cultured in cysteine-deficient medium (−Cys) vs. cysteine-supplemented medium (+Cys). The number of genes identified in each comparison and their overlap are shown in the Venn diagram. Numbers are the number of genes that were differentially expressed at a significance level of $P < 0.0001$ for the treatments shown. A total of 979 genes (1,109 probe ids) were identified for the 3 treatments combined.

selected, yielding a list of 979 genes (1,109 probe ids). Of these 979 genes, 532 were upregulated in response to cysteine deprivation (fold differences of 1.2–14.9) and 447 were downregulated in response to cysteine deprivation (fold differences of 0.95–0.07). A total of 823 genes were selected as differentially expressed in cells with Long exposures (i.e., 36 h + 6 h = 42 h) to cysteine-deficient vs. cysteine-supplemented medium, with 52% of these being upregulated. On the other hand, the number of genes selected as differentially expressed in cells with a Short exposure (i.e., 6 h, following 36 h in the opposite medium) to the opposite medium was 241 for cells switched to −Cys medium (with 65% of these being upregulated in response to cysteine deprivation) and 86 for cells switched to +Cys medium (with 84% of these being downregulated in response to +Cys vs. −Cys or, reciprocally, upregulated in response to cysteine deprivation, i.e., −Cys vs. +Cys). As shown in Table 1, pathway analysis of these 979 genes showed that differentially expressed genes were distributed among many functions and diseases and were especially associated with cancer, cell cycle, and DNA replication, recombination, and repair.

To focus on the genes most likely to be truly differentially expressed in response to cysteine deprivation, we used the stringent criterion of a gene being differentially expressed with a statistical significance of $P < 0.0001$ in all three comparisons. With this strategy, 25 genes were identified as being consistently differentially expressed in response to cysteine concentration. All 25 of the genes selected by this criterion were upregulated in response to cysteine deprivation; none of the downregulated genes met the statistical cutoff for inclusion in all three comparisons. These 25 genes and the Affymetrix probe id numbers are listed in Table 2. Fold differences for expression of these genes between treatments ranged from 1.3 to 14.9 and, in most cases, were somewhat greater in cells with Long or Short exposures to −Cys medium than in cells with Short exposure to +Cys medium. Of the subset of 25 genes that were highly significantly upregulated in response to cysteine deprivation in all three treatment comparisons, five genes (*ASNS*, *ATF3*, *CEBPB*, *SLC7A11*, and *TRIB3*) are known to contain amino acid response elements (AAREs) and to respond to amino acid deprivation, suggesting that cysteine deprivation activated the GCN2/ATF4-mediated pathway. In addition to *SLC7A11*, two others (*CARS*, *GCLM*) are also genes involved directly in cysteine uptake or metabolism and two (*GCLM*, *TXNRD1*) are also genes that would be predicted to be upregulated in response to oxidative stress. Three (*SLC7A11*, *GCLM*, and *TXNRD1*) of these four are known to contain EpREs and to be induced by oxidative or chemical stress (25, 42). Of the other genes in this subset, several (*STC2*, *FOXO3A*, *GADD45A*, *LNK*, and *INHBE*) encode proteins that act to inhibit proliferation and to suppress growth, which may also serve to protect cells when an essential amino acid is limiting for protein synthesis. Other genes included in the subset are *ARFGEF2*, *CBX4*, *CEBPG, DAF*, *MAP1LC3B*, *TUBE1*, *VLDLR*, and *ZFAND1*, along with four genes of unknown function. When the criterion for selection of highly regulated genes was slightly relaxed to include those differentially expressed at $P < 0.001$ in all three of the comparisons plus at $P < 0.0001$ in at least one comparison, 80 additional genes were selected. These additional genes are shown in Supplemental Table S1.[1]

Table 1. *Identification of functions and diseases of differentially expressed genes in response to cysteine deprivation in HepG2/C3A cells*

Functions and Diseases	No. of Genes
Cancer	128
Cell cycle	107
DNA replication, recombination, and repair	88
Reproductive system disease	61
Cell morphology	53
Small molecule biochemistry	51
Cell death	47
Cellular assembly and organization	42
Cellular growth and proliferation	39
Connective tissue disease	35
Connective tissue development and function	27
Respiratory disease	26
Gene expression	25
Cellular movement	24
Cellular development	23
Lipid metabolism	23
Amino acid metabolism	21

Analysis was done with Ingenuity Pathway Analysis 3.0 on the 979 genes that were differentially expressed with a $P < 0.0001$ in at least 1 treatment comparison.

Cysteine deprivation induced expression of genes involved in sulfur amino acid metabolism. To further explore the effect of cysteine deprivation on expression of genes involved in sulfur amino acid metabolism, and because of our goal to identify all components of the sulfur amino acid metabolic pathway that were induced by cysteine deprivation, we made a list of genes involved in methionine and cysteine metabolism, identified Affymetrix probe sets for genes in this list, and obtained expression results from the Affymetrix array data in all cases where reliable data were available (see MATERIALS AND METHODS). Expression array results for these genes are shown in Table 3. In addition to *CARS* (cysteinyl-tRNA synthetase), *GCLM*, and *SLC7A11* (cystine-glutamate transporter subunit xCT), which were all among the 25 highly differentially expressed genes (Table 2), *GCLC*, *CTH* (cystathionase), *SLC1A4* (glutamate/neutral amino acid transporter that is also known as ASCT1), and *SLC3A2* (4F2hc/CD98, the activator subunit of dimeric amino acid transporters including xCT) were upregulated in all three comparisons at $P < 0.001$ and in at least one of the three treatment comparisons at $P < 0.0001$ (see Supplemental Table S1). Expressions of *PAPSS1* (3′-phosphoadenosine 5′-phosphosulfate synthase 1) and *SUOX* (sulfite oxidase) were significantly downregulated ($P < 0.001$) in cells cultured long term in cysteine-deficient medium vs. long term in cysteine-adequate medium, perhaps related to decreased inorganic sulfur production from cysteine in these cells.

Cysteine deprivation induced expression of genes involved in the amino acid deprivation pathway mediated via the AARE/ATF4 system. Because depletion of an essential amino acid is known to activate the GCN2/ATF4-mediated amino acid deprivation response, we also used the expression array data to look specifically at the effects of cysteine deprivation on expression of genes known to be regulated by this pathway. We compiled a list of genes known to contain AAREs (i.e.,

[1] The online version of this article contains supplemental material.

Table 2. *Differential expression of genes in response to cysteine deprivation*

Probe id	Gene Symbol	Fold Difference: Long − Cys/ Short + Cys	Fold Difference: Long − Cys/ Long + Cys	Fold Difference: Short − Cys/ Long + Cys	Gene Title
218098_at	ARFGEF2	1.61	1.97	1.75	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)
205047_s_at	ASNS	2.43	1.56	1.35	asparagine synthetase
202672_s_at	ATF3	3.24	10.28	7.34	activating transcription factor 3
212971_at	CARS	1.98	2.69	2.28	cysteinyl-tRNA synthetase
227558_at	CBX4	1.94	2.49	2.05	chromobox homolog 4 (Pc class homolog, *Drosophila*)
212501_at	CEBPB	2.98	4.60	4.46	CCAAT/enhancer binding protein (C/EBP), β
204203_at	CEBPG	1.90	2.56	2.48	CCAAT/enhancer binding protein (C/EBP), γ
201925_s_at	DAF	2.75	4.20	3.61	decay accelerating factor for complement (CD55, Cromer blood group system)
201919_at	FLJ10618	1.90	2.65	2.57	hypothetical protein FLJ10618
204131_s_at	FOXO3A	1.80	2.84	3.11	forkhead box O3A
203725_at	GADD45A	1.78	5.59	3.79	growth arrest and DNA-damage-inducible, α
203925_at	GCLM	1.88	3.25	1.97	glutamate-cysteine ligase, modifier subunit
210587_at	INHBE	4.47	6.76	10.59	inhibin, βE
203320_at	LNK	2.65	3.41	4.40	lymphocyte adaptor protein
208786_s_at	MAP1LC3B	2.08	3.68	1.70	microtubule-associated protein 1 light chain 3β
236285_at	MGC16635	2.18	11.32	5.35	hypothetical protein BC009980
204538_x_at	NPIP /// LOC339047 /// LOC440341	2.39	2.76	2.09	nuclear pore complex interacting protein /// hypothetical protein LOC339047 /// similar to hypothetical protein LOC339047
207528_s_at	SLC7A11	2.92	5.61	4.93	solute carrier family 7 (cationic amino acid transporter, y^+ system), member 11
209921_at		4.56	13.77	14.86	
217678_at		4.98	9.88	11.41	
203438_at	STC2	3.00	5.99	8.30	stanniocalcin 2
218145_at	TRIB3	2.79	4.03	5.38	tribbles homolog 3 (*Drosophila*)
226181_at	TUBE1	3.40	4.52	5.88	tubulin, ε1
201266_at	TXNRD1	1.69	3.62	2.04	thioredoxin reductase 1
209822_s_at	VLDLR	3.09	3.52	3.06	very low-density lipoprotein receptor
218919_at	ZFAND1	2.11	2.42	2.46	zinc finger, AN1-type domain 1
227755_at		2.31	5.86	6.03	CDNA clone IMAGE:4077090, partial cds

For inclusion in this table, a gene had to be differentially expressed with a significance level of $P < 0.0001$ for all 3 treatment comparisons.

ATF4-C/EBP composite sites, or amino acid response elements), which have been shown to bind ATF4 heterodimers that act as enhancer elements (10, 34, 43). Then, as for the sulfur amino acid metabolism-related genes, we identified probe sets for this list of genes and included all reliable expression array data in Table 4. Five of these genes [*ASNS* (asparagine synthetase), *ATF3* (activating transcription factor 3), *CEBPB* (CCAAT/enhancer binding protein, β), *SLC7A11*, and *TRIB3* (tribbles homolog 3)] were identified in Table 2 as differentially expressed at $P < 0.0001$ for all three treatment comparisons. Two additional genes, *ATF4* and *SLC7A1* (system y^+ cationic amino acid transporter CAT1) were identified as being differentially expressed at $P < 0.001$ for all three comparisons. In addition, *DDIT3* (DNA-damage-inducible transcript 3, also commonly known as *CHOP*) and *SLC38A2* (system A sodium-coupled neutral amino acid transporter, SNAT) were differentially expressed ($P < 0.001$) in some, although not all, of the treatment comparisons. Thus all of the genes known to contain an AARE and to respond to amino acid deprivation via ATF4 binding were significantly upregulated in some, if not all, of the treatment comparisons. In addition, although its expression is not directly regulated by ATF4, *PPP1R15A* (protein phosphatase 1, regulatory subunit 15A, also known as *GADD34*) was examined because it is a well-established downstream target of the eIF2α kinase/ATF4-dependent stress response pathways (26, 28, 29), and *PPP1R15A* was significantly upregulated in two of the three comparisons.

Cysteine deprivation did not induce expression of many genes known to be regulated in response to oxidative stress signaling via the EpRE/Nrf2 system. We compiled a list of genes known to contain EpRE elements that interact with Nrf2 heterodimers (25, 37) and, as for the sulfur amino acid metabolism-related genes and amino acid deprivation pathway-regulated genes, identified probe sets for this list of genes and included all reliable data in Table 5. Of a list of 23 genes, only 5, *GCLC*, *GCLM*, *SLC7A11*, *HMOX1* (heme oxygenase 1), and *TXNRD1* (thioredoxin reductase 1), were differentially expressed at $P < 0.001$ for all three comparisons; only four (*GCLC*, *GCLM*, *SLC7A11*, and *TXNRD1*) were differentially expressed at $P < 0.0001$ in at least one of the three comparisons. Three of these five genes are involved in sulfur amino acid metabolism (*GCLC*, *GCLM*, and *SLC7A11*) or are known to be regulated via the amino acid deprivation pathway (*SLC7A11*). In addition, the fold differences in expression of *FTH1* (ferritin heavy chain 1), *HERPUD1* (homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein, also known as *HERP*), and *MT1E* and *MT1H* (metallothionein 1E and 1H) were significantly greater ($P < 0.001$) in cysteine-deprived cells for one or two of the three comparisons, with increases being <50%. Notably, however, many of the genes that are known to be highly responsive to oxidative stress (37), including *NQO1* [NAD(P)H dehydrogenase, quinone 1], *PRDX1* (peroxiredoxin 1), *TXN* (thioredoxin), and *MGST1* (microsomal glutathione *S*-transferase 1), were not upregulated. Furthermore, several of the genes known to

Table 3. *Differential expression of genes involved in sulfur amino acid metabolism*

Gene Symbol	Fold Difference: Long − Cys/ Short + Cys	Fold Difference: Long − Cys/ Long + Cys	Fold Difference: Short − Cys/ Long + Cys	Gene Title	Probe id
AHCY	1.05	0.73	0.88	*S*-adenosylhomocysteine hydrolase	200903_s_at
C10orf22	1.01	1.28	1.14	chromosome 10 open reading frame 22; recently	212500_at
	1.06	1.24	1.04	identified as cysteamine dioxygenase (Ref. 7a)	212502_at
	0.94	0.98	1.00		229409_s_at
CARS	**2.03**	**2.69**	**2.45**	**cysteinyl-tRNA synthetase**	**202402_s_at**
	1.98	**2.69**	**2.28**		**212971_at**
	1.75	**2.48**	**2.30**		240983_s_at
CBS	1.32	1.08	1.19	cystathionine-β-synthase	1553972_a_at
CDO1	1.01	0.88	0.95	cysteine dioxygenase, type I	204154_at
CTH	**3.31**	**2.90**	**2.17**	**cystathionase (cystathionine γ-lyase)**	**206085_s_at**
	3.59	**2.86**	**2.19**		**217127_at**
GCLC	**1.68**	**2.57**	**2.13**	**glutamate-cysteine ligase, catalytic subunit**	**202922_at**
	1.69	**2.47**	**1.76**		**202923_s_at**
GCLM	**1.87**	**3.24**	**1.97**	**glutamate-cysteine ligase, modifier subunit**	**203925_at**
	2.19	**3.46**	**2.75**		236140_at
GGT1	1.13	0.64	0.84	γ-glutamyltransferase 1	207131_x_at
	1.08	0.74	0.95		208284_x_at
	1.14	0.70	0.98		209919_x_at
	1.05	0.61	0.76		211417_x_at
GSS	1.01	1.10	1.11	glutathione synthetase	201415_at
	1.05	1.10	1.08		211630_s_at
MAT1A	1.10	0.65	0.79	methionine adenosyltransferase I, α	205813_s_at
MAT2A	0.84	1.13	0.59	methionine adenosyltransferase II, α	200768_s_at
	0.78	1.30	0.69		200769_s_at
MAT2B	1.00	0.86	1.00	methionine adenosyltransferase II, β	217993_s_at
PAPSS1	0.96	**0.62**	0.82	3¢-phosphoadenosine 5¢-phosphosulfate synthase 1	209043_at
SLC1A4	**2.01**	**2.17**	**2.88**	**solute carrier family 1 (glutamate/neutral amino**	**209610_s_at**
	1.87	1.68	2.27	**acid transporter), member 4**	209611_s_at
	2.23	**2.51**	**2.93**		**212810_s_at**
	2.01	**2.17**	**2.94**		212811_x_at
	1.20	1.45	1.36		235875_at
	1.23	1.12	1.21		244377_at
SLC3A2	**2.46**	**3.65**	**2.98**	**solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2**	**200924_s_at**
SLC7A11	**2.92**	**5.61**	**4.93**	**solute carrier family 7 (cationic amino acid**	**207528_s_at**
	4.56	**13.77**	**14.86**	**transporter, y^+ system), member 11;**	**209921_at**
	4.98	**9.88**	**11.41**	**cystine-glutamate exchanger**	**217678_at**
SUOX	0.88	**0.53**	0.68	sulfite oxidase	1553030_a_at

Fold changes significant at $P < 0.001$ are in bold. Names of genes and probes for which fold changes were $P < 0.001$ in all 3 experiments are also in bold.

be induced by oxidative stress were actually expressed at a lower level in cells cultured in the cysteine-deficient medium: expressions of *GPX3* and *GPX4* (glutathione peroxidase 3 and 4) and of *SOD1* (superoxide dismutase 1) were significantly lower ($P < 0.001$) in the Long − Cys/Long + Cys comparison.

Verification of expression array results. The array results for several upregulated genes (*ATF4*, *ASNS*, *ATF3*, *GCLC*, *GCLM*, *SLC7A11*, and *DDIT3*), an example of a gene that was not differentially expressed (*NQO1*), and an example of a downregulated gene (*VSNL1*) were verified by qRT-PCR, as shown in Table 6. These results were further supported for *GCLC* and *GCLM* by Northern blotting, which demonstrated that both the 3.2- and 4.1-kb transcripts of *GCLC* and both the 1.4- and 4.1-kb transcripts of *GCLM* were strongly upregulated in response to cysteine deprivation (not shown) and that the fold differences determined by quantitative Northern blotting were similar to those obtained by qRT-PCR. We further determined whether differential mRNA levels were associated with changes in protein levels for several proteins by running Western blots (Table 6). GCLM, SLC7A11, ATF3, ASNS, ATF4, and DDIT3 protein levels were strongly upregulated in cysteine-deprived C3A cells, consistent with upregulation of their mRNA abundance, although fold differences in protein levels were generally less than those for mRNA levels. Despite an increase in GCLC mRNA, the GCLC protein level was not different for cells cultured in −Cys vs. +Cys medium at the 42 h time point. No effect of cysteine deprivation on NQO1 protein levels was observed, which is consistent with the absence of differential expression of its mRNA.

Comparison of selected gene expression in C3A cells deprived of leucine vs. cysteine. To further explore the possibility that the observed response to cysteine deprivation was mediated by the amino acid deprivation/GCN2 signaling pathway, we compared the response of cells to leucine deprivation vs. that of cells to cysteine deprivation. As shown in Table 7, the fold changes in gene expression for cells cultured in leucine-devoid medium were all in the same direction as those observed for cells cultured in cysteine-devoid medium. Additionally, the ratio of phosphorylated eIF2α to total eIF2α was increased by four- to fivefold in cells cultured in either −Cys or −Leu medium (Western blot results; data not shown). The

Table 4. *Differential expression of genes known to be regulated in response to the amino acid deprivation pathway mediated via the AARE/ATF4 system*

Gene Symbol	Fold Difference: Long − Cys/ Short + Cys	Fold Difference: Long − Cys/ Long + Cys	Fold Difference: Short − Cys/ Long + Cys	Gene Title	Probe id
ATF3	**3.24**	**10.28**	**7.34**	**activating transcription factor 3**	**202672_s_at**
	2.04	**4.30**	**3.72**		**1554980_a_at**
ATF4	**1.58**	**1.49**	**3.19**	**activating transcription factor 4**	**200779_at**
ASNS	**2.43**	**1.56**	**1.35**	**asparagine synthetase**	**205047_s_at**
CEBPB	**2.98**	**4.60**	**4.46**	**CCAAT/enhancer binding protein (C/EBP), β**	**212501_at**
DDIT3	1.96	**4.15**	**2.73**	DNA-damage-inducible transcript 3; C/EBP homologous protein (CHOP); growth arrest and DNA-damage-inducible transcript 153 (GADD153)	209383_at
SLC38A2	1.09	**1.49**	1.23	solute carrier family 38, member 2; amino acid transporter 2; system A amino acid transporter; sodium-dependent neutral amino acid transporter 2 (SNAT2)	222982_x_at
	1.11	**1.68**	1.22		220924_s_at
	1.07	**1.70**	1.22		218041_x_at
	0.98	1.04	0.96		1559924_at
SLC7A1	**2.27**	**3.37**	**2.78**	**solute carrier family 7 (cationic amino acid transporter, y^+ system), member 1 (CAT1)**	**212295_s_at**
	2.33	**3.25**	**2.53**		**212290_at**
	2.12	2.45	2.11		212292_at
	1.60	2.54	2.35		206566_at
SLC7A11	**4.98**	**9.88**	**11.41**	**solute carrier family 7 (cationic amino acid transporter, y^+ system), member 11 (xCT)**	**217678_at**
	4.56	**13.77**	**14.86**		**209921_at**
	2.92	**5.61**	**4.93**		**207528_s_at**
TRIB3	**2.79**	**4.03**	**5.38**	**tribbles homolog 3 (*Drosophila*)**	**218145_at**
	3.94	**5.80**	**7.54**		**1555788_a_at**
PPP1R15A (downstream target)	1.58	**6.17**	**4.73**	protein phosphatase 1, regulatory (inhibitor) subunit 15A; growth arrest and DNA-damage-inducible transcript 34 (GADD34)	37028_at
	1.76	**8.56**	**4.90**		202014_at

AARE, amino acid response element; ATF4, activating transcription factor 4. Fold changes significant at $P < 0.001$ are in bold. Names of genes and probes for which fold changes were $P < 0.001$ in all 3 experiments are also in bold.

likelihood that this response is mediated by GCN2, rather than another eIF2α kinase such as PERK, is further supported by the observation that the level of the endoplasmic reticulum (ER) chaperone protein HSPA5 (heat shock 70-kDa protein 5; also known as BiP and as glucose-regulated protein 78 kDa), which would be induced in response to ER stress, was not upregulated by either leucine or cysteine deprivation (Western blot results shown in Supplemental Fig. S1). However, leucine deprivation was much less effective in the induction of *GCLM*, *SLC7A11*, and *ATF3* expression and much more effective in the induction of *ASNS* and *DDIT3* expression than cysteine deprivation, whereas both cysteine and leucine deprivation similarly induced ATF4 expression levels.

DISCUSSION

With regard to our objective to further define the list of genes that are differentially expressed in response to cysteine deprivation, we identified 979 highly differentially expressed genes that were associated with many different functions, including cell cycle and DNA recombination and repair. Interestingly, despite the prediction that cysteine deprivation might lead to an amino acid deficiency, affecting protein synthesis, or to a lack of GSH synthesis, giving rise to oxidative stress, a relatively small fraction of the differentially expressed genes encoded proteins that would clearly be involved in either of these two processes.

A small subset of genes were highly differentially expressed in all three treatment comparisons, yielding a set of 25 genes (i.e., those in Table 2) that were differentially expressed at $P < 0.0001$ in both short-term treatments as well as in the long-term treatment. The differential expression of these genes in multiple treatment comparisons provides a higher level of confidence that they are truly responding to cysteine deprivation. However, genes that responded more slowly or only transiently to cysteine deprivation, which could have been part of the larger set of differentially expressed genes, would have been eliminated by this subsequent selection criterion. As a compromise and to identify other possible genes of interest, we defined an intermediate list of 105 genes (i.e., the 25 reported in Table 2 plus 80 reported in Supplemental Table S1) that were differentially expressed at $P < 0.0001$ in at least one treatment comparison and at $P < 0.001$ in all three treatment comparisons. The most striking observation from pathway analysis of these smaller sets of genes was a high degree of inclusion of genes involved in eIF2α kinase/ATF4-mediated stress responses.

The short list of 25 genes consistently highly upregulated in response to cysteine deprivation did include three genes (*CARS*, *GCLM*, and *SLC7A11*) involved in cysteine metabolism or transport. The intermediate list of 105 genes included four additional upregulated genes involved in cyst(e)ine transport and metabolism (*SLC1A4*, *SLC3A2*, *GCLC*, and *CTH*).

Table 5. *Differential expression of genes known to be regulated in response to oxidative stress mediated via the EpRE (ARE)/Nrf2 system*

Gene Symbol	Fold Difference: Short − Cys/ Long + Cys	Fold Difference: Long − Cys/ Long + Cys	Fold Difference: Long − Cys/ Short + Cys	Gene Title	Probe id
ABCC2	1.18	1.47	1.01	ATP-binding cassette, subfamily C (CFTR/MRP), member 2	206155_at
ABCC3	0.87	1.18	1.06	ATP-binding cassette, subfamily C (CFTR/MRP), member 3	208161_s_at
	1.24	1.05	0.93		230682_x_at
	1.07	0.99	0.87		239217_x_at
	1.00	1.06	1.00		242553_at
FTH1	1.04	1.38	0.98	ferritin, heavy polypeptide 1	200748_s_at
	1.15	**1.94**	1.01		214211_at
FTL	0.99	0.94	0.95	ferritin, light polypeptide	212788_x_at
	0.98	0.98	0.95		213187_x_at
GCLC	**1.68**	**2.56**	**2.12**	**glutamate-cysteine ligase, catalytic subunit**	**202922_at**
	1.68	**2.46**	**1.76**		**202923_s_at**
GCLM	**1.87**	**3.24**	**1.97**	**glutamate-cysteine ligase, modifier subunit**	**203925_at**
	2.18	**3.45**	2.74		236140_at
GPX1	0.97	0.80	0.78	glutathione peroxidase 1	200736_s_at
GPX2	0.97	0.70	0.78	glutathione peroxidase 2 (gastrointestinal)	202831_at
	1.06	0.89	0.89		239595_at
GPX3	0.91	**0.48**	0.63	glutathione peroxidase 3 (plasma)	201348_at
	0.93	**0.51**	0.67		214091_s_at
GPX4	0.92	**0.61**	0.77	glutathione peroxidase 4 (phospholipid hydroperoxidase)	201106_at
GSTA2	0.91	1.24	1.21	glutathione *S*-transferase A2	242478_at
GSTA4	0.89	0.62	0.85	glutathione *S*-transferase A4	202967_at
	0.86	0.85	0.67		235405_at
HERPUD1	**1.52**	**2.44**	1.87	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERPUD1, or HERP)	217168_s_at
HMOX1	**1.59**	**3.63**	**3.00**	heme oxygenase (decycling) 1	**203665_at**
MGST1	0.91	1.25	0.98	microsomal glutathione *S*-transferase 1	1565162_s_at
	0.94	1.30	0.99		224918_x_at
	0.91	1.26	0.95		231736_x_at
	0.91	1.31	1.31		239001_at
MT1E	1.50	**1.47**	**1.94**	metallothionein 1E (functional)	212859_x_at
MT1H	**1.78**	1.23	**1.62**	metallothionein 1H	206461_x_at
NQO1	1.04	0.94	0.91	NAD(P)H dehydrogenase, quinone 1	201467_s_at
	1.10	1.06	1.03		201468_s_at
	1.03	0.97	0.93		210519_s_at
PRDX1	0.94	0.85	0.86	peroxiredoxin 1	208680_at
SLC7A11	**2.92**	**5.60**	**4.93**	**solute carrier family 7 (cationic amino acid transporter, y^+ system), member 11**	**207528_s_at**
	4.55	**13.76**	**14.85**		**209921_at**
	4.98	**9.87**	**11.41**		**217678_at**
SOD1	0.91	**0.56**	0.68	superoxide dismutase 1, soluble [amyotrophic lateral sclerosis 1 (adult)]	200642_at
TXN	0.94	0.90	0.84	thioredoxin	208864_s_at
	0.86	0.81	0.95		216609_at
TXNRD1	**1.69**	**3.62**	**2.03**	**thioredoxin reductase 1**	**201266_at**

ARE, antioxidant response element; EpRE, electrophile response element; Nrf2, NF-E2-related factor 2. Fold changes significant at $P < 0.001$ are in bold. Names of genes and probes for which fold changes were $P < 0.001$ in all 3 experiments are also in bold.

Further lowering the criterion to include genes differentially expressed at $P < 0.001$ in all three of the comparisons did not result in the identification of any additional genes in the sulfur amino acid metabolic or transport pathways. Thus these seven genes appear to comprise a near-complete set of those genes in the sulfur amino acid metabolic or transport pathways that are upregulated in response to cysteine deprivation. Upregulation of expression of these seven genes would favor cyst(e)ine uptake by cells; an increased capacity to use the limiting substrate, cysteine, for synthesis of GSH and for aminoacylation of $tRNA^{Cys}$ to allow synthesis of critical proteins; the sustained production of sulfide from cyst(e)ine due to the β-cleavage activity of CTH; and possibly some effect on net transsulfuration flux via increased hydrolysis of cystathionine due to the γ-cleavage activity of CTH.

A number of other genes involved in amino acid uptake and metabolism were differentially expressed in response to cysteine. Genes involved in uptake and metabolism of amino acids other than cysteine that were differentially expressed at $P < 0.0001$ in at least one treatment comparison (i.e., were in the long list of 979 genes) included *ASNS*, which was also identified in the short list of 25 highly upregulated genes; *SLC7A1*, *GARS* (glycyl-tRNA synthetase), *SARS* (seryl-tRNA synthetase), and *TARS* (threonyl-tRNA synthetase), which were also identified in the expanded list of 105 differentially expressed genes and all of which were upregulated; *AMD1*

Table 6. *Fold changes in mRNA determined by different methods for C3A cells cultured in −Cys for 42 h (−Cys Long) compared to control (+Cys Long)*

	GeneChip (mRNA)	qRT-PCR (mRNA)	Northern Blot (mRNA)	Western Blot (protein)
ATF4	1.5	5.0±1.1		3.1
ATF3	4.3, 10.3	72.5±6.4		11.7
ASNS	1.6	5.1±0.8		2.0
DDIT3	4.1	9.2±0.1		2.6
SLC7A11	5.6-13.8	35±5		>4.5*
GCLC	2.5	5.7±0.3	5.1±0.3	1.0
GCLM	3.4	8.4±0.2	9.9±0.6	2.8
NQO1	1.0	1.3±0.5		1.3
VSNL1	0.18	0.24±0.2		ND

Values are fold differences for cells cultured in cysteine-deficient medium (−Cys) vs. cysteine-sufficient medium (+Cys). Values for microarray (GeneChip) are ratios of the mean for cells cultured in −Cys vs. the mean for cells cultured in +Cys ($n = 3$ for each mean). Quantitative reverse transcription PCR (qRT-PCR) and Northern blots were done by comparing 1 −Cys sample to 1 +Cys sample (random assignment), and values are means ± SE for the 3 comparisons. ND, not determined. *Control soluble protein level was below limits of detection.

(adenosylmethionine decarboxylase 1), *HAL* (histidine ammonia-lyase), *ARG2* (arginase type 2), *KYNU* (kynureninase), *WARS* (tryptophanyl-tRNA synthetase), and *SLC7A2* (CAT-2 amino acid transporter), which were all identified as upregulated; and *PAH* (phenylalanine hydroxylase), *AMT* (aminomethyltransferase; glycine cleavage system protein T), and *GCSH* (glycine cleavage system protein H), which were all identified as downregulated. Thus the differentially expressed genes involved in amino acid uptake and metabolism were comprised of genes involved in cysteine uptake and metabolism as well as genes involved in uptake and metabolism of different amino acids.

Our second objective was to evaluate the potential roles of amino acid deprivation vs. oxidative stress pathways in the regulation of gene expression in response to cysteine deprivation. Although it is frequently assumed that low cellular cysteine and GSH levels will result in oxidative stress, the cysteine-deprived C3A cells did not appear to have enhanced EpRE/Nrf2-mediated gene transcription in response to oxidative stress. This is consistent with our previous report (18) that C3A cells cultured in cysteine-deficient medium did not have elevated levels of ROS or lipid peroxidation products. The majority of genes that have been shown to be upregulated via EpRE/Nrf2-dependent pathways in various models of oxidative stress (i.e., 18 of 23 genes) were not upregulated in this study, and some target genes were actually downregulated in cysteine-deprived cells. Nevertheless, three genes known to contain EpREs (*SLC7A11*, *GCLM*, and *TXNRD1*) were identified in the list of 25 most significantly upregulated genes, and two others (*HMOX1* and *GCLC*) were differentially expressed at $P < 0.001$ for all three comparisons. Upregulation of *SLC7A11*, *HMOX1*, and *TXNRD1* in the cysteine-deprived cells could possibly be due to activation of the amino acid deprivation pathway, as discussed below, but the potential mechanisms involved in the induction of *GCLC* and *GCLM* in the cysteine-deprived C3A cells are not obvious and require further study.

In contrast to the apparent absence of activation of the Nrf2/EpRE-mediated gene transcription, cysteine deprivation clearly activated the eIF2α kinase/ATF4-mediated ISR pathway. We found evidence for upregulation of all nine of the well-established, direct targets of ATF4/AARE-mediated gene expression in C3A cells exposed to cysteine deprivation as well as for upregulation of the downstream target *PPP1R15A*. Five of these target genes (*ASNS*, *ATF3*, *CEBPB, SLC7A11*, and *TRIB3*) were significantly upregulated at $P < 0.0001$ in all three treatment comparisons, and two additional ones (*ATF4* and *SLC7A1*) were included with the slightly relaxed criterion of differential expression at $P < 0.001$ in all three treatment comparisons plus at $P < 0.0001$ in at least one treatment comparison.

In addition to the nine well-documented target genes of the amino acid deprivation response pathway, a number of other genes that were upregulated in cysteine-deprived C3A cells are genes that have been shown to be dependent upon ATF4 and/or eIF2α phosphorylation for their induction in murine cells (10, 13–16, 23). Upregulated genes whose expression has been reported to be reduced in ATF4-deficient cells include *CARS*, *CEBPG* (CCAAT/enhancer-binding protein, γ), *GADD45A* (growth arrest- and DNA-damage-inducible transcript 45A), and *STC2* (stanniocalcin 2), which were differentially upregulated by cysteine deprivation ($P < 0.0001$) in all three of our treatment comparisons; *CTH*, *GARS*, *SARS*, *SLC3A2*, and *SLC1A4*, which were differentially expressed at $P < 0.0001$ in at least one of our comparisons and at $P < 0.001$ in all three of our treatment comparisons; *WARS*, which was differentially expressed at $P < 0.0001$ with long-term cysteine deprivation in C3A cells; and *HERPUD1* and *HMOX1*, which were upregulated at $P < 0.001$ in two or three of the treatment comparisons for cysteine-deprived C3A cells. Phosphorylation of an artificial eIF2α under conditions in which upstream stress pathways were not activated also resulted in upregulation of these same genes, except *HMOX1*, providing further evidence that these genes are downstream targets of the eIF2α kinase/ATF4 pathway (23). Phosphorylation of the artificial eIF2α (23) also resulted in upregulation of *CARS, CBX4*, and *TXNRD*, which were also on our short list of 25 highly differentially expressed genes; of *SARS* and *TARS*, which were also upregulated in our cysteine-deprived C3A cells ($P < 0.0001$ in at least 1 comparison and $P < 0.001$ in all 3 treatment comparisons); and of *HERPUD1,* which was upregulated at $P < 0.001$ in two

Table 7. *Comparison of fold changes in mRNA for C3A cells in response to leucine deprivation versus cysteine deprivation*

	−Leu/+Leu	−Cys/+Cys
ATF4	5.8±0.4	5.0±1.1
ATF3	15.6±1.1	72.5±6.4
ASNS	23.1±1.0	5.1±0.8
DDIT3	46.1±6.3	9.2±0.1
SLC7A11	6.3±0.6	35±5
GCLC	3.4±0.4	5.7±0.3
GCLM	2.9±0.5	8.4±0.2
NQO1	1.0±0.1	1.3±0.5
VSNL1	0.58±0.1	0.24±0.2

Values are fold differences in mRNA ratio for cells cultured in −Cys vs. +Cys (complete) or in −Leu vs. +Leu (complete) medium. qRT-PCR was done by comparing 1 −Cys sample to 1 +Cys sample or 1 −Leu sample to 1 +Leu sample (random assignment), and values are means ± SE for the 3 comparisons.

treatment comparisons for cysteine-deprived C3A cells. The ATF4-dependent upregulation of several of these genes, including *CTH*, *GARS*, *STC2*, and *HERPUD1*, in response to leucine deprivation was further shown to be blocked by overexpression of *TRIB3*, which appears to act as a negative feedback regulator of ATF4-dependent transcription (16, 30–32).

Although many genes involved in amino acid biosynthesis are upregulated in yeast (50), *ASNS* is the only amino acid biosynthetic gene that has been shown so far to be a direct target of the GCN2/ATF4 pathway in mammals (8, 45). Mammals clearly have a more limited capacity for amino acid synthesis and, in contrast to yeast, appear to cope with amino acid deprivation largely by upregulating amino acid transporters and aminoacyl-tRNA synthetases. This is consistent with 7 of the 15 new genes we identified as likely targets of the GCN2/ATF4 pathway being ones that encode amino acid transporters or aminoacyl-tRNA synthetases. Furthermore, our identification of *CTH* as a potential target is of interest because only one mammalian gene encoding an enzyme involved in amino acid biosynthesis (*ASNS*) has so far been established as a target of the GCN2/ATF4 pathway in mammals. CTH functions both in cysteine biosynthesis via the methionine transsulfuration pathway and in cysteine catabolism with H_2S production by the β-cleavage of cystine. H_2S production from cysteine has been shown to have inhibitory effects on cell proliferation (53), and it is possible that this latter function of CTH plays a role in the ISR. In addition to *CTH*, *GADD45A* and *STC2* could also be target genes that encode proteins that act to inhibit proliferation and suppress growth during amino acid deprivation, and upregulation of *HMOX1* may serve to prevent heme accumulation when protein synthesis is globally inhibited.

Further work, obviously, is needed to determine whether these 15 genes represent direct or downstream targets of eIF2α phosphorylation, because the presence of AAREs and activation by ATF4 heterodimer binding to these elements have not yet been demonstrated for the promoter regions of these genes. However, an ATF4 binding site in *HERPUD1* has been identified (20), and ATF4, perhaps as an Nrf2-ATF4 heterodimer, has been shown to regulate basal and induced expression of *HMOX1* in mouse hepatoma (Hepa) cells (11).

Although our work seems to rule out EpRE/Nrf2-mediated gene transcription in response to oxidative stress as the mechanism responsible for upregulation of *GCLC* and *GCLM* expression in cysteine-deprived C3A cells, available data are insufficient to determine whether the GCN2/ATF4-mediated amino acid deprivation response pathway is responsible for *GCLC* and *GCLM* upregulation. These two genes have not been reported to be dependent upon ATF4 and/or eIF2α phosphorylation for their induction in murine cells (10, 13, 16, 23). However, we found that leucine deprivation of C3A cells also resulted in upregulation of *GCLC* and *GCLM* expression, supporting the hypothesis that the cellular response to cysteine deprivation is at least partly mediated by a general response to amino acid deprivation mediated by GCN2/ATF4. Thus an important basic component of the general GCN2/ATF4-mediated amino acid deprivation/ISR pathway could be the upregulation of GSH synthesis, which along with an increase in cyst(e)ine uptake by the cystine-glutamate exchanger X_C^- and the neutral amino acid transporter ASCT1 and an increase in TXNRD1 could increase the cell's antioxidative and protective capacities.

Regardless of the mechanism involved in upregulation of *GCLC* and *GCLM* gene expression, the 2.8-fold increase in GCLM protein level, along with the >4.5-fold increase in SLC7A11 concentration, observed in cysteine-deprived C3A cells would be expected to favor cystine uptake and its incorporation into GSH, which in turn could serve to protect cells against oxidative and chemical stress. Our observation that GCLC protein level was not increased in cysteine-deprived C3A cells, despite upregulation of its mRNA level, is consistent with our previous observations (18) of a greater fold increase in GCLM than in GCLC in cysteine-deprived C3A cells and in liver of rats fed a low-protein/cysteine-deficient (10% casein) diet. However, because hepatic GCLC is normally not saturated with GCLM, upregulation of GCLM alone can markedly increase the capacity for GSH synthesis because the holoenzyme has a much higher k_{cat} (18).

The extent to which the cellular response to amino acid deprivation varies with the particular amino acid that is deficient is not clear; the limited data shown here for leucine vs. cysteine deprivation suggest that, while the overall response may require an intact GCN2/ATF4 signaling pathway, the magnitude of differential expression of particular target genes may vary with the specific amino acid that is deficient. For example, although both cysteine and leucine deprivation similarly induced *ATF4* expression in C3A cells, cysteine deprivation was much more effective in the induction of *GCLM*, *SLC7A11*, and *ATF3* expression and much less effective in the induction of *ASNS* and *DDIT3* expression than leucine deprivation. One explanation might be that some GCN2 signaling is independent of ATF4. The likelihood that some aspects of GCN2 signaling are independent of ATF4 upregulation is supported by the recent report that GCN2 but not ATF4 expression was required for repression of fatty acid synthase gene expression and prevention of steatosis in liver of mice fed a leucine-devoid diet (8). Another possibility is that the GCN2/ATF4 pathway is amplified or supplemented by other signaling mechanisms to provide a degree of specificity for the transcriptional response to deficiencies of particular indispensable amino acids. This would, in some ways, be analogous to the interactions of signaling from ATF6 and IRE1 with those from eIF2α kinase PERK signaling to generate the specific response to ER stress. Although relatively little is known about the role of other *cis* and *trans* regulatory factors in mediating or modifying the response to amino acid deprivation, it is likely that other factors are involved. For example, phosphorylation of ATF2 and binding to the C/EBP-ATF4 composite site was required for *DDIT3* and *ATF3* induction, but not for *ASNS* induction, in amino acid starvation (1, 3), and ATF3 has been shown to have both positive and negative effects on transcription of targets of the amino acid deprivation pathway and may, in some cases, serve in a feedback role to limit target gene transcription (5, 21, 36).

It is interesting to consider that *GCLC*, *GCLM*, *SLC7A11*, *SLC3A2* and other stress-related genes might be induced by an amino acid deficiency before GSH depletion, as well as by oxidative events occurring subsequent to cellular thiol/GSH depletion. It is possible that the beneficial effects of dietary protein or sulfur amino acid restriction on longevity, which are associated with decreases in mitochondrial ROS production

and oxidative tissue damage (35), could be related to upregulation of protective genes [including certain antioxidant enzymes, cyst(e)ine transporters, and GSH synthetic enzymes] in response to a cysteine deficiency in anticipation of, rather than in response to, oxidative or electrophilic stress. Such a regulatory mechanism would have clear benefits in terms of favoring cell survival and minimizing tissue damage. The responses to cysteine deficiency observed in this study support the existence of regulatory pathways that respond to amino acid deficiency, or at least to cysteine deficiency, by downregulation of global protein synthesis and growth/proliferation along with upregulation of the expression of a group of antioxidative and protective genes that favor cell survival.

Overall, this work has identified most, if not all, of the genes for cyst(e)ine metabolism or uptake that are differentially expressed in C3A cells in response to cysteine deprivation. The robust upregulation of *GLCM* expression relative to *GCLC* expression, at both the mRNA and protein levels, also underscores our previous observations that *GCLM* expression plays a dominant role in regulation of hepatic GCL activity. In addition, it has demonstrated the strong induction of the amino acid deprivation pathway in C3A cells deprived of cyst(e)ine. Finally, this work provides further support to our earlier observations (18) indicating that cysteine deprivation does not necessarily induce an oxidative state in unstressed cells. Rather, it suggests that expression of genes for cyst(e)ine uptake and GSH synthesis may be induced as part of the cell's normal response to amino acid deficiency or other stress that activates one of the eIF2α kinases. Additional studies are clearly required to conclusively demonstrate the role of GCN2 (eIF2α kinase 4) in initiating the response to cysteine deprivation and to elucidate the specific role of the amino acid deprivation/ISR pathway in bringing about each of the observed responses to cysteine deprivation.

Nevertheless, it is clear from this study that the overall response of cells to cysteine deprivation, despite its association with low tissue GSH levels, is similar to the response of cells to deficiencies of other amino acids or other stressors that activate eIF2α kinases. The cysteine-deprived cells responded by initiating a response that appears to be protective and anticipatory of possible oxidative stress in the absence of an actual increase in ROS levels or redox state. This response appears to be quite different from the EpRE/Nrf2-mediated responses of cells to treatments that induce oxidative/chemical stress, which appear to be mediated largely by changes in the thiol-disulfide redox state of Keap1, a cytoplasmic protein that associates with both Nrf2 and the actin cytoskeleton, and lead to induction of a largely different set of genes. The responses to cysteine deprivation appear to be protective, depressing cysteine utilization for protein synthesis, enhancing the capacity for cyst(e)ine uptake from the medium, enhancing the capacity for cysteinyl-tRNA synthesis, and enhancing the capacity for GSH synthesis and maintenance of a reduced intracellular milieu. Together, these responses serve to conserve cysteine and support critical cellular functions while limiting nonessential protein synthesis or growth.

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