Initial Characterization of the Glutamate-Cysteine Ligase Modifier Subunit Gclm(−/−) Knockout Mouse

NOVEL MODEL SYSTEM FOR A SEVERELY COMPROMISED OXIDATIVE STRESS RESPONSE*

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Yi Yang, Matthew Z. Dieter†, Ying Chen, Howard G. Shertzer, Daniel W. Nebert, and Timothy P. Dalton§

From the Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0056

Glutamate-cysteine ligase (GCL) is the rate-limiting enzyme in the GSH biosynthesis pathway. In higher eukaryotes, this enzyme is a heterodimer comprising a catalytic subunit (GCLC) and a modifier subunit (GCLM), which change the catalytic characteristics of the holoenzyme. To define the cellular function of GCLM, we disrupted the mouse Gclm gene to create a null allele. Gclm(−/−) mice are viable and fertile and have no overt phenotype. In liver, lung, pancreas, erythrocytes, and plasma, however, GSH levels in Gclm(−/−) mice were 9–16% of that in Gclm(+/+) littermates. Cysteine levels in Gclm(−/−) mice were 9, 35, and 40% of that in Gclm(+/+) mice in kidney, pancreas, and plasma, respectively, but remained unchanged in the liver and erythrocytes. Comparing the hepatic GCL holoenzyme with GCLC in the genetic absence of GCLM, we found the latter had an ~2-fold increase in Km for glutamate and a dramatically enhanced sensitivity to GSH inhibition. The major decrease in GSH, combined with diminished GCL activity, rendered Gclm(−/−) fetal fibroblasts strikingly more sensitive to chemical oxidants such as H2O2. We conclude that the Gclm(−/−) mouse represents a model of chronic GSH depletion that will be very useful in evaluating the role of the GCLM subunit and GSH in numerous pathophysiological conditions as well as in environmental toxicity associated with oxidant insult.

Glutathione (GSH) is the most abundant non-protein thiol found in most aerobic organisms (1, 2). GSH is an antioxidant and, by way of direct scavenging and enzymatic reduction reactions, participates in cellular protection against both endogenous and exogenous electrophiles. GSH also regulates the cellular redox balance by maintaining the essential thiol status of proteins (3). Moreover, GSH serves as a storage depot for cysteine and as a cofactor for the enzymic reduction of ribonucleotides (4, 5). Because of one or more of these functions, GSH has been shown to be involved in decisions concerning the fate of the cell, including cellular proliferation, differentiation, and apoptosis (6, 7).

GSH is synthesized from its precursor amino acids in two sequential enzymatic reactions. Glutamate-cysteine ligase (GCL) catalyzes the formation of γ-glutamylcysteine (γ-GC) from glutamate and cysteine. Glutathione synthetase then couples glycine to γ-GC to form GSH. The reaction catalyzed by GCL is rate-limiting in GSH biosynthesis, and the product GSH is a feedback inhibitor of GCL activity. Higher eukaryotes contain GCL as a heterodimer comprised of a 72.8-kDa catalytic subunit (GCLC) and a 30.8-kDa modifier subunit (GCLM), which are encoded by genes on different chromosomes (8–10). Studies with both native and recombinant GCL protein demonstrate that the catalytic subunit is necessary and sufficient for γ-GC biosynthesis (11, 12). Targeted disruption of the Gclc gene in mice demonstrated that GCLC and most likely GSH are essential for embryonic development but not survival of cells in culture (13, 14).

Much of our understanding about the function of the modifier subunit has been the result of studies examining the catalytic activity of the purified GCLC protein versus the GCL holoenzyme. Experiments with both rat and human preparations indicated that GCLM modifies GCLC catalytic properties by decreasing the Km for glutamate and diminishing the inhibition by GSH (15, 16). Because intracellular levels of glutamate are typically lower and those of GSH are typically higher than the Km and Ks for GCLC, respectively, GCLC alone has been predicted to function poorly in maintaining cellular GSH levels (15). The experimental support for this hypothesis, however, is mixed. Overexpression of GCLM increases the cellular GSH by 2-fold, rendering cells resistant to oxidative stress (17). In agreement with this result, down-regulation of GCLM mRNA by ribozyme expression leads to a decrease of GSH levels in cultured pancreatic islet cells (18). On the other hand, up-regulation of GCLC alone has also been reported to support high levels of intracellular GSH (19, 20). Furthermore, despite a decrease in GCLM following antisense RNA inhibition of GCLM translation, no changes in GSH were noted in human hepatoblastoma HepG2 cell cultures (21, 22).

To define the cellular function of GCLM, we have generated a mouse line with a targeted disruption of the Gclm gene. Mice homozygous for the null allele are viable and fertile; we show here that this mouse appears to be an excellent model system for studying animals that are severely compromised in their response to oxidative stress.

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† Present address: Dept. of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160-7417.
§ To whom correspondence should be addressed: Dept. of Environmental Health, University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati, OH 45267-0056. Tel.: 513-558-0973; Fax: 513-558-0974; E-mail: tim.dalton@uc.edu.

3 The abbreviations used are: GCL, glutamate-cysteine ligase; GCLC, GCL catalytic subunit; GCLM, GCL modifier subunit; γ-GC, γ-glutamylcysteine; GSH, glutathione; GSH-EE, GSH monoethyl ester; MFF, mouse fetal fibroblast; HPLC, high pressure liquid chromatography.
EXPERIMENTAL PROCEDURES

Materials—Hydrogen Peroxide (H₂O₂) and phorone were purchased from Sigma. The GSH monoethyl ester (GSH-EE) was prepared as described previously (23).

Preparation of Targeting Construct, Targeting, and Generation of Gclm(−/−) Mice—A 5′-F11 (Stratagene, La Jolla, CA) 129/SvJ mouse genomic library was screened with a GCLM cDNA probe (24), and several overlapping clones were mapped. A targeting construct was then designed using portions of these clones (Fig. 1). The Gclm gene was targeted in the same J1 and D embryonic stem cell lines as described previously (14). C57BL/6J blastocysts were used for embryonic stem cell injection (14). Resultant male chimeric mice were mated with C57BL/6J females, and the offspring were of mixed (C57BL/6J and 129/SvJ) genetic background. All mouse experiments were conducted on littersmates having the three possible genotypes following the targeted construct (Fig. 1). Following targeting, the mice were routinely genotyped using PCR. The targeted allele was detected as a 250-bp fragment using primers OL183a (5′-AACGTTGCAAGCTACTGC-3′) external to the targeted region of intron 1 and OL184a (5′-ATATGGCAAGTGACCTG-3′) within the target region of intron 1 and OL184a (5′-ATATGGCAAGTGACCTG-3′) within the N-terminal gene. The wild-type allele was detected as a 200-bp fragment using primers OL83a 5′-AACGTTGCAAGCTACTGC-3′) and OL818s (5′-AGTTGAGAGCTTCACTG-3′) within the deleted region of intron 1.

Northern Blot and Western Immunoblot Analysis—Mice were killed by CO₂ asphyxiation, and tissues were frozen immediately on dry ice and stored at −70 °C until use. For Northern blot analysis, total RNA was isolated from various tissues and analyzed for the presence of GCLC and GCLM mRNA as described previously (25). Equal loading was determined by rehybridizing blots with a probe for β-actin mRNA. For detection of GCLC protein, a rabbit polyclonal antibody was raised against the synthetic peptide QFEGRTTPHNHIC (amino acids 79–91 of the mouse GCLC) coupled to keyhole limpet hemocyanin. The cytosolic fractions were resolved by 12% SDS-PAGE and blotted with the above antibody (1:50,000). The amount of GCLM protein was determined as described previously (14) using anti-GCLM antibody (a gift of T. Kavanagh).

Gel Filtration Chromatography—Liver samples were homogenized immediately in 10 volumes of homogenization buffer (154 mM KCl, 5 mM dithiothreitol, 10 mM potassium phosphate buffer, 10 mM MgCl₂, and 5 mM β-mercaptoethanol, pH 6.5) using a Teflon homogenizer. The cytosolic fractions were prepared as described previously (14) and then separated by fast protein liquid chromatography (FPLC) (Amersham Biosciences). Samples were applied to a Superose-6 gel filtration column pre-equilibrated with the homogenization buffer, and the protein-containing fractions were collected, lyophilized, and stored at −80 °C. "GSH, Oxidized Glutathione (GSX), and Cysteine Measurements—GSH, GSX, and cysteine levels were determined as described previously (26, 27).

Analysis of GCL Activity—The formation of γ-GC from l-glutamate and L-cysteine was performed at 37 °C under the conditions described previously (28). The reaction was stopped by adding a 50-μl aliquot of the reaction mixture to 50 μl of 10 mM diethyletheraminepentacetic acid in 40 mM HCl and 10% trichloroacetic acid. A fluorescent derivative of γ-GC was generated using o-phthalaldehyde (26), and the derivative was separated from GSH and quantified using HPLC. The reverse-phase HPLC utilized isocratic elution from a Nova-Pak (4 μm, 60 A, 3.9 × 300 mm) column (Waters Corp., Milford, MA) with a mobile phase consisting of 7.5% methanol, 92.5% 150 mM ammonium acetate, pH 7.0. Fluorescence was determined at 385 nm excitation and 430 nm emission against standards of known concentration. Under the conditions utilized, the assay was linear with protein concentration and with time for at least 60 min.

Preparation of Mouse Fetal Fibroblasts (MFFs)—MFFs were prepared from gestational day 14.5 fetuses (29) derived from the Gclm(+/−) × Gclm(+/−) intercross. The cells from individual fetuses were cultured until the Gclm genotype was determined by PCR (as above) following which the cells from three or four fetuses were pooled for subsequent analyses.

Cell Treatments—For toxicity measurements, MFF cells were seeded in 24-well plates (4 × 10⁴ cells/well) 14 h before treatment. Cells were then administered by direct addition into the culture medium, 100-fold concentrated stocks of the indicated compound in phosphate-buffered saline. The cultures were then incubated for an additional 8 h before the toxicity measurements; longer incubations did not lead to greater toxicity.

Cytotoxicity Assay—H₂O₂-induced cell death was determined by both the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega, Madison, WI) and neutral red assay (Sigma) according to the manufacturers’ protocols.

Statistical Analysis—All data are expressed as the means ± S.E. Group means were compared by one-way analysis of variance using the Systat software (Windows version 8.0). p values < 0.05 were considered statistically significant. When the overall test of significance led to rejection of the null hypothesis, a multiple comparison was performed to determine the source of the effect.

RESULTS

Generation of Gclm(−/−) Mice—We constructed a targeting vector in which the NEO minigene cassette disrupts Gclm exon 1 and removes the 3′ splice donor site of intron 1 (Fig. 1A). Transcripts initiated at either of the two previously identified transcriptional start clusters of the Gclm gene (24) would be expected to encode only the N-terminal 61 amino acids of GCLM and terminate in the out-of-phase NEO gene, producing, if any, a nonfunctional protein. Of the 270 (agouti, 129/SvJ-derived) embryonic stem cell clones resistant to both G418 and ganciclovir, four homologous recombinant clones were identified (data not shown); these clones were used for injecting into the (non-agouti C57BL/6J) blastocyst to generate chimeric mice. Such mice were mated with C57BL/6J female mice, and agouti animals were tested for germ line transmission of the targeted Gclm(−/−) allele, which would result in Gclm(+/+/-) heterozygous mice. Gclm(+/+) mice were then intercrossed, and the resultant offspring were genotyped by Southern blot (Fig. 1B) and PCR analysis (Fig. 1C). Because the probe used was outside the targeting construct, Southern blots confirmed Gclm targeting as opposed to random integration of the targeting construct.

Of 126 mice genotyped, 28 (23%) were Gclm(+/+), 63 (50%) were Gclm(+/−), and 35 (27%) were Gclm(−/−). These numbers approximate the expected Mendelian percentages of 25:50:25 and indicate that no embryonic lethality occurred as the result of the Gclm targeting.

Fig. 2, A and B, confirmed that we could detect neither GCLM mRNA nor GCLM protein, respectively, in the Gclm(−/−) homozygote. Tissues surveyed included liver, kidney, pancreas, lung, heart, and spleen (liver and kidney shown). Taken together these data indicate that the targeted Gclm(−/−) allele, as experimentally planned, is a null allele.

Phenotype of the Gclm(−/−) Mouse—Gclm(−/−) mice are viable and appear overtly healthy up to 6 months of age. Moreover, we have crossed Gclm(−/−) mice and found both sexes to be fertile. Female Gclm(+/+), Gclm(+/−), and Gclm(−/−) mice show no statistical differences in body weights from 2 to 11 weeks of age; male Gclm(+/+) mice weigh ~10% less than Gclm(+/−) or Gclm(+/+) littersmates, however, beginning at week 5 (Fig. 3). A histological survey performed at 5 weeks of age revealed no evidence for abnormalities, however, in tissues of knockout mice, including liver, kidney, lung, pancreas, and spleen (data not shown). The reason for the sex-linked weight difference has not yet been investigated further.

GSH and Cysteine Levels—GCLM is known to bind to GCLC and change the catalytic characteristics of GCLC in vitro. In the context of cellular glutamate and GSH concentrations, GCLC alone (in a Gclm(−/−) mouse) is predicted to function poorly in synthesizing γ-GC. Would GCLC alone lead to lowered levels of GSH? We therefore measured the intracellular...
levels of GSH in several tissues (Table I). In liver, kidney, pancreas, red cells, and plasma, GSH levels were dramatically decreased in Gclm−/− mice (9–16% of that in wild-type animals) and significantly diminished in Gclm+/− heterozygotes (43–82% of that in wild-type animals). Alterations in GSSG levels in Gclm−/− mice followed a trend similar to that of GSH (data not shown); consequently, there were no significant differences in the ratio of GSSG/GSH + GSSG among the three genotypes. Lower GSH synthesis, presumably by liver, resulted in a dramatic decrease of GSH in plasma, and compared with that in Gclm+/+ mice, plasma GSH levels in Gclm−/− and Gclm+/− mice dropped to 16 and 48%, respectively, of that in Gclm+/+ wild-type mice.

Circulating GSH is believed to replenish the intracellular cysteine pools via the membrane-bound enzymes γ-glutamyltranspeptidase and dipeptidase, which are especially abundant in kidney and pancreas (30). Correspondingly, we found that cysteine levels in the kidney, pancreas, and plasma of Gclm−/− mice were markedly decreased, whereas no significant changes in cysteine levels were seen in liver or erythrocytes (Table I).

**FIG. 1. Targeted disruption of the mouse Gclm gene.** A, targeting scheme for Gclm disruption. Although the Gclm gene has seven exons spanning 22.3 kb (24), only exon 1 (closed box) is illustrated here. NEO, neomycin-resistance minicassette, and HSV-TK, herpes simplex virus thymidine kinase minicassette, represent genes used as selection markers. The arrow pointing left above NEO represents the 5′ to 3′ orientation of that minigene. B, Southern blot analysis of genomic DNA from offspring of the three Gclm genotypes; the DNA was digested with BglII and hybridized with the probe shown in A. C, PCR genotype analysis of the three Gclm genotypes.

**FIG. 2. Expression of GCLC and GCLM in liver and kidney.** Northern (A) and Western (B) analyses for GCLM and GCLC mRNA and protein, respectively, from liver and kidney of offspring of the three Gclm genotypes.
The apparent $K_m$ for l-cysteine does not differ significantly (Table II). Except for the large difference between the rat and both mouse and human with regard to a change in the $K_m$ for glutamate, the kinetic constants are similar between species. A striking difference between the GCL holoenzyme and GCLC is the dramatic inhibition of the latter by GSH (15, 16, 31). Using partially purified mouse GCL holoenzyme versus GCLC alone, we obtained a similar result. GCLC was more sensitive than the GCL holoenzyme to GSH inhibition (Fig. 5). Taken together these data suggest that GSH deficiency in the $\text{Gclm}(−/−)$ mouse is at least in part due to differences in catalytic characteristics between the GCL holoenzyme and the GCLC subunit.

**Sensitivity of $\text{Gclm}(−/−)$ Cells to Oxidant Stress**—To obtain further insight into the cellular function of Gclm, we studied cultured primary MFFs derived from $\text{Gclm}(+/+)$, $\text{Gclm}(+/−)$, and $\text{Gclm}(−/−)$ mice. GCL activity (measured with saturating glutamate and no GSH), GCLC, and cysteine levels did not differ significantly in MFFs of any genotype (data not shown).

GSH levels in $\text{Gclm}(−/−)$ cells, however, were about 20% of that in $\text{Gclm}(+/+)$. The differences are strikingly more sensitive (10-fold) than $\text{Gclm}(+/+)$. GCLC was more sensitive than the GCL holoenzyme to cell killing by H$_2$O$_2$. In an effort to normalize toxicity between $\text{Gclm}(+/+)$ and $\text{Gclm}(−/−)$ MFFs, GSH was depleted in the former using phorone (32, 33) and replenished in the latter using the cell-permeable GSH-EE (1, 23). Preliminary dose-response experiments first defined the concentrations of phorone and GSH-EE needed to normalize within 5% the GSH concentrations between $\text{Gclm}(+/+)$. MFFs. Depletion of GSH by phorone enhanced the sensitivity of $\text{Gclm}(+/+)$. MFFs treated with phorone contained GSH levels similar to that in $\text{Gclm}(−/−)$ MFFs, and the $\text{Gclm}(−/−)$ MFFs treated with GSH-EE contained GSH levels similar to that in $\text{Gclm}(+/+)$. MFFs. Depletion of GSH by phorone enhanced the sensitivity of $\text{Gclm}(+/+)$. MFFs treated with GSH-EE (1, 23). Preliminary dose-response experiments first defined the concentrations of phorone and GSH-EE needed to normalize within 5% the GSH concentrations between $\text{Gclm}(−/−)$ and $\text{Gclm}(+/+)$. MFFs (data not shown).

Thus, at the time MFFs were exposed to H$_2$O$_2$, the $\text{Gclm}(+/+)$ MFFs treated with phorone contained GSH levels similar to that in $\text{Gclm}(+/+)$. MFFs, and the $\text{Gclm}(−/−)$ MFFs treated with GSH-EE contained GSH levels similar to that in $\text{Gclm}(+/+)$. MFFs. Depletion of GSH by phorone enhanced the sensitivity of $\text{Gclm}(+/+)$. MFFs treated with GSH-EE (1, 23). Preliminary dose-response experiments first defined the concentrations of phorone and GSH-EE needed to normalize within 5% the GSH concentrations between $\text{Gclm}(−/−)$ and $\text{Gclm}(+/+)$. MFFs (data not shown).

To test the possibility that cells without GCLM might be sensitive to oxidant stress, we measured MFF viability following exposure to the cellular oxidant H$_2$O$_2$ (Fig. 6A). This dose-response analysis demonstrated that $\text{Gclm}(−/−)$ cells are strikingly more sensitive than $\text{Gclm}(+/+)$. The $\text{Gclm}(−/−)$ and $\text{Gclm}(+/+)$. MFFs. Depletion of GSH by phorone enhanced the sensitivity of $\text{Gclm}(+/+)$. MFFs treated with GSH-EE (Fig. 6B); however, these cells were still significantly more resistant than $\text{Gclm}(−/−)$ cells. On the other hand, increases of intracellular GSH level by GSH-EE offered only moderate protection against H$_2$O$_2$ for the $\text{Gclm}(−/−)$ cells.

**DISCUSSION**

In this study, we have described the generation and initial characterization of the $\text{Gclm}(−/−)$ mouse line. $\text{Gclm}(−/−)$ mice are viable and fertile and show no overt phenotype compared with their $\text{Gclm}(+/+)$. On the other hand, increases of intracellular GSH level by GSH-EE offered only moderate protection against H$_2$O$_2$ for the $\text{Gclm}(−/−)$ cells.

![Graph showing body weights of mice of the three $\text{Gclm}$ genotypes as a function of age. The data shown are the mean ± S.E. for 10–25 mice at each age.](image)

The major, if not sole, dimerization partner for GCLC.

Measurement of GCL specific activity from the hepatic cytosol of $\text{Gclm}(+/+)$ and $\text{Gclm}(−/−)$ mice (1.4 ± 0.2 and 2.0 ± 0.3 nmol/h/mg of protein, respectively) revealed a modest increase in the knockout mouse. This result is not surprising because we used saturating glutamate concentrations (15 mM) and no GSH in our assay system. Thus, the measured activity actually reflects the amount of GCLC present in the hepatic cytosol, which is somewhat elevated in $\text{Gclm}(−/−)$ mice (Fig. 2B).

Following gel filtration, partially purified fractions containing only the GCL holoenzyme or GCLC alone were isolated (Fig. 4), and the catalytic characteristics of these fractions were tested to investigate the role of GCLM in mouse GCL holoenzyme function. These analyses were performed on GCL holoenzyme partially purified as shown in Fig. 4 or on GCLC prepared by three different methods: 1) GCLC from a $\text{Gclm}(−/−)$ hepatic cytosol without purification, 2) GCLC from a $\text{Gclm}(+/+)$ hepatic cytosol chromatographically separated from GCLM (Fig. 4), and 3) partially purified GCLC from a $\text{Gclm}(−/−)$ hepatic cytosol (Fig. 4). All preparations provided similar results. Shown are data from GCLC prepared by method 3. Under all conditions for kinetic analysis the production of γ-GC was linear for at least 1 h, and data were collected for these analyses 30 min following the start of enzymatic reactions. Shown for comparison are results from two previous studies that examined the $K_m$ for glutamate and cysteine for the recombinant rat and human GCL holoenzyme and GCLC alone (Table II). The apparent $K_m$ for l-glutamate is increased for GCLC compared with that for the GCL holoenzyme, whereas the apparent $K_m$ for l-cysteine does not differ significantly (Table II). Exceptions for the large difference between the rat and both mouse and human with regard to a change in the $K_m$ for glutamate, the kinetic constants are similar between species. A striking difference between the GCL holoenzyme and GCLC is the dramatic inhibition of the latter by GSH (15, 16, 31). Using partially purified mouse GCL holoenzyme versus GCLC alone, we obtained a similar result. GCLC was more sensitive than the GCL holoenzyme to GSH inhibition (Fig. 5). Taken together these data suggest that GSH deficiency in the $\text{Gclm}(−/−)$ mouse is at least in part due to differences in catalytic characteristics between the GCL holoenzyme and the GCLC subunit.
**Phenotype of the Gclm(−/−) Knockout Mouse**

GSH and cysteine levels in tissues of mice of the three Gclm genotypes

| Thiol levels | Gclm | Liver | Kidney | Pancreas | Erythrocytes | Plasma |
|--------------|------|-------|--------|----------|--------------|--------|
| GSH          | (+/+)| 6.1 ± 0.1 | 2.2 ± 0.1 | 0.9 ± 0.1 | 67.5 ± 3.7 | 62.1 ± 4.8 |
|              | (+/−)| 4.7 ± 0.1** (77%) | 1.8 ± 0.1** (82%) | 0.7 ± 0.1*** (78%) | 29.0 ± 2.4*** (43%) | 29.7 ± 1.9*** (48%) |
|              | (−/−)| 0.8 ± 0.0** (13%) | 0.3 ± 0.0** (14%) | 0.1 ± 0.0** (11%) | 6.0 ± 0.4** (9%) | 10.0 ± 0.6** (16%) |
| Cysteine     | (+/+)| 0.1 ± 0.0 | 1.3 ± 0.1 | 0.4 ± 0.0 | 2.2 ± 0.5 | 26.7 ± 0.9 |
|              | (+/−)| 0.1 ± 0.0 | 1.6 ± 0.2 | 0.3 ± 0.0 | 2.2 ± 0.2 | 20.0 ± 3.2 |
|              | (−/−)| 0.1 ± 0.0 | 0.1 ± 0.0** (9%) | 0.1 ± 0.0** (35%) | 2.2 ± 0.3 | 10.8 ± 1.3** (40%) |

* a p < 0.01 when comparing (+/+) and (+/−) mice.
* b p < 0.01 when comparing (+/+) and (−/−) mice.

**FIG. 4. Separation of the GCL holoenzyme and GCLM by gel filtration.** Liver cytosol from Gclm(+/+) (A) or Gclm(−/−) (B) mice was subjected to gel filtration chromatography. Fractions were analyzed by Western immunoblot analysis using antibodies that detect either the 72.8-kDa GCLC or the 30.8-kDa GCLM subunit.

**TABLE II**

**Kinetic constants for the GCL holoenzyme or the GCLC alone**
The Km for glutamate was determined at saturating cysteine and several glutamate concentrations (0, 0.2, 0.4, 0.6, 1, 2, 3, 5, 8, 10, and 15 mM). The Km for cysteine was determined at saturating glutamate and several cysteine concentrations (0.02, 0.05, 0.1, 0.15, 0.2, 0.5, 1, 2, and 3 mM). Data represent the mean ± S.E. for three independent determinations.

| Enzyme         | Km for L-Glu (mM) | Km for L-Cys (mM) |
|----------------|------------------|------------------|
| Mouse GCL      | 0.86 ± 0.06      | 0.17             |
| Mouse GCLC     | 1.70 ± 0.10      | 0.14             |
| Rat GCL<sup>a</sup> | 1.4              | 0.2              |
| Rat GCLC<sup>b</sup> | 18.2            | 0.2              |
| Human GCL<sup>b</sup> | 1.9 ± 0.2      | 0.10 ± 0.02     |
| Human GCLM<sup>b</sup> | 3.2 ± 0.1      | 0.13 ± 0.01     |

<sup>a</sup> Values from Huang et al. (15).
<sup>b</sup> Values from Misra and Griffith (16).

**FIG. 5. GSH inhibition of activity derived from the GCL holoenzyme or from GCLC alone.** Partially purified GCL holoenzyme or GCLC was assayed for γ-GC synthesis in the presence of saturating glutamate and cysteine and the indicated concentrations of GSH. Assays were conducted in triplicate, and data are expressed as the percentage of the control activity in the absence of GSH (means ± S.E.). In some instances, the circle denoting the mean is larger than the brackets denoting the S.E.

TABLE I

GSH and cysteine levels in tissues of liver, kidney, and pancreas; nmol/mmol of hemoglobin in erythrocytes; and μM in plasma. Values are means ± S.E. of three or four mice. Numbers in parentheses are percentage of the wild type.

| GSH | Liver | Kidney | Pancreas | Erythrocytes | Plasma |
|-----|-------|--------|----------|--------------|--------|
| (+/+) | 6.1 ± 0.1 | 2.2 ± 0.1 | 0.9 ± 0.1 | 67.5 ± 3.7 | 62.1 ± 4.8 |
| (+/−) | 4.7 ± 0.1** (77%) | 1.8 ± 0.1** (82%) | 0.7 ± 0.1*** (78%) | 29.0 ± 2.4*** (43%) | 29.7 ± 1.9*** (48%) |
| (−/−) | 0.8 ± 0.0** (13%) | 0.3 ± 0.0** (14%) | 0.1 ± 0.0** (11%) | 6.0 ± 0.4** (9%) | 10.0 ± 0.6** (16%) |

**TABLE II**

Kinetic constants for the GCL holoenzyme or the GCLC alone

The Km for glutamate was determined at saturating cysteine and several glutamate concentrations (0, 0.2, 0.4, 0.6, 1, 2, 3, 5, 8, 10, and 15 mM). The Km for cysteine was determined at saturating glutamate and several cysteine concentrations (0.02, 0.05, 0.1, 0.15, 0.2, 0.5, 1, 2, and 3 mM). Data represent the mean ± S.E. for three independent determinations.

| Enzyme | Km for L-Glu (mM) | Km for L-Cys (mM) |
|--------|------------------|------------------|
| Mouse GCL | 0.86 ± 0.06 | 0.17             |
| Mouse GCLC | 1.70 ± 0.10 | 0.14             |
| Rat GCL<sup>a</sup> | 1.4  | 0.2              |
| Rat GCLC<sup>b</sup> | 18.2 | 0.2              |
| Human GCL<sup>b</sup> | 1.9 ± 0.2 | 0.10 ± 0.02     |
| Human GCLM<sup>b</sup> | 3.2 ± 0.1 | 0.13 ± 0.01     |

<sup>a</sup> Values from Huang et al. (15).
<sup>b</sup> Values from Misra and Griffith (16).

dremely low in cells, one cannot dismiss the possibility that γ-GC serves some critical cellular function(s) aside from GSH biosynthesis.

Various studies suggest that intracellular GCLC and GCLM do not exist in equimolar amounts (21, 34). The lowered GSH levels in tissues from Gclm(+/−) mice further support this premise. One must be cautious with the interpretation of these data, however, because diminished GSH concentrations may also be attributed to lower levels of the GCL substrate, cysteine. This is very likely to be the case for kidney and pancreas where cysteine levels are well below normal; yet, this is unlikely for liver and erythrocytes. Further, the chromatographic separation of GCL in Gclm(+/+) hepatic cytosol shows an excess of GCLC (Fig. 4) and little evidence for free GCLM. On the other hand, free GCLM was detected when Gclm(+/+) MFFs were chromatographed, and this may explain why the heterozygous Gclm(+/−) MFFs do not exhibit intermediate toxicity when challenged with H2O2 (Fig. 6A).

Support for the idea that GCLM may be limiting can also be gleaned from the literature. For example, in HIV-1 Tat transgenic mice, a 59% decrease of GCLM leads to lowered hepatic GSH levels (35). In addition, a mutation in the 5′-flanking region of the human GCLM gene results in a diminished reporter gene response to oxidant insult and is associated with susceptibility to ischemic heart disease (36). Since GCLC and GCLM are regulated at the transcriptional level by oxidative stress and regulation of the two proteins might not be coordi-
of the indicated genotype were pretreated for 1 h with phorone (0.6 mM) and then treated for 8 h with H2O2 (0.5 mM). Data are expressed as percentage of untreated control MFFs and represent the means ± S.E. of three independent experiments. B, cells of the indicated genotype were pretreated for 1 h with phorone (0.6 mM) or GSH-EE (2 mM) as indicated, washed with complete medium, and then treated for 8 h with H2O2 (0.5 mM). Data are expressed as percentage of the cells surviving compared with that of untreated controls and represent the means ± S.E. of three independent experiments.

DMSO, Me2SO.

nated, it is intriguing to speculate that some cell types may respond by up-regulating GCLM and others by up-regulating GCLC in contexts where their respective dimerization partner is in excess. Such regulation remains to be rigorously demonstrated.

The present study clearly shows that GCLM is not essential for viability but that loss of GCLM severely lowers GSH and renders cells susceptible to oxidative stress. Is lower GSH, per se, a principle reason for sensitivity to oxidants? Several studies have shown that the resistance to drug and/or radiation treatment of tumor cells does not necessarily correlate with cellular GSH levels but rather with GCL activity (33, 37). Consistent with this notion, decreasing GSH in Gclm(+/-) MFFs or elevating GSH in Gclm(-/-) MFFs is only modestly effective in normalizing the phenotypes of these cells (Fig. 6B). This finding may suggest that recovery from oxidative insult requires a robust GSH synthetic potential and that the GCL holoenzyme is necessary for this capacity. Thus, a major function of GCLM may be to improve GSH synthetic capacity as a defense against oxidative insult. Such a role may be predictable based on kinetic properties of the GCL holoenzyme versus GCLC provided by this and previous reports (15, 16).

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