Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase

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Abstract

Glutathione (GSH) is a tripeptide composed of glutamate, cysteine, and glycine. The first and rate-limiting step in GSH synthesis is catalyzed by glutamate cysteine ligase (GCL, previously known as γ-glutamylcysteine synthetase). GCL is a heterodimeric protein composed of catalytic (GCLC) and modifier (GCLM) subunits that are expressed from different genes. GCLC catalyzes a unique γ-carboxyl linkage from glutamate to cysteine and requires ATP and Mg^{++} as cofactors in this reaction. GCLM increases the V_{max} and K_{cat} of GCLC, decreases the K_m for glutamate and ATP, and increases the K_i for GSH-mediated feedback inhibition of GCL. While post-translational modifications of GCLC (e.g. phosphorylation, myristoylation, caspase-mediated cleavage) have modest effects on GCL activity, oxidative stress dramatically affects GCL holoenzyme formation and activity. Pyridine nucleotides can also modulate GCL activity in some species. Variability in GCL expression is associated with several disease phenotypes and transgenic mouse and rat models promise to be highly useful for investigating the relationships between GCL activity, GSH synthesis, and disease in humans.

Keywords

Glutamate cysteine ligase; GCL; GCLC; GCLM; Glutathione; GSH; Post-translational

1. Introduction

Glutathione (GSH) is an endogenously synthesized tripeptide thiol (γ-glutamylcysteinylglycine) with important biochemical and antioxidant properties. GSH is part of numerous basic cellular processes including protein synthesis, DNA synthesis and repair, cell proliferation, and redox signaling (Meister, 1983; Wu et al., 2004; Townsend, 2007). GSH is utilized in phase-II metabolism of many drugs and xenobiotics via glutathione *S*-transferase (GST)-mediated GSH conjugation reactions (Hayes and Pulford, 1995). As an antioxidant, GSH scavenges ROS, RNS, and other free radicals produced in association with electron transport, xenobiotic metabolism, and inflammatory responses (Rahman et al., 2004; Haddad

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and Harb, 2005; Rahman et al., 2005). GSH appears to be especially important in protecting mitochondria from xenobiotic- and ROS-induced toxicity (Reed, 2004; Fernandez-Checa and Kaplowitz, 2005). Importantly, although most cells have the capacity for *de novo* GSH biosynthesis, GSH is transported from the liver into the blood for use by other organs in the body, and into the bile for use by the gastrointestinal system (Griffith and Meister, 1979a; Meister, 1983; Martensson et al., 1990).

Cellular GSH homeostasis is regulated by the rate of GSH synthesis, utilization, and export from the cell (Griffith, 1999; Griffith and Mulcahy, 1999). The GSH biosynthetic capacity of various cells and tissues throughout the body is also controlled by multiple factors, including substrate availability (especially cysteine) and the activity of glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis (Griffith, 1999; Griffith and Mulcahy, 1999). GCL carries out the first of two ATP-dependent steps in GSH synthesis, forming γ-glutamylcysteine (γ-GC) from glutamate and cysteine (Fig. 1). The second step is catalyzed by glutathione synthetase (GSS), which ligates glycine to γ-GC, thus forming GSH.

Because GCL is a major determinant of cellular GSH levels, many laboratories have investigated the factors that regulate GCL expression and activity (for a review, see Lu, 2008, this issue). In many lower organisms the GCL enzyme is a single polypeptide, but most eukaryotic GCL enzymes are heterodimeric complexes consisting of two distinct gene products. The catalytic subunit (GCLC) is the larger of the two subunits (637 amino acids, approximately 73 kDa) and contains the active site responsible for the ATP-dependent bond formation between the amino group of cysteine and the γ-carboxyl group of glutamate. The modifier subunit (GCLM) is smaller (274 amino acids, approximately 31 kDa) and through direct interaction with GCLC acts to increase the catalytic efficiency of GCLC. GCLM lowers the K_m for glutamate and ATP, and increases the K_i for GSH feedback inhibition (Meister, 1983; Griffith, 1999; Yang et al., 2007). GSH competitively inhibits GCL in a non-allosteric fashion by competing with glutamate at the active site of GCLC. Interestingly, under some circumstances alternative forms of GCL can be observed that migrate at a higher molecular weight than a GCLC/GCLM heterodimer upon SDS–PAGE. Furthermore, gel filtration analysis of *Drosophila* GCL suggests a putative heterotrimeric structure (Fraser et al., 2002). Whether these more highly complexed forms of GCL have unique biochemical properties is not known.

Some interesting differences in GCL composition and function exist among non-mammalian organisms. For instance, while there is apparently no GCLM counterpart in plants, GCL can still dimerize (i.e. two GCLC subunits) in a redox-dependent manner, which dramatically affects enzymatic activity (Hicks et al., 2007). Yeast also have a second related enzyme (YdbK) that can carry out this first step in GSH synthesis, albeit with much lower efficiency (approximately 500-fold) than yeast GCL (Lehmann et al., 2004). Finally, in some bacteria, GCL and GS activities are present in a single polypeptide, allowing for the complete synthesis of GSH to be carried out by a single gene product (GCL-GS) (Janowiak and Griffith, 2005; Janowiak et al., 2006; Vergauwen et al., 2006; Kino et al., 2007).

2. GCLC and GCLC gene structure and polymorphisms

GCLC and GCLM are expressed from separate genes on distinct chromosomes (6p12, and 1 p22.1 in humans; and 9 D–E and 3 H1–3 in mice, for *GCLC* and *GCLM*, respectively) (Sierra-Rivera et al., 1995; Tsuchiya et al., 1995; Walsh et al., 1996). In humans, the *GCLC* gene is composed of 16 exons and spans approximately 48 kb of DNA sequence, whereas *GCLM* is composed of 7 exons stretching over 22 kb. The coding regions of these genes are highly conserved among eukaryotes, showing only small differences in amino acid sequence among mammalian species (e.g. the human GCLC amino acid sequence is 97.6% similar to the mouse

GCLC sequence; and the human GCLM amino acid sequence is 98.2% similar to the mouse GCLM sequence).

Trans-acting regulatory transcription factors that are known to interact with the promoters of these genes include members of the Nrf2 family of transcription factors, and AP1, AP3, NFκB, Maf family proteins, JunD, Fra, CREB, and others (for a review of this literature see Lu, 2008, this issue).

3. GCL subunit protein structures

Limited data exist on the three-dimensional structure of mammalian GCL. However, the crystal structures for bacterial (*Escherichia coli*) and plant (*Brassica juncea*) GCL have been solved (Hibi et al., 2004; Hothorn et al., 2006). Such studies have revealed similarities between the active site of bacterial GCL and that of glutamine synthase, which has a similar catalytic mechanism and cofactor requirements (ATP and Mg^{++}). The similarity in active sites between GCL and glutamine synthase underlies the observation that L-methionine sulfoximine, a potent inhibitor of glutamine synthase also inhibited rat GCL (Richman et al., 1973). This ultimately led to the discovery by Griffith and Meister that L-buthionine-(SR)-sulfoximine (BSO) is a highly selective mechanism-based inhibitor of GCL (Griffith and Meister, 1979b). BSO is widely used to investigate the roles of GSH and its synthesis in many biological processes.

3.1. GCLC protein structure

In eukaryotes, the GCLC amino acid sequence appears to be rather unique to this class of proteins, with little homology to other proteins. However, as with the bacterial GCL enzyme, there are apparent similarities between the active site of trypanosome GCLC and that of glutamine synthase (Abbott et al., 2001).

Using an intensive search of structures present in the protein database, Hamilton and colleagues used computer-based modeling to predict the structure of human GCLC and its catalytically active site (Hamilton et al., 2003). This permitted a structure-based explanation for the markedly attenuated activity of a mutant form of the human GCL enzyme. A subsequent *in silico* screen of the NCI compound library identified 10 novel inhibitors of GCL, some of which were capable of depleting cellular GSH levels and sensitizing cells to the chemotherapeutic agent melphalan (Hamilton et al., 2007). Using computer modeling software and the yeast YbdK protein as a reference structure, Mañú Pereira et al. have also proposed a three-dimensional structure for human GCLC (Mañú Pereira et al., 2007). In a similar approach, using the *B. juncea* GCLC crystal structure as a reference, Pieper and colleagues have also proposed two structures for human GCLC (Pieper et al., 2007a).

3.2. GCLM protein structure

Mammalian GCLM appears to possess a highly conserved domain homologous to aldo-keto reductases (AKR) (Soltanin-assab et al., 2000). This is interesting because NADPH is a cofactor for AKRs and Toroser et al. have shown a putative role for NADPH in regulating the activity of *Drosophila* and mouse GCL (see Section 4.3) (Toroser et al., 2006). Pieper et al. have also proposed several structures for human GCLM using aldo-keto reductases as reference structures (Pieper et al., 2007b). It remains to be determined whether any of the proposed structures for human GCLC or GCLM are indeed correct.

4. Post-translational regulation of GCL activity

4.1. Oxidative stress and redox-dependent changes in GCL activity

The relative levels of the GCL subunits are a major determinant of cellular GCL activity and are highly regulated at the transcriptional and post-transcriptional level in response to oxidative stress (Griffith, 1999; Griffith and Mulcahy, 1999; Rahman and MacNee, 2000; Wild and Mulcahy, 2000). The GCL subunits are often coordinately induced in response to oxidative stress, but distinct transcriptional and post-transcriptional mechanisms mediate their differential rates and levels of induction (Yao et al., 1995; Cai et al., 1997; Galloway et al., 1997; Tian et al., 1997; Liu et al., 1998). While these transcriptional events invariably lead to increased protein translation, only recently have direct and functional quantification of the GCL subunit protein levels demonstrated that GCLM is limiting for GCL holoenzyme formation in most cell types and tissues (Krzywanski et al., 2004; Chen et al., 2005; Lee et al., 2006). Thus, while GCLC levels are clearly important in dictating cellular GCL activity, increased expression of GCLM alone is likely an effective mechanism for enhancing cellular GCL activity (Tipnis et al., 1999; Neurohr et al., 2003; Lee et al., 2006; Dasgupta et al., 2007). GCL activity can also be rapidly regulated by post-translational modification of pre-existing GCLC and/or GCLM protein (see Fig. 2 for a summary of the major post-translational modifications and their effects). In this regard, oxidative stress has been shown to stimulate GCL activity prior to, or in the absence of, an increase in GCL subunit protein expression. Sub-toxic concentrations of hydrogen peroxide (H_2O_2), menadione, phorone, or other oxidative agents, lead to the transient stimulation of GCL activity without detectable increases in GCL subunit protein levels (Ochi, 1995, 1996; Krejsa C.M., et al., unpublished observations). The rapid time-course of GCL activation (10 min – 1 h) and the inability of *de novo* protein synthesis inhibition to prevent menadione- or H_2O_2-induced GCL activity also precludes the involvement of increased GCL subunit protein expression in these responses (Ochi, 1995, 1996). Thus, in addition to transcriptional regulation of GCL subunit expression, oxidative stress appears to enhance cellular GCL activity via direct post-translational modification of one or both GCL subunits (Fig. 2, #5).

Reducing agents reversibly dissociate a large portion of GCL holoenzyme, highlighting the importance of intermolecular disulfide bonds in GCL holoenzyme formation (Seelig et al., 1984; Tu and Anders, 1998; Fraser et al., 2002, 2003). This finding has given rise to the concept that cellular GCL activity may be regulated through a redox switch mechanism whereby oxidizing conditions stimulate GCL activity by enhancing GCL holoenzyme formation, while reducing conditions inhibit GCL activity via dissociation of the GCL subunits (Fig. 2, #2) (Huang et al., 1993). Studies employing sulfhydryl modifying agents and mutational analysis of human GCLC (Tu and Anders, 1998) and *Drosophila melanogaster* GCLM (Fraser et al., 2003) demonstrate that cysteine residues in both GCL subunits play a role in regulating GCL holoenzyme formation and activity. The cysteine modifier cystamine inactivates GCL from most species presumably via interaction with a critical cysteine residue within the GCL active site (Seelig and Meister, 1982; Huang et al., 1988; Lueder and Phillips, 1996; Fraser et al., 2002; Jez et al., 2004). However, *E. coli* GCL is insensitive to cystamine (Huang et al., 1988) and mutation of an invariant cysteine within *Trypanasoma brucei* (Cys[319]), while preventing cystamine inactivation, had no effect on GCL activity or enzyme kinetics (Brekken and Phillips, 1998). In contrast, the cysteine modifier *N*-ethylmaleimide abolishes the enzymatic activity of both monomeric murine GCLC and GCL holoenzyme (Backos et al., unpublished observation).

Mutational analysis has begun to identify the specific cysteine residues mediating these responses. While mutation of several conserved cysteine residues within human GCLC decreased GCLC enzymatic activity, only mutation of Cys[553] significantly reduced GCL holoenzyme activity (Tu and Anders, 1998). However, mutation of Cys[553] did not prevent

GCL holoenzyme formation, suggesting the existence of additional intersubunit covalent interactions (Tu and Anders, 1998). Three cysteine residues within *Drosophila* GCLM (Cys[213], Cys[214], Cys[267]), two of which are conserved within human GCLM (Cys[193], Cys[194]), have also been determined to be important for intersubunit disulfide bond formation (Fraser et al., 2003). Mutation of these residues inhibited intersubunit disulfide bond formation under non-reducing conditions, but did not prevent subunit association. In fact, this mutant GCLM was still capable of enhancing GCLC activity, albeit at a reduced level, and exhibited enhanced sensitivity to GSH feedback inhibition.

GSH feedback inhibition of GCL (Fig. 2, #4) is competitive with glutamate and the reduction of an intersubunit disulfide bond is thought to result in a conformational change that affects accessibility to the substrate binding site (Huang et al., 1993;Fraser et al., 2002,2003). However, GSH does not induce subunit dissociation even at high concentrations (5–10 mM) (Tu and Anders, 1998;Yang et al., 2007). Furthermore, while GCL holoenzyme is largely dissociated in the presence of 50 mM dithiothreitol, 10–25% of the enzyme is resistant to dissociation (Seelig et al., 1984;Tu and Anders, 1998). A fraction of GCL holoenzyme formed in response to cysteine deprivation is also resistant to reduction and heat denaturation (Lee et al., 2006). In aggregate, these findings suggest that multiple covalent and non-covalent intermolecular interactions are important in the formation, stability, and activity of the GCL holoenzyme complex.

In contrast to mammalian GCL, plant GCL activity is regulated by a unique post-translational redox switch mechanism involving the reversible oxidation/reduction of specific intramolecular sulfhydryl bonds (Hothorn et al., 2006). While mammalian GCL is a heterodimeric holoenzyme, plant GCL is composed of a single gene product that is regulated and functions as a monomeric protein, though it exists as a homodimer (Jez et al., 2004; Hothorn et al., 2006). The crystal structure of *B. juncea* GCL and mass spectrometry data from *Arabidopsis thaliana* GCL revealed two redox-sensitive intramolecular disulfide bonds (CC1 and CC2) located at the homodimer interface that regulate plant GCL activity (Hicks et al., 2007; Gromes et al., 2008). Reduction of CC1 causes a conformational change that shields the active site and impairs substrate binding, while reduction of CC2 causes a reversible dimer to monomer transition that nearly abolishes GCL activity. In contrast to mammalian GCL (Ochi, 1995, 1996), H_2O_2- and menadione-induced *Arabidopsis* GCL activity is associated with the oxidation of GCL and increased homodimer formation (Hicks et al., 2007). While determination of the crystal structures of monomeric GCL from *B. juncea* (Hothorn et al., 2006) and *E. coli* (Hibi et al., 2004) have greatly enhanced our knowledge of the molecular mechanisms regulating GCL activity, the lack of sequence homology and structural similarity with mammalian GCL subunits limits the application of these findings to higher species. Elucidation of the tertiary/quaternary structure of mammalian GCL will improve our understanding of this crucial enzyme.

4.2. Phosphorylation

γ-Glutamylcysteine synthesis involves the GCL-mediated phosphorylation of L-glutamate creating the activated enzyme-bound γ-glutamylphosphate intermediate (Orlowski and Meister, 1971). GCL also mediates the phosphorylation of BSO, which is required for its tight and irreversible binding to the active site of GCL (Griffith and Meister, 1979b). In addition to this substrate/inhibitor kinase activity, purified rat kidney GCL holoenzyme is capable of undergoing autophosphorylation (Sekhar and Freeman, 1999). This phosphorylation is specific for the GCLC subunit as no phosphorylation of the GCLM subunit was detected. GCLC autophosphorylation decreased the V_{max} of GCL activity by 50%, but had no effect on the K_m for glutamate (Fig. 2, #4). While the phosphorylation site(s) and target residue(s) (Ser, Thr, or Tyr) were not identified, the ability of GCL substrates (L-glutamate and L-α-aminobutyrate)

to competitively inhibit autophosphorylation suggests that phosphorylation occurs within or near the enzyme active site. The physiological relevance and potential regulation of GCLC autophosphorylation remain to be determined.

The first indication that phosphorylation may play an important role in regulating GCL activity *in vivo* was the ability of various hormones and agents that activate protein kinase C (PKC), cAMP-dependent protein kinase (PKA), or Ca^{2+}-calmodulin-dependent protein kinase II (CMKII) to inhibit GCL activity in cultured rat hepatocytes (Lu et al., 1991). GCLC was subsequently found to be inducibly phosphorylated in response to dibutyryl cAMP or phenylephrine in this cultured cell system (Sun et al., 1996). *In vitro* studies confirmed that PKC, PKA, and CMKII were capable of directly phosphorylating GCLC and inhibiting GCL activity (Fig. 2, #4). Phosphoamino acid analysis revealed that phosphorylation of GCLC occurred on serine and threonine residues *in vitro* and the phosphorylation site(s) were likely identical for all three kinases (Sun et al., 1996). Similar to GCLC autophosphorylation, kinase-mediated phosphorylation of rat kidney GCLC decreased the V_{max} of GCL activity, but had no effect on the K_m for cysteine or glutamate. GCLC phosphorylation did not cause dissociation of the GCL holoenzyme into its monomeric subunits, suggesting a molecular mechanism involving a conformational change that suppresses specific activity, but does not affect holoenzyme formation or substrate affinity. While the degree of phosphorylation correlated with the extent of GCL inhibition, only a modest reduction in activity was observed in rat hepatocytes. However, cell type- and species-specific effects may account for this, as *in vitro* manipulation of conditions purported to affect GCLC phosphorylation/dephosphorylation had a much greater effect on GCL activity in extracts from *Drosophila* and mouse liver, brain, and heart (Toroser et al., 2006). In these studies, preincubation of cell or tissue extracts in the presence of MgATP (phosphorylating conditions) nearly abolished GCL activity, while dephosphorylating conditions (absence of ATP and phosphatase inhibitors) resulted in the "spontaneous activation" of GCL. While the phosphorylation status of GCLC was not assessed, these findings suggest that GCL activity may be constitutively suppressed by GCLC phosphorylation in some species and tissues. This would be consistent with the basal level of GCLC phosphorylation detected in cultured rat hepatocytes and may explain the minimal effects of subsequent kinase-mediated phosphorylation on rat kidney GCL activity *in vitro* (Sun et al., 1996). Further work is required to identify and functionally characterize the relevant protein kinases and/or phosphatases that reversibly regulate GCL activity *in vivo*

4.3. Pyridine dinucleotide phosphates (NAD(P)H)

A screen of various small molecular weight compounds related to redox signaling revealed that nicotinamide adenine dinucleotide phosphates ($NADP^+$ and NADPH) are capable of regulating *Drosophila* GCL activity *in vitro* (Toroser et al., 2006). Direct addition of reduced NADPH to a GCL assay mixture containing *Drosophila* extract increased GCL activity ~two-fold, while oxidized $NADP^+$ suppressed GCL activity (Fig. 2, #5). There was specificity amongst nicotinamide adenine dinucleotides as NAD^+ and NADH had no effect on GCL activity. The stimulatory effects of NADPH were due to an increase in V_{max} with no detectable change in substrate affinity. While there are no clear NADPH-binding sites in GCLC, a BLAST search using the human or mouse GCLM sequences revealed some homology to several members of the aldo-keto reductase (AKR) family (Soltaninassab et al., 2000). AKRs contain a highly conserved NAD(P)H-binding pocket (Jez and Penning, 2001), which is partially conserved in GCLM. If $NADP^+$ and NADPH directly affect GCL activity, it seems likely that these effects are mediated via interaction with the GCLM subunit. To test this hypothesis, we included NADPH in a GCL assay mixture containing recombinant murine GCLC or GCL holoenzyme purified as reported previously (Krzywanski et al., 2004). In contrast to the stimulatory effects observed in *Drosophila* extracts (Toroser et al., 2006), NADPH did not increase monomeric GCLC or GCL holoenzyme activity and even suppressed GCL activity at

higher concentrations (Fig. 3). While the functional effects of $NADP^+$ were not examined, these findings suggest that either the effects of NADPH are species-specific or NADPH-induced activation of GCL in *Drosophila* extracts is due to indirect effects on a factor(s) that influences GCL activity (Toroser et al., 2006). Should NADPH prove to be an authentic activator of GCL, this would provide an additional NADPH-dependent means of regulating cellular levels of reduced GSH in addition to that mediated by the GSH reductase salvage pathway (Griffith, 1999).

4.4. Caspase-mediated cleavage of GCLC

Intracellular GSH levels are rapidly depleted in many models of apoptotic cell death and this is often accompanied by the efflux of reduced GSH and the post-translational cleavage of GCLC protein (Fig. 2, #6) (Ghibelli et al., 1995;Coppola and Ghibelli, 2000;Franklin et al., 2002). Both GCLC cleavage and GSH depletion are caspase-dependent (z-VAD-fmk-inhibitable), yet they are functionally and mechanistically distinct as GSH depletion/efflux can occur in the absence of GCLC cleavage in caspase-3-deficient cells (Franklin et al., 2002). While members of both the multidrug resistance protein (ABCC/MRP) and organic anion transporter protein (SLCO/OATP) families have been implicated in the efflux of reduced GSH during apoptotic cell death (van den Dobbelsteen et al., 1996;Oda et al., 1999;Ballatori et al., 2005;Franco and Cidlowski, 2006), the functional effects of GCLC cleavage are not clear. It is has been postulated that GCLC cleavage inhibits GCL activity and prevents the repletion of depleted cellular GSH pools during apoptosis, but little direct evidence supports this contention. Caspase-dependent GCLC cleavage has been reported in response to multiple apoptotic stimuli, including chemotherapeutics (Siitonen et al., 1999;Franklin et al., 2002) death receptor agonists TNF-α and anti-Fas antibody (Franklin et al., 2002), TGFβ1 (Franklin et al., 2003), UV irradiation (Zhu and Bowden, 2004;Franklin et al., 2002, p. 45), and arsenicals (Sumi et al., 2007, and our unpublished results). GCLC cleavage generates N- and C-terminal fragments of 60 and 13 kDa, respectively (Fig. 2, #6) (Franklin et al., 2002). The GCLC cleavage site (Asp^{499}) is located N-terminal to a cysteine residue (Cys^{553}) reportedly involved in disulfide bond formation with GCLM (Tu and Anders, 1998) and removal of this interaction domain would likely suppress GCL enzymatic activity either directly or by affecting GCL holoenzyme formation (Griffith, 1999;Griffith and Mulcahy, 1999). However, GCLC cleavage did not result in a significant reduction in GCL activity during apoptotic cell death (Siitonen et al., 1999;Zhu and Bowden, 2004).

Interestingly, *in vitro* studies utilizing purified recombinant full-length and caspase-cleaved GCLC indicate that cleavage does not result in the dissociation of the small 13 kDa fragment containing the putative GCLM-binding domain from the larger 60 kDa cleavage fragment (unpublished results) (Fig. 2, #6). This likely provides the molecular basis by which cleaved GCLC retains full enzymatic activity since cleavage of GCLC does not affect its ability to associate with GCLM and form fully functional GCL holoenzyme (Fig. 4). In this regard, GCLC cleavage has no effect on the V_{max} or the K_m for glutamate, cysteine or ATP, of either monomeric GCLC or GCL holoenzyme (unpublished results). The failure to detect any clear effect of GCLC cleavage on GCL enzyme kinetics suggests that any functional effects of this post-translational modification are likely to be subtle and/or complex in nature.

4.5. Myristoylation and regulation of GCL subunit subcellular localization

Caspase-mediated subcellular redistribution can also alter protein function during apoptosis. A classic example of this is the translocation of the caspase-8-cleaved 15 kDa truncated BID (tBID) fragment to the outer mitochondrial membrane leading to mitochondrial dysfunction and the release of cytochrome C (Li et al., 1998; Luo et al., 1998). Mitochondrial targeting of tBID results from the post-translational *N*-myristoylation of the tBID fragment (Zha et al., 2000). While co-translational *N*-myristoylation occurs at penultimate N-terminal glycine

residues after removal of the initiator methionine, post-translational myristoylation can occur at internal glycine residues exposed by caspase-mediated cleavage (de Jonge et al., 2000). Caspase-mediated cleavage of GCLC occurs at AVVD^{499}G creating a 13 kDa C-terminal fragment with an N-terminal glycine residue (Franklin et al., 2002). Analysis of the C-terminal sequences downstream of the cleavage site utilizing The MYR Predictor indicates that this exposed N-terminal glycine residue is a "reliable" myristoylation site (Eisenhaber et al., 2003). To date, 48 caspase targets have been identified that contain a glycine residue immediately C-terminal to the cleavage site and 9 are predicted to be candidates for myristoylation, including GCLC (Fig. 2, #7) (Fischer et al., 2003; Martin et al., 2008). To examine GCLC post-translational myristoylation, Martin et al. developed a novel nonradioactive approach that is six orders of magnitude more sensitive than radioactive labeling techniques (Martin et al., 2008). By employing this methodology, an EGFP fusion protein containing the first 10 amino acids downstream of the GCLC cleavage site (AVVD^{499}GCGLAQNSTE) was found to be myristoylated in transiently transfected COS7 cells. Furthermore, this myristoylation was sufficient to confer partial nuclear exclusion of the GCLC–EGFP chimera and association with intracellular membranes as judged by confocal microscopy. These findings imply that myristoylation of caspase-cleaved GCLC may result in its subcellular redistribution during apoptosis (Fig. 2, #6 and #7). However, only the first ten amino acids downstream of the GCLC cleavage site were utilized in this study and it is not known whether cleavage of *bona fide* full-length endogenous GCLC undergoes post-translational myristoylation during apoptosis. Furthermore, the small 13 kDa and large 60 kDa GCLC fragments remain associated after caspase-mediated cleavage (see above and Fig. 2, #6) and it is not clear whether the N-terminus of the small C-terminal fragment would be accessible for post-translational myristoylation. This approach also measures co-translational myristoylation of the GCLC–EGFP chimera as a surrogate marker for post-translational myristoylation and this may not accurately reflect myristoylation of cleaved GCLC protein in the cytosol of apoptotic cells. While myristoylation alone is sufficient for targeting to intracellular membranes, additional lipid modification or a vicinal polybasic domain is required for localization to the plasma membrane (McCabe and Berthiaume, 1999). The lack of basic amino acids downstream of the GCLC caspase cleavage site suggests that GCLC is either not redistributed to the plasma membrane or undergoes an additional covalent lipid modification. While it remains to be determined how GCLC cleavage might affect GCL activity, the ability of caspase cleavage to target other caspase substrates for post-translational myristoylation and altered subcellular redistribution (Zha et al., 2000; Utsumi et al., 2003; Vilas et al., 2006) makes this an intriguing and previously unrecognized mechanism for regulating GCL activity during apoptotic cell death.

While GCLC and GCLM are generally considered to be cytosolic proteins there is evidence that they may exhibit altered subcellular localization in certain circumstances (Fig. 2, #7). *In vivo* immunohistochemical analysis indicates that hepatocyte-specific deletion of GCLC leads to the nuclear accumulation of GCLM (Chen et al., 2007). This is consistent with *in silico* analysis of the GCL subunits using PSORT II, which predicts a nuclear localization for GCLM and a cytoplasmic localization for GCLC. Deletion of GCLC ultimately leads to punctate cytoplasmic GCLM staining (Chen et al., 2007), suggesting either the altered compartmentalization and/or aggregation of GCLM in the absence of GCLC for extended periods of time (post-natal day 21/28). It cannot be delineated from this study whether GCLC *per se* dictates the subcellular localization of GCLM or the subsequent change in cellular redox homeostasis resulting from deletion of GCLC mediates these responses, although there is *in vitro* evidence supporting both contentions. We and others have found that GCLM is quite sensitive to aggregation *in vitro* in the absence of GCLC (Fraser et al., 2003; Yang et al., 2007; unpublished observations). Recombinant monomeric GCLM also undergoes a dose-dependent, H_2O_2-induced multimerization *in vitro* (Yang et al., 2007). However, it should be noted that all cultured cell lines and tissues examined to date (except kidney) contain a molar

excess of GCLC and only under extreme conditions of GCLC down-regulation would GCLM exist as a monomer *in vivo* (Krzywanski et al., 2004;Chen et al., 2005;Lee et al., 2006). Additional research will reveal how the subcellular distribution and biophysical properties of the GCL subunits are regulated in response to various cellular conditions (i.e. apoptosis, GSH depletion, and oxidative stress).

4.6. Regulation of GCL subunit protein stability

Caspase-dependent apoptosis is usually associated with the cleavage of GCLC to a stable 60 kDa fragment (Siitonen et al., 1999; Franklin et al., 2002; Botta et al., 2004; Zhu and Bowden, 2004). However, TGFβ1- and diphenylarsinic acid (DPAsV)-induced apoptosis in murine hepatocytes is associated with the cleavage and loss of GCLC protein expression and GCL activity (Franklin et al., 2003; Sumi et al., 2007). TGFβ1 also suppresses *Gclc* gene transcription (Arsalane et al., 1997; De Bleser et al., 1999; Jardine et al., 2002; Franklin et al., 2003; Bakin et al., 2005; Hosler et al., 2006; Fu et al., 2008), although it is unlikely that transcriptional repression alone accounts for the observed decrease in GCLC protein and GCL activity as the GCL subunits are extremely stable proteins in unstimulated conditions. In fact, little or no change in steady-state GCLC and/or GCLM protein expression is observed upon inhibition of *de novo* protein synthesis with cycloheximide for up to 72 h (Franklin et al., 2002, 2003; Sumi et al., 2007). Given the stability of GCLC, it is intriguing to speculate that cleavage targets GCLC for ubiquitination and proteasomal degradation during TGFβ1- and DPAsV-induced apoptosis (Fig. 2, #8). Cleavage-dependent degradation has been reported for the caspase targets Bid (Breitschopf et al., 2000) and the atypical protein kinase C ζ(Smith et al., 2000), whereby caspase-mediated cleavage is necessary and sufficient for their degradation by the ubiquitin–proteasome system. However, while proteasome inhibitors have been shown to affect *Gclc* gene expression (Sekhar et al., 2000; Hosler et al., 2006), there is no evidence that GCLC protein undergoes ubiquitination and/or proteasome-mediated degradation. The GCLC protein also contains a putative PEST sequence between residues 210–229, however, it is not clear whether this sequence is functional as there was no degradation of GCLC detected within 6 h in an *in vitro* cell-free system (Sekhar et al., 1997; Soltaninassab et al., 2000).

Several rare GCLC missense mutations have also been identified that affect GCL activity and/ or protein stability leading to low erythrocyte GSH levels and hemolytic anemia (Fig. 2, #8) (Beutler et al.,1990,1999;Ristoff et al., 2000;Hamilton et al., 2003). In one case, a patient homozygous for an A-T transversion at nucleotide 1109, which produced a His370Leu substitution, expressed extremely low erythrocyte GCLC protein levels presumably due to the instability of the mutant GCLC protein (Beutler et al., 1999). GCLM levels were also compromised in this patient, suggesting that GCLC may be required for GCLM stabilization which is reminiscent of the altered biophysical characteristics of GCLM upon targeted deletion of GCLC (Chen et al., 2007).

5. GCLC and GCLM polymorphisms and disease susceptibility in humans

There exists variability among humans with respect to induction of GCL after exposure to GSH-depleting drugs (O'Dwyer et al., 1996). These differences in GCL activity and expression among individual humans are not likely to be due to common polymorphisms that affect the amino acid sequence of either subunit, although rare mutations do exist that are associated with disease (Beutler et al., 1990, 1999; Ristoff et al., 2000; Hamilton et al., 2003; Mañú Pereira et al., 2007). In those cases where such changes have resulted in significantly diminished GCL expression and activity, the disease phenotype can be quite severe, including hemolytic anemia, aminoaciduria and spinocerebellar degeneration (Ristoff et al., 2000; Mañú Pereira et al., 2007, and references therein). Walsh and colleagues have shown that a common trinucleotide repeat (GAG) polymorphism in the 5′-untranslated region (5′-UTR) of the human *GCLC* mRNA is functionally important, with the number of repeats affecting GSH levels and drug

sensitivities in multiple human tumor cell lines (Walsh et al., 2001). We have found that the number of GAG repeats in *GCLC* is directly correlated with lung function (FEV1) in patients with cystic fibrosis (CF) having weak CF mutations (McKone et al., 2006). We have also demonstrated that *GCLC* and *GCLM* polymorphisms are associated with chronic beryllium disease pathogenesis (Bekris et al., 2006). In addition, a number of studies indicate that polymorphisms in both *GCLC* and *GCLM* gene promoters influence the risk of cardiovascular disease (Nakamura et al., 2002, 2003; Koide et al., 2003; Campolo et al., 2007); however see (Muehlhause et al., 2007). These studies report lower plasma GSH levels or impaired GCL induction by oxidants, as well as compromised vasodilation in response to acetylcholine administration in patients having *GCLM* -588 C/T or *GCLC* -129 C/T or T/T genotypes. Other diseases in which polymorphisms in the promoters of these genes are likely to play a role in disease risk include schizophrenia (Tosic et al., 2006; Gysin et al., 2007), stroke (Baum et al., 2007), drug abuse (Hashimoto et al., 2008), carbon disulfide metabolism (Jönsson et al., 2007), mercury burden (Custodio et al., 2005), diabetes mellitus (Bekris et al., 2007), and asthma (Polonikov et al., 2007). In several of these latter studies, the effects of these polymorphisms on *GCLM* and *GCLC* mRNA expression, GCL activity and/or GSH synthesis have been investigated in various cell types and tissues from study patients, including cultured fibroblasts (Tosic et al., 2006; Gysin et al., 2007), blood monocytes (Nakamura et al, 2002), plasma and blood (Nakamura et al, 2002, 2003; Nichenametla et al, 2008). Although there is some variability depending on the cell and tissue types examined, the *GCLC* 7 GAG repeat allele is generally associated with higher GCLC expression and GSH levels than the 8 and 9 GAG repeat alleles, and the *GCLM* -588T and *GCLC* -129T alleles are associated with lower expression or inducibility and GSH levels. Interestingly, the *GCLC* C-129T polymorphism is apparently in linkage disequilibrium with the *GCLC* GAG repeat polymorphism (Bekris et al., 2007). In light of the functional significance of these polymorphisms, it is clear that transgenic animals having variable GCL subunit expression are needed to model GCL expression in humans and to predict what effects such variability might have on disease susceptibility.

6. Transgenic and knock-out models with altered GCL subunit expression

We and others have developed animal models of GCL over expression and insufficiency to begin to address the role of GSH synthesis in xenobiotic toxicity and in various diseases characterized by oxidative stress (Dalton et al., 2000; Shi et al., 2000; Yang et al., 2002; Dalton et al., 2004; Botta et al., 2006; Chen et al., 2007; McConnachie et al., 2007). We have developed *Gclc* and *Gclm* transgenic mice designed to conditionally over express GCL in the liver. We recently showed that conditional *Gcl* transgene expression in these mice promotes resistance to acetaminophen (APAP)-induced liver injury (Botta et al., 2006). While *Gclc* null mice do not survive beyond embryonic day 9, heterozygotes express only 50% of normal GCLC protein levels and appear to have a normal phenotype (Dalton et al., 2000; Shi et al., 2000). Using Cre recombinase technology, Dalton and co-workers developed transgenic mice having a liver specific, albumin promoter directed and Cre mediated deletion of the *Gclc* gene (Chen et al., 2007). These mice survive for approximately one month postnatally after the *Gclc* gene is deleted and die of extensive liver failure secondary to hepatic steatosis, necrosis and inflammation. In an interesting and related series of studies, Akai et al. found that transgenic rats carrying an inducible short hair-pin RNA directed against *Gclc* showed decreased GSH levels and susceptibility to APAP-induced liver injury (Akai et al., 2007).

While deletion of *Gclc* is an embryonic lethal phenotype in mice, deletion of *Gclm* has little effect on survival or development (Yang et al., 2002; McConnachie et al., 2007). As expected *Gclm* null mice do have the ability to synthesize GSH in limited amounts (about 10–20% of normal levels). We have found that *Gclm* null mice are sensitive to APAP-induced liver injury and confirmed that *N*-acetylcysteine mediated protection against APAP is dependent upon its being metabolized to GSH (McConnachie et al., 2007). These various GCL transgenic and

knock-out mice and rats will be highly useful for modeling the role of GSH synthesis in human disease susceptibility.

7. Conclusions

GSH has many critical functions in the cell including maintaining redox status, scavenging free radicals and electrophilic intermediates, conjugation/detoxification reactions, apoptosis, and cell signaling. Because of its important role in regulating the synthesis and intracellular concentration of GSH, GCL has received a great deal of attention by those working in the areas of biochemistry, toxicology, free radical biology, embryonic/fetal development, reproduction, and cancer research. The many factors that regulate GCL expression and activity make it a fascinating enzyme to study. The molecular cloning and crystal structures of GCL from various species have dramatically advanced our understanding of this enzyme. Additional structural information on the mammalian enzymes and the availability of GCL transgenic and knock-out models will substantially advance this field, and the molecular mechanisms by which GCL and its subunits are regulated will continue to be actively investigated in the years to come. While much work has been done to elucidate mechanisms regulating GCL activity, our appreciation of its many roles in human physiology and disease is just beginning.

Acknowledgements

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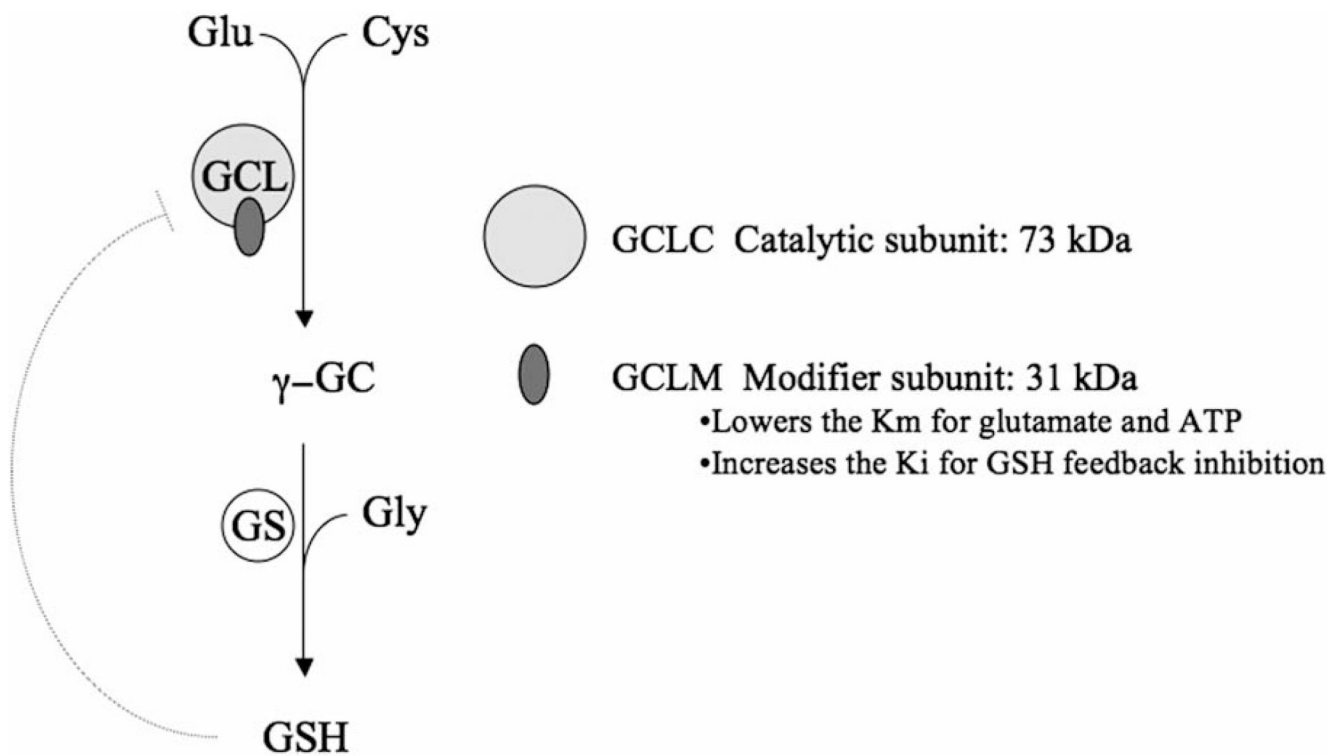
Fig. 1.
Glutathione synthesis. The first step in glutathione (GSH) synthesis is carried out by glutamate cysteine ligase (GCL), which is composed of catalytic (GCLC) and modifier (GCLM) subunits. In this rate-limiting step, glutamate and cysteine are ligated to form γ-glutamylcysteine (γ-GC). The second step is carried out by glutathione synthetase (GS), which ligates glycine to γ-GC. Both steps are ATP dependent.
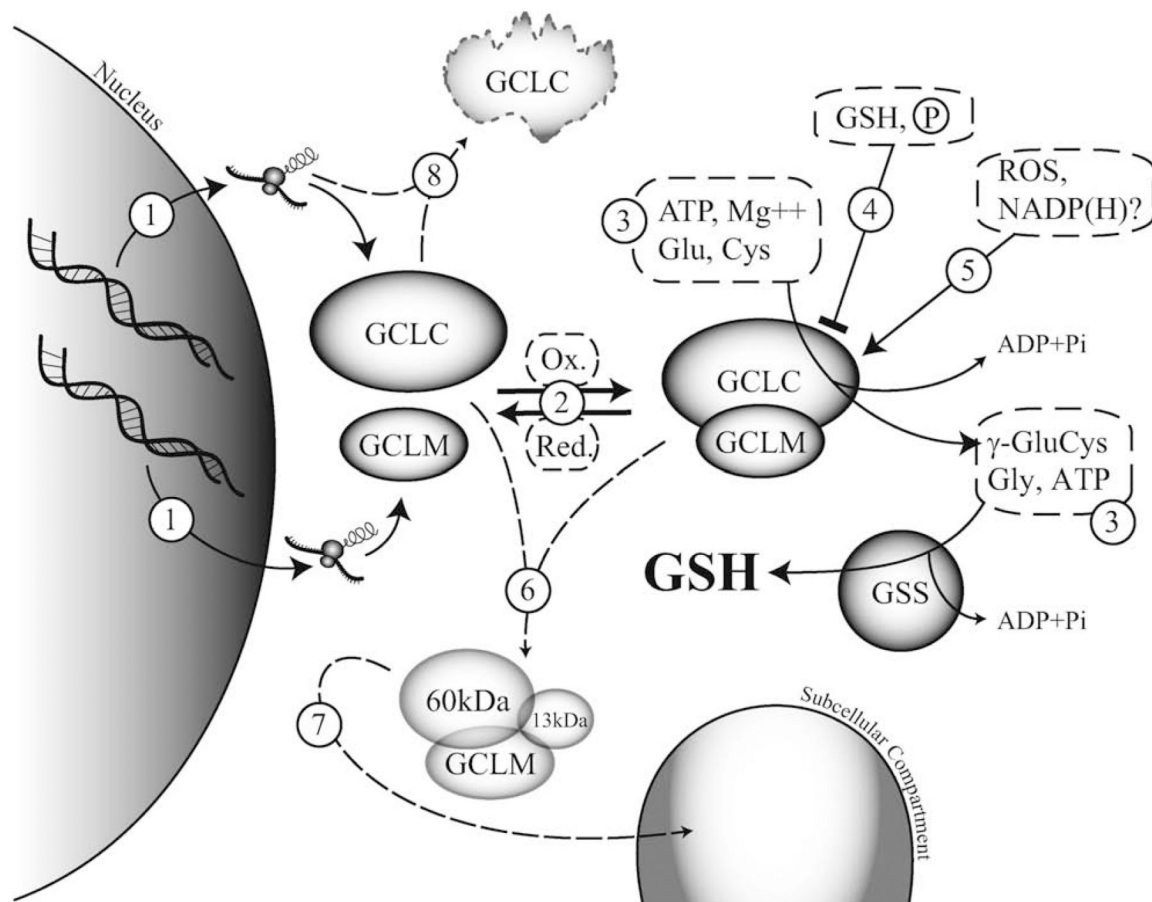
Fig. 2.
Post-translational regulation of glutamate cysteine ligase activity. Glutathione (GSH) is synthesized by a two-step ATP-dependent process. The first and rate-limiting step is the formation of γ-glutamylcysteine (γ-GluCys) by glutamate cysteine ligase (GCL), which is composed of catalytic (GCLC) and modifier (GCLM) subunits. The second step is the ligation of glycine (Gly) to γ-GluCys, which is catalyzed by glutathione synthetase (GSS), to form GSH. Cellular regulation of GCL activity can occur by a number of mechanisms. (1) Transcriptional or post-transcriptional: activation or suppression of gene transcription [Sections 2, 4.1 and 4.6] and/or mRNA stability, and intra- and 5′UTR *GCLC* polymorphisms [Sections 4.1 and 5]. (2) Reversible GCL holoenzyme formation: redox-sensitive [Section 4.1], dependent on molar ratios of GCL subunits (GCLM is usually limiting) [Section 4.1], subunit stability [Section 4.6]. (3) Substrate (Glu, Cys, Gly) and co-factor (ATP, Mg++) availability [Section 1]. (4) Inhibition of GCLC activity: negative feedback regulation by GSH [Sections 1 and 4.1], autophosphorylation and kinase-mediated phosphorylation of GCLC [Section 4.2]. (5) Activation of GCL: oxidative stress [Section 4.1], NADPH (activation or inhibition) [Section 4.3]. (6) Caspase-mediated cleavage of GCLC [Sections 4.4 and 4.6]. (7) Myristoylation of the 13 kDa C-terminal fragment of caspase-cleaved GCLC and/or altered subcellular localization [Section 4.5]. (8) Inducible or mutational regulation of GCLC protein stability [Section 4.6].
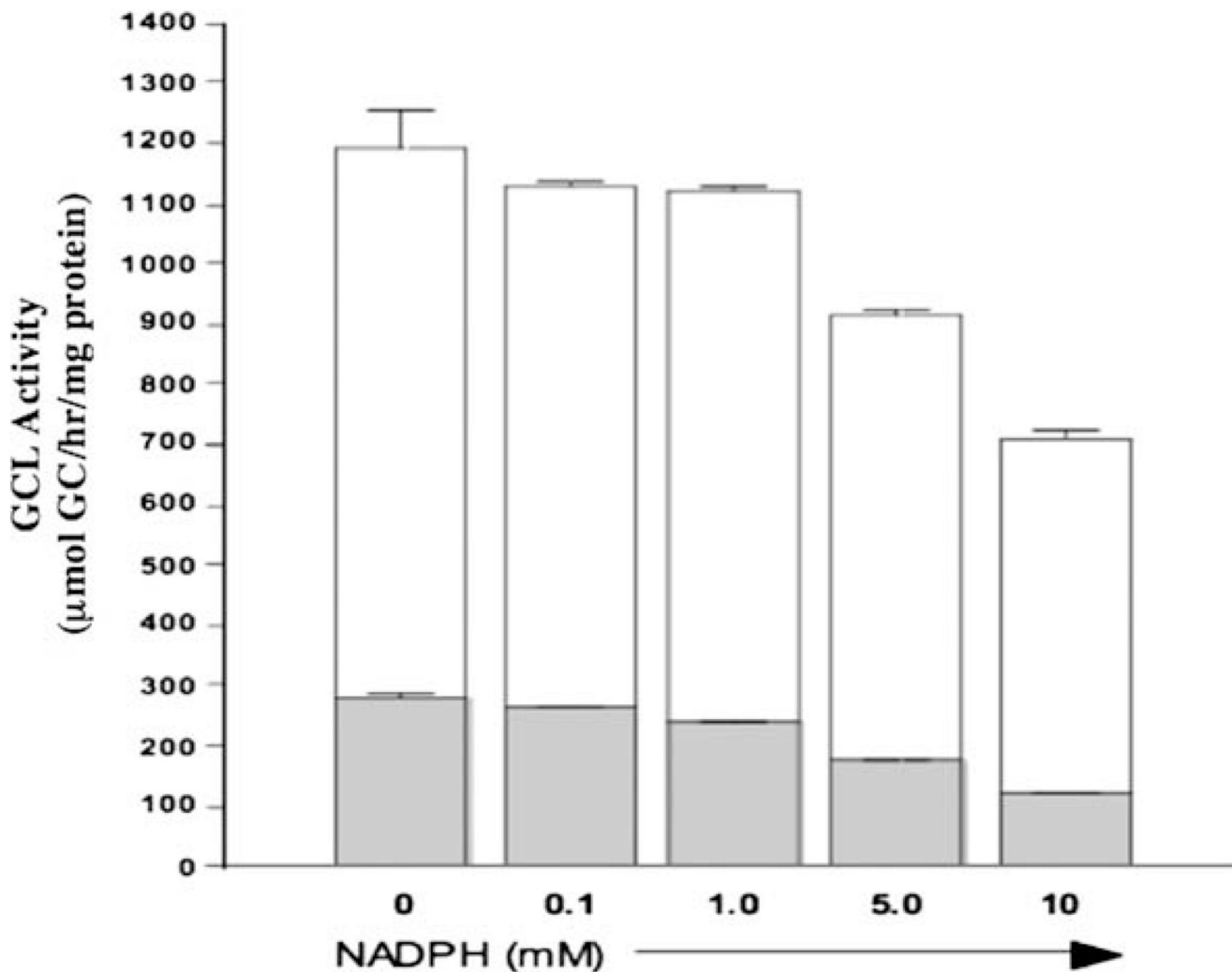
Fig. 3.
NADPH does not directly enhance the activity of recombinant GCLC or GCL holoenzyme *in vitro*. The enzymatic activity of purified recombinant murine GCLC and GCL holoenzyme was assessed in the absence or presence of increasing concentrations of NADPH. Open bars indicate recombinant GCL holoenzyme, and closed bars are the GCLC subunit alone. For GCL holoenzyme, GCLC-His and GCLM-His fusion proteins (Krzywanski et al., 2004) were mixed at a molar ratio of 1:4 and preincubated for 10 min at 37 °C prior to addition of NADPH and initiation of the GCL assay (White et al., 2003).
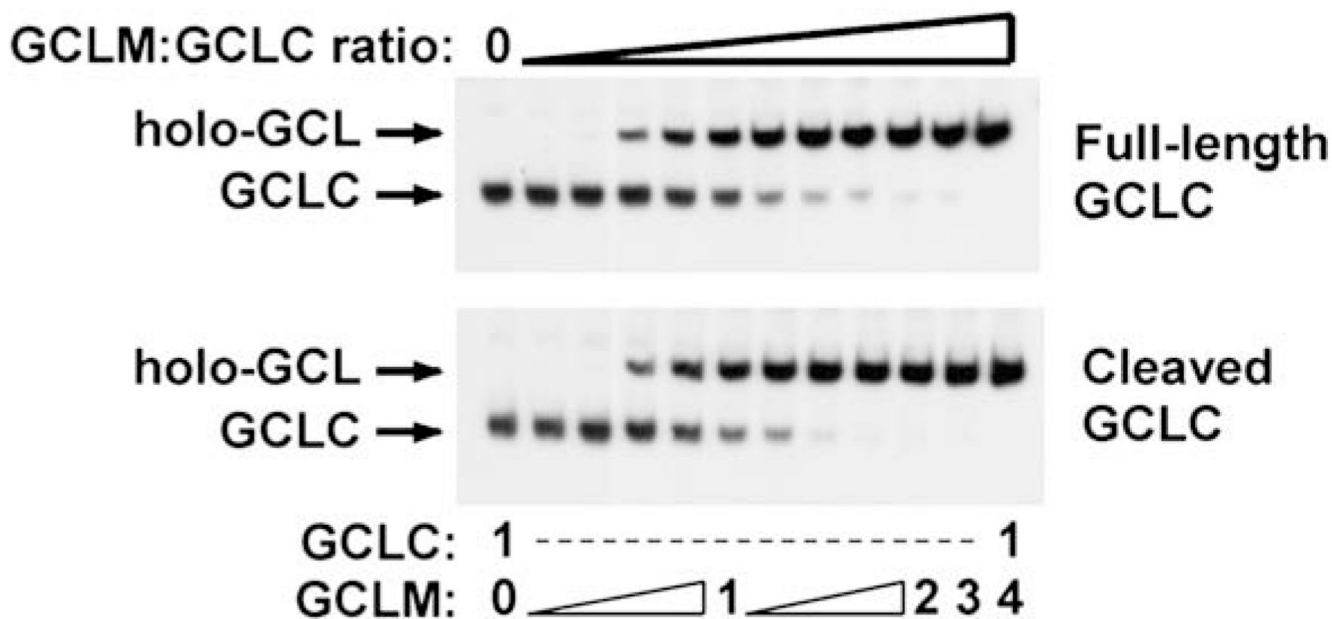
Fig. 4.
Caspase-mediated cleavage of GCLC does not alter GCL holoenzyme formation *in vitro*.
Murine GCLC-His or GCLM-His fusion proteins were synthesized in BL21(DE3) *E. coli* in
the absence or presence of pGEX2T-caspase-3 (Deak et al., 1998) and purified as previously
described (Krzywanski et al., 2004). Purified full-length and caspase-cleaved GCLC-His were
incubated in the absence or presence of increasing molar equivalents of purified recombinant
GCLM-His as indicated and GCL holoenzyme formation was assessed by native gel
electrophoresis and immunoblotting for GCLC.