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Manipulation of cellular GSH biosynthetic capacity via TAT-mediated protein transduction of wild-type or a dominant-negative mutant of glutamate cysteine ligase alters cell sensitivity to oxidant-induced cytotoxicity

Donald S. Backos[a], **Chad N. Brocker**[a], and **Christopher C. Franklin**[a,b]

[a]Department of Pharmaceutical Sciences, Graduate Program in Toxicology, School of Pharmacy, University of Colorado Denver, Aurora, CO 80045

[b]University of Colorado Cancer Center, University of Colorado Denver, Aurora, CO 80045

Abstract

The glutathione (GSH) antioxidant defense system plays a central role in protecting mammalian cells against oxidative injury. Glutamate cysteine ligase (GCL) is the rate-limiting enzyme in GSH biosynthesis and is a heterodimeric holoenzyme composed of a catalytic (GCLC) and a modifier (GCLM) subunit. As a means of assessing the cytoprotective effects of enhanced GSH biosynthetic capacity, we have developed a protein transduction approach whereby recombinant GCL protein can be rapidly and directly transferred into cells when coupled to the HIV TAT protein transduction domain. Bacterial expression vectors encoding TAT fusion proteins of both GCL subunits were generated and recombinant fusion proteins were synthesized and purified to near homogeneity. The TAT-GCL fusion proteins were capable of heterodimerization and formation of functional GCL holoenzyme *in vitro*. Exposure of Hepa-1c1c7 cells to the TAT-GCL fusion proteins resulted in the time- and dose-dependent transduction of both GCL subunits and increased cellular GCL activity and GSH levels. A heterodimerization-competent, enzymatically deficient GCLC-TAT mutant was also generated in an attempt to create a dominant-negative suppressor of GCL. Transduction of cells with a catalytically inactive GCLC(E103A)-TAT mutant decreased cellular GCL activity in a dose-dependent manner. TAT-mediated manipulation of cellular GCL activity was also functionally relevant as transduction with wild-type GCLC(WT)-TAT or mutant GCLC(E103A)-TAT conferred protection or enhanced sensitivity to H_2O_2-induced cell death, respectively. These findings demonstrate that TAT-mediated transduction of wild-type or dominant-inhibitory mutants of the GCL subunits is a viable means of manipulating cellular GCL activity to assess the effects of altered GSH biosynthetic capacity.

Keywords

glutathione; glutamate cysteine ligase; GCLC; GCLM; TAT; protein transduction

To whom correspondence should be addressed: Christopher C. Franklin, University of Colorado Denver, Department of Pharmaceutical Sciences, School of Pharmacy, C238-P15, Research-2, 12700 E. 19th Avenue, Room 3009, Aurora, CO 80045, phone: 303-724-6124, FAX: 303-724-7266, christopher.franklin@ucdenver.edu.

Introduction

Mammalian cells possess a number of antioxidant defense mechanisms to counteract the deleterious effects of oxidative stress. The tripeptide antioxidant glutathione (GSH) is a particularly effective defense mechanism against oxidative injury (Griffith and Mulcahy, 1999). GSH is the most abundant non-protein thiol antioxidant within the cell and plays an important role in combating oxidative stress, preserving protein thiol status, and maintaining cellular redox homeostasis. The antioxidant cytoprotective effects of GSH are derivative of its role as a co-factor for selenium-dependent glutathione peroxidases (GPx, EC 1.11.1.9) that reduce hydrogen peroxide, and lipid and phospholipid peroxides. These reactions lead to the oxidation of GSH and formation of glutathione disulfide (GSSG), which can be readily salvaged via reduction by glutathione reductase (GR, EC 1.8.1.7) (Griffith and Mulcahy, 1999; Hayes and McLellan, 1999). GSH also serves as a co-factor in the detoxification of various xenobiotics via GST-mediated GSH-conjugation reactions and transporter-mediated efflux (Griffith and Mulcahy, 1999; Hayes and McLellan, 1999). GSH homeostasis is dependent on the rate of GSH synthesis, utilization, and export (Griffith, 1999). Most cells do not import significant quantities of GSH, highlighting the importance of *de novo* GSH biosynthesis in maintaining intracellular GSH levels during periods of enhanced GSH utilization.

GSH is synthesized by two sequential ATP-dependent reactions catalyzed by glutamate cysteine ligase (GCL, EC 6.3.2.2) (Sekura and Meister, 1977) and glutathione synthetase (GS, EC 6.3.2.3) (Griffith and Mulcahy, 1999). The first and rate-limiting step in GSH biosynthesis is the GCL-mediated formation of γ-glutamylcysteine (γ-GC). GCL is a heterodimeric holoenzyme consisting of catalytic (GCLC, 73 kDa) and modifier (GCLM, 31 kDa) subunits (Griffith and Mulcahy, 1999). GCLC contributes all the enzymatic activity and contains all the substrate and co-factor binding sites associated with GCL (Seelig *et al.*, 1984; Chen *et al.*, 2005). While GCLM possesses no enzymatic activity *per se*, formation of GCL holoenzyme via heterodimerization with GCLC dramatically increases the Vmax and Kcat of GCLC (Griffith and Mulcahy, 1999). GCLM also lowers the Km for glutamate and ATP and increases the Ki for GSH-mediated feedback inhibition of GCLC (Griffith and Mulcahy, 1999; Franklin *et al.*, 2009). Thus, while GCLM does not retain any catalytic activity, GCL holoenzyme formation is required for optimal GCLC enzymatic activity under physiological concentrations of substrates and inhibitors.

While all mammalian cells are equipped with the cellular machinery for *de novo* GSH biosynthesis, GSH biosynthetic capacity varies significantly between tissues and is mainly influenced by substrate availability and GCL enzymatic activity. Perhaps the most important determinant of cellular GCL activity is the relative levels of the GCL subunits, which are highly regulated at the transcriptional, post-transcriptional, and translational levels (Franklin *et al.*, 2009; Lu, 2009). Many approaches have been employed to overexpress one or both of the GCL subunits in order to assess the role of the GCL subunits and GSH biosynthetic capacity in dictating cellular responses to xenobiotic exposure or oxidative stress. This has classically involved either treatments that induce endogenous protein expression, or ectopic expression via transient or stable transfection of cells with plasmid- or viral-based expression vectors. However, there are difficulties inherent to each of these methodologies. Recently there have been great strides in protein transduction technology whereby recombinant proteins are able to cross the plasma membrane and rapidly enter cells when covalently linked to small peptide domains termed “protein transduction domains” (PTDs) (Wadia and Dowdy, 2003; Wadia and Dowdy, 2005). These transduction domains include cell-penetrating peptide sequences from the HIV Tat protein, the HSV VP22 protein, the Antennapedia protein from *Drosophila*, as well as short synthetic polycationic transduction sequences (Gupta *et al.*, 2005). The advantages of protein transduction over conventional methods of transgene expression (both

viral and non-viral) are numerous, including high efficiency and rapid expression both *in vitro* and *in vivo* and the alleviation of potential toxicity and pro-inflammatory responses *in vivo* (Schwarze and Dowdy, 2000; Wadia and Dowdy, 2005).

Several approaches have also been employed to selectively downregulate one or both GCL subunits to examine their role in regulating both cellular GSH biosynthetic capacity and sensitivity to xenobiotics and/or oxidative stress. Constitutive and/or conditional knockout mice have now been established for both GCL subunits for *in vivo* studies (Dalton *et al.*, 2004; Botta *et al.*, 2008) and successful knockdown of GCL subunit expression has been achieved in cultured cell systems utilizing RNAi and hammerhead ribozyme methodologies (Iida *et al.*, 2001; Nagata *et al.*, 2001; Fojo and Bates, 2003; Huynh *et al.*, 2003; Diaz-Hernandez *et al.*, 2005; Akai *et al.*, 2007; Luchak *et al.*, 2007). While the knockout models have proven extremely useful for *in vivo* studies, they are expensive and limited to certain cell type-specific applications *in vitro*. There are also problems associated with *in vitro* gene knockdown studies in cultured cell systems, including the efficiency of cellular transfection, the extent of transcriptional suppression, and the species-specific nature of the reagents employed. To develop a novel means of selectively inhibiting GSH biosynthesis in a species-independent manner, we have attempted to create a dominant-negative mutant of GCLC. We hypothesized that a dimerization-competent, enzymatically-deficient GCLC mutant might act in a dominant-inhibitory manner by preventing formation of functionally active GCL holoenzyme. Several artificial or naturally occurring point mutations of *GCLC* have been identified that significantly reduce or eliminate GCL activity in various species (Chang, 1996; Beutler *et al.*, 1999; Ristoff *et al.*, 2000; Abbott *et al.*, 2001; Hamilton *et al.*, 2003; Yang *et al.*, 2007). However, it is not known whether these GCLC mutants maintain the ability to heterodimerize with GCLM or function as dominant-inhibitory suppressors of wild-type endogenous GCLC.

In this study, we have developed a novel protein transduction approach to manipulate cellular GSH biosynthetic capacity, whereby recombinant GCL protein can be rapidly and directly transferred into cells when fused to a TAT protein transduction domain. The recombinant TAT-GCL subunit fusion proteins formed functional GCL holoenzyme *in vitro*, were efficiently transduced in cultured Hepa-1c1c7 cells, and enhanced cellular GCL activity. In contrast, a mutant GCLC-TAT fusion protein that was devoid of GCL enzymatic activity functioned in a dominant-inhibitory manner to suppress cellular GCL activity. Importantly, transduction of wild-type or mutant GCLC-TAT fusion protein decreased or enhanced cellular sensitivity to H_2O_2-induced toxicity, respectively. In aggregate, TAT-mediated transduction of wild-type and dominant-negative GCL mutants represents a novel and versatile means of manipulating cellular GCL activity to assess the effects of altered GSH biosynthetic capacity on cellular responses to oxidative stress and its role in human disease.

Materials and Methods

Chemicals and reagents

Except where noted, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO). Sulfosalicylic acid (SSA) and Tris base were obtained from Fisher Scientific (Fair Lawn, NJ). Non-fat powdered milk was obtained from Bio-Rad (Hercules, CA). α-GCLC and α-GCLM antibodies were generously provided by Dr. Terrance Kavanagh, University of Washington.

Production of TAT-GCL fusion proteins

Bacterial expression plasmids were constructed for the synthesis of the TAT-GCL fusion proteins (see Figure 1). An expression vector encoding a GCLM fusion protein containing an

N-terminal 6His-TAT-HA tag (TAT-GCLM) was created by subcloning a full-length murine GCLM PCR product in-frame into the NcoI/XhoI sites of the pTAT-HA vector (Nagahara *et al.*, 1998) (graciously provided by Dr. Steven Dowdy, UCSD). To generate a C-terminally tagged GCLC TAT fusion protein, a double-stranded oligonucleotide insert encoding the HA-TAT sequence (YPYDVPDYARKKRRQRRR) (Invitrogen, Carlsbad, CA) was subcloned in-frame into the HindII/XhoI sites upstream of the 6His sequence in the pET29a(+) vector (Novagen, Madison, WI), yielding pET29a(+)-HA-TAT-6His. The oligonucleotide insert was designed to destroy the original XhoI site and reestablish the original multiple cloning sites of pET29a(+). A full-length murine GCLC PCR product was then subcloned in-frame into the NdeI/XhoI sites upstream of the TAT-containing sequence to yield a GCLC fusion protein containing a C-terminal HA-TAT-6His tag (GCLC-TAT). All constructs were verified by DNA sequencing. BL21(DE3) bacteria (Stratagene, San Diego, CA) expressing these plasmids were grown at 37°C to an OD_{600} of 0.5 and then induced for 6 h with 1.0 mM (GCLC-TAT) or 0.25 mM (TAT-GCLM) isopropyl-β-D-1-thiogalactopyranoside (IPTG). Bacteria expressing GCLC-TAT were lysed using BugBuster® protein extraction reagent (Novagen) and bacteria expressing TAT-GCLM were lysed by sonication in TALON Metal Affinity Resin wash buffer (Clontech, Mountain View, CA). All lysis buffers contained 1× Complete EDTA-free Protease Inhibitor Cocktail (Roche, Indianapolis, IN), 25 U/mL benzonase nuclease (Novagen), and 0.1 uL/mL lysozyme (Novagen). Recombinant proteins were recovered from clarified lysates using TALON Metal Affinity Resin (Clontech). The immobilized fusion proteins were washed extensively and eluted in PBS containing 150 mM imidazole. Protein aliquots were stored with 10% glycerol at −80 °C. Just prior to use, protein aliquots were thawed on ice and the glycerol and imidazole removed using PD-10 protein desalting spin columns (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined by Bradford assay and purity assessed by SDS-PAGE analysis and Coomassie-staining. 6His-only tagged GCL subunit fusion proteins were produced as previously described (Krzywanski *et al.*, 2004).

Cell culture and treatments

Murine Hepa-1c1c7 cells (ATCC, Manassas, VA) were cultured in DMEM/F12 (50/50) media (Mediatech, Herndon, VA) supplemented with 2 mM L-glutamine, 10% FBS, and 1% penicillin/streptomycin. Cells were seeded at a density of 5.5×10^4 cells/cm^2 and cultured for 24 h prior to transduction in fresh media with recombinant protein or vehicle alone (PBS). Cells were 75–80% confluent at the time of transduction. Except where indicated, cells were incubated continuously in the presence of TAT-GCL fusion protein. After the indicated time period, cells were washed three times with ice-cold PBS, collected by scraping on ice with a rubber policeman, centrifuged at 16,000 × g for 15 s, and the cell pellets stored at −80°C until use. For washout experiments, cells were incubated with TAT-GCL fusion protein for 24 h, washed three times with warm media and then placed in fresh media for the time period indicated. For MTS assays, Hepa-1c1c7 cells were seeded in 96-well plates (10,000 cells/well) and cultured for 24 h prior to transduction with the indicated TAT-fusion proteins for 36 h. Cells were then treated in triplicate for 18 h with various concentrations of H_2O_2 and cell viability was measured by MTS assay. Cells were incubated with 20 µl CellTiter 96® AQueous One Solution (1.9 mg/ml MTS) (Promega, Madison, WI) in culture medium at 37°C for 2 h and absorbance was measured at 490 nm on a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA).

Preparation of cell extracts and analysis of GCL activity and GSH levels

Cell pellets were lysed by a brief sonication on ice in TES/SB buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 250 mM sucrose, 20 mM boric acid, and 1 mM L-serine) containing 1× Complete Protease Inhibitor Cocktail (Roche) as previously described (Franklin *et al.*, 2002; Thompson *et al.*, 2009). Lysates were clarified by centrifugation at 16,000 × g at 4°C for 15 min and

soluble protein concentrations determined by Bradford assay. Total cellular glutathione levels (GSH + GSSG) were determined by a modified Tietze assay as described previously (Baker *et al.*, 1990; Thompson *et al.*, 2009). GCL specific activities of recombinant proteins and cell extracts were determined utilizing a plate-reader assay that employs the fluorophore naphthalene dicarboxaldehyde (NDA) which fluoresces upon reaction with GSH or γ-GC (White *et al.*, 2003). Recombinant protein (1 μg) or cell extract (~150 μg) were pre-incubated for 5 min at 37°C in 96-well plates prior to initiation of the GCL activity assay by addition of GCL assay buffer (10 mM L-glutamate, 2 mM L-cysteine, and 5 mM ATP) (White *et al.*, 2003). Assays were allowed to proceed for 10 min (recombinant proteins) or 30 min (cell extracts) and the reaction was halted by the addition of 50 mM SSA and protein precipitated for 20 min on ice. The plates were centrifuged at 1500 rpm for 10 min at 4°C to remove the protein precipitate and an aliquot of the supernatant from each well was derivatized with NDA in the dark for 30 min at room temperature. Fluorescence was measured (472 nm excitation, 528 nm emission) on a SpectraMax Gemini EM plate reader (Molecular Devices, Sunnyvale, CA). Baseline activity (measured in the absence of cysteine) was subtracted and GCL specific activity was calculated against a standard curve of GSH and expressed as the rate of γ-GC formation.

PAGE and immunoblot analysis

Recombinant GCLC-TAT and/or TAT-GCLM fusion protein (~150 ng) or cell extract (20 μg soluble protein) was resolved by PAGE under either non-denaturing, non-reducing (Native), or denaturing, reducing (SDS) conditions. For analysis of purified recombinant fusion proteins, GCLC-TAT was incubated alone or in combination with the indicated molar ratio(s) of TAT-GCLM in TES/SB buffer at 37°C for 15 min. Samples for native analysis were mixed with ice-cold protein loading buffer without 2-mercaptoethanol or boiling, resolved in 8% polyacrylamide gels without stacking gels or SDS at 70 V and 4°C for 3 h, and transferred overnight at 4°C onto Immobilon-P PVDF membranes (Millipore, Billerica, MA). Samples for denaturing SDS-PAGE analysis were mixed with protein loading buffer containing 2-mercaptoethanol, heated at 95°C for 5 min, and resolved in 10% polyacrylamide gels with 5% stacking gels both containing 0.1% SDS at 100 V at room temperature for 90 min, and transferred overnight at 4°C onto Immobilon-P PVDF membranes. Membranes were blocked in TBS-T containing 5% powdered milk for 1 h, probed with the indicated antibodies, and immune complexes detected with Western Lightning Chemoluminescent Reagent (PerkinElmer, Boston, MA).

Densitometry and statistical analysis

All studies were performed a minimum of three times and the error bars in each figure represent the standard error of the mean. Results were considered statistically significant if $p < 0.05$ as determined by one-way ANOVA with Tukey's multiple comparison test using GraphPad Prism 4.0 software. Densitometry was performed using ImageJ software (NIH, Bethesda, MD) and the results expressed as the ratio of GCLC:β-Actin or GCLM:β-Actin, relative to the control.

RESULTS

TAT-mediated protein transduction is an effective means of rapidly increasing cellular levels of proteins both *in vivo* and in a wide variety of cultured cell systems *in vitro* (Schwarze *et al.*, 1999; Wadia and Dowdy, 2005). To determine whether cellular GSH biosynthetic capacity could be manipulated *in vitro* by TAT-mediated protein transduction of the GCL subunits we generated bacterial expression vectors encoding TAT fusion proteins of murine GCLC and GCLM. The TAT-fusion proteins were engineered to contain both a 6His sequence and an HA tag to assist in protein purification and detection, respectively. The GCLC and GCLM fusion proteins employed for these studies contained C- and N-terminal HA-TAT-6His tags,

respectively (Figure 1A). GCLC tagged at its C-terminus was utilized for these studies as preliminary studies indicated that placement of the HA-TAT-6His at the N-terminus of GCLC compromised both monomeric GCLC and GCL holoenzyme activity *in vitro* (data not shown). Similar inhibitory effects have been reported previously for N-terminal tagged recombinant human GCLC protein *in vitro* (Tu and Anders, 1998a). In contrast, the location of the HA-TAT-6His tag on GCLM had no effect on the ability of GCLM to heterodimerize with GCLC and form fully functional GCL holoenzyme (data not shown). The TAT-GCL fusion proteins were synthesized in BL21(DE3) *E. coli* and purified to near homogeneity by metal affinity chromatography (Figure 1B).

We and others have demonstrated that recombinant 6His-tagged GCL subunit fusion proteins from various species are capable of forming functional GCL holoenzyme *in vitro* (Tu and Anders, 1998b; Tu and Anders, 1998a; Fraser *et al.*, 2002; Fraser *et al.*, 2003; Lee *et al.*, 2006; Franklin *et al.*, 2009), data not shown). To determine whether insertion of the highly basic HIV TAT protein transduction domain between the 6His tag and the coding region of the GCL subunit altered GCLC function and/or GCL holoenzyme formation, GCLC-HA-TAT-6His (GCLC-TAT) was incubated with increasing molar equivalents of 6His-TAT-HA-GCLM (TAT-GCLM) and GCL holoenzyme formation assessed by native PAGE and immunoblotting against GCLC. Increasing the TAT-GCLM:GCLC-TAT molar ratio led to a dose-dependent increase in GCL holoenzyme formation with maximal GCL holoenzyme complex detected in the presence of a 1–2 fold molar excess of TAT-GCLM (Figure 2A). Formation of the holoenzyme complex was associated with a concomitant increase in GCL activity, with a 4–5 fold increase in activity observed upon complete heterodimerization of GCLC-TAT in the presence of a 1–2 fold molar excess of TAT-GCLM (Figure 2B). Importantly, GCL holoenzyme formation and activity of the TAT-GCL fusion proteins was comparable to that observed for 6His-only tagged recombinant GCL fusion proteins (GCLC-His and GCLM-His). A similar 2-fold molar excess of GCLM-His was sufficient to quantitatively shift monomeric GCLC-His to GCL holoenzyme (Figure 2C). Furthermore, the enzymatic activities of the monomeric and holoenzyme TAT-GCL fusion proteins were nearly identical to those observed for 6His-only tagged GCL fusion proteins (Figure 2D). In aggregate, these findings demonstrate that the TAT-GCL fusion proteins form functional GCL holoenzyme *in vitro* and exhibit enzyme kinetics similar to those of recombinant GCL fusion proteins lacking the TAT moiety.

To determine whether the TAT-GCL fusion proteins were efficiently transduced into cultured cells, murine Hepa-1c1c7 (Hepa) cells were exposed to various concentrations of GCLC-TAT or TAT-GCLM for 18 h and cellular levels of the GCL fusion proteins assessed by immunoblotting with the corresponding antibody. Transduction of both TAT-GCL subunits was dose-dependent with near maximal uptake occurring at 50 μg/ml for GCLC-TAT and 25 ug/ml for TAT-GCLM (Figure 3A). These concentrations represent an approximately 1:1 molar ratio of GCLC:GCLM and were utilized in all subsequent experiments. Interestingly, exposure to the TAT-GCL fusion proteins resulted in distinct time-dependent kinetics of transduction. Transduction of Hepa cells with GCLC-TAT led to a gradual time-dependent increase in uptake with maximal transduction observed within 16 h (Figure 3B, top panel). In contrast, while uptake of TAT-GCLM did show a degree of time dependency, transduction was extremely rapid with a dramatic increase in TAT-GCLM protein levels observed within 30 min and maximal transduction observed by 16 h (Figure 3B, bottom panel). The cellular levels of both TAT-GCL fusions remained elevated for at least 48 h post-transduction. These findings demonstrate that the TAT-GCL fusion proteins are efficiently transduced in cultured Hepa cells *in vitro*.

While the TAT-GCL fusion proteins were functionally active *in vitro* and readily transduced in Hepa cells, it was important to demonstrate that increased protein levels enhanced cellular

GCL enzymatic activity. To test this hypothesis, Hepa cells were transduced in the absence or presence of TAT-GCLM and/or GCLC-TAT and cell extracts examined for protein levels and GCL activity. As shown previously, transduction of Hepa cells with either subunit individually resulted in significantly elevated levels of the TAT-GCL fusion proteins (Figure 4A). A similar level of uptake of both subunits was also observed upon co-transduction of the TAT-GCL subunits (Figure 4A, lane 4). In contrast, transduction of Hepa cells with GCLC and/or GCLM fusion proteins containing only a 6His tag did not increase cellular protein levels of either subunit (Figure 4B). While transduction of Hepa cells with TAT-GCLM led to a modest increase cellular GCL activity, transduction with GCLC-TAT increased GCL activity >2-fold (Figure 4C). Furthermore, co-transduction with TAT-GCLM and GCLC-TAT increased cellular GCL activity above that observed with GCLC-TAT alone. In these studies, increased GCL activity correlated with increased cellular GSH levels. In this regard, TAT-GCLM alone was sufficient to induce a modest increase in cellular GSH, while transduction with GCLC-TAT alone or in combination with TAT-GCLM increased cellular GSH by 50% and 75%, respectively (Figure 4D). These cellular effects were absolutely dependent on the TAT PTD as GCL fusion proteins containing a 6His tag alone had no effect on cellular GCL activity or GSH levels (Figure 4C & 4D). Transduction of Hepa cells with TAT-GFP fusion protein also had no effect on cellular GCL activity or GSH levels (data not shown).

Exposure of Hepa cells to the individual TAT-GCL subunits led to an apparent steady-state level of fusion protein transduction within 24 h (Figure 3B). However, those studies were performed in the continuous presence of TAT-GCL fusion protein. To determine the persistence of cellular TAT-GCL fusion protein transduction in the absence of extracellular TAT fusion protein, Hepa cells were incubated with GCLC-TAT or TAT-GCLM for 24 h, washed three times with warm media, and then incubated in fresh media lacking TAT fusion protein. While the relative levels of both TAT-GCL subunits decreased over time, GCLC-TAT and TAT-GCLM levels remained elevated (~3-fold over control) for up to 24 h in the absence of extracellular TAT fusion protein (Figure 5). However, little or no cellular TAT-GCL subunit protein was detectable by 48 h.

While TAT-mediated transduction of the GCL subunits enhanced cellular GCL activity in Hepa cells, we were also interested in creating a dominant-inhibitory GCLC protein as a novel means of suppressing cellular GSH biosynthetic capacity. We hypothesized that a heterodimerization-competent, enzymatically-deficient GCLC protein would function in a dominant-inhibitory manner by competing with endogenous wild-type GCLC for heterodimerization with GCLM and resulting in the formation of functionally inactive GCL holoenzyme While several naturally occurring mutations have been identified in human GCLC that suppress GCLC enzymatic activity, none of these mutations abolish GCL activity (Beutler *et al*., 1999; Ristoff *et al.*, 2000; Hamilton *et al.*, 2003). However, mutational analysis of trypanosome GCLC has identified several mutations that result in the complete abrogation of trypanosome GCLC enzymatic activity, including a glutamate to alanine mutation at amino acid position 100 (E100A) (Abbott *et al.*, 2001). The E100 residue of trypanosome GCLC is highly conserved across multiple species and corresponds to E103 in both human and mouse GCLC (Figure 6A) (Abbott *et al.*, 2001). Site-directed mutagenesis was performed to determine whether mutation of this residue abolished murine GCLC activity. The E103A mutation was incorporated into the murine GCLC-TAT fusion protein for *in vitro* analysis and subsequent use in cell transduction studies (Figure 6B). Similar to the wild-type TAT-tagged GCLC, GCLC(WT)-TAT, the mutant GCLC(E103A)-TAT fusion protein was capable of heterodimerization with TAT-GCLM and formation of GCL holoenzyme *in vitro* (Figure 6C). In contrast to wild-type GCLC, the GCLC(E103A)-TAT mutant was devoid of enzymatic activity as either monomeric GCLC or as GCL holoenzyme (Figure 6D). These findings demonstrate that the E103A mutation not only abolished the enzymatic activity of murine GCLC, but also had no effect on the ability of GCLC to heterodimerize with GCLM. This ability to interact with GCLM is

essential for the GCLC(E103A) mutant to competitively inhibit heterodimerization of endogenous GCLC and GCLM and prevent formation of functional GCL holoenzyme.

To determine whether the GCLC(E103A)-TAT mutant functioned as a dominant-inhibitory suppressor of cellular GCL activity, Hepa cells were transduced with various concentrations of either GCLC(WT)-TAT or GCLC(E103A)-TAT for 36 h. As shown in Figure 7A, GCLC (E103A)-TAT was transduced in a dose-dependent manner similar to that observed for GCLC (WT)-TAT. Importantly, while transduction of GCLC(WT)-TAT enhanced cellular GCL activity, the GCLC(E103A)-TAT fusion protein reduced cellular GCL activity to <50% of control at the highest dose examined (Figure 7B). However, while transduction of GCLC(WT)-TAT significantly increased GSH levels, the suppression of GCL activity via transduction of GCLC(E103A)-TAT did not reduce cellular GSH levels *per se* (Figure 7C). The partial inhibition of GCL activity by the GCLC(E103A)-TAT fusion protein likely accounts for this apparent discrepancy, whereby the residual GCL activity is sufficient to maintain cellular GSH homeostasis in the absence of GSH utilization.

Stable changes in GCL subunit expression levels, GCL activity, and GSH homeostasis are known to have profound effects on H_2O_2s-induced cytotoxicity (Yang *et al.*, 2002; Orr *et al.*, 2005; Shi *et al.*, 2007; Cortes-Wanstreet *et al.*, 2009). To determine whether TAT-mediated manipulation of GSH biosynthetic capacity affected Hepa cell sensitivity to oxidative stress, untransduced cells and cells transduced with either GCLC(WT)-TAT or GCLC(E103A)-TAT were treated with increasing concentrations of H_2O_2. While H_2O_2 caused a dose-dependent loss in cell viability in all cases, transduction with GCLC(WT)-TAT decreased sensitivity to H_2O_2-induced toxicity as judged by a shift in the dose curve to the right (Figure 8A, dashed line). In contrast, suppression of cellular GCL activity by transduction of the dominant-negative GCLC(E103A)-TAT mutant enhanced Hepa cell susceptibility to H_2O_2-induced toxicity as judged by a shift in the dose curve to the left (Figure 8A, dotted line). Furthermore, transduction with GCLC(WT)-TAT or GCLC(E103A)-TAT significantly increased or decreased cell viability relative to untransduced control cells, respectively, when cells were treated with H_2O_2 at concentrations near its IC_{50} in untransduced cells (Figure 8B). Importantly, these changes in cellular susceptibility to oxidative stress correlated closely with cellular GSH levels and GSH biosynthetic capacity. In this regard, H_2O_2-induced toxicity in Hepa cells was associated with a dramatic reduction in cellular GSH levels (Figure 8C). Transduction with GCLC(WT)-TAT not only elevated GSH levels in untreated cells, but also significantly attenuated H_2O_2-induced GSH depletion. In contrast, transduction with GCLC(E103A)-TAT, while having no effect on GSH levels in untreated cells, significantly enhanced H_2O_2-induced depletion of cellular GSH levels. In aggregate, these findings validate the use of GCLC(WT)-TAT and/or GCLC(E103A)-TAT fusion proteins as novel and effective means of manipulating cellular GCL activity to assess the effects of altered GSH biosynthetic capacity on cellular sensitivity to oxidative stress.

Discussion

Alterations in cellular antioxidant defenses in general, and GSH biosynthetic capacity in particular, are observed in a variety of diseases associated with chronic oxidative stress (Townsend *et al.*, 2003). Such findings highlight the need for further investigation into the role of altered antioxidant defenses in regulating cellular susceptibility to oxidative stress, which may lead to the rational design of effective therapies for a wide range of human diseases. In this study we describe a new and novel means of manipulating cellular GSH biosynthetic capacity via TAT-mediated protein transduction of purified recombinant GCL protein. The functional validation of TAT-mediated delivery of the GCL subunits together with our creation of a dominant-negative GCLC mutant provide a highly versatile means of rapidly assessing the functional effects of enhanced or suppressed cellular GSH biosynthetic capacity.

Most cells do not import significant quantities of GSH and many approaches have been employed to increase cellular GSH levels, including the use of membrane permeable GSH analogs and supplementation with precursors to GSH biosynthesis such as N-acetylcysteine (NAC). While NAC is routinely used to rapidly elevate intracellular GSH levels both *in vitro* and *in vivo*, this increase is often transient as NAC and GSH stores are susceptible to depletion in the absence of continued *de novo* GSH biosynthesis. The use of GSH precursors and prodrugs also provide no information on the functional effects of altered GSH biosynthetic capacity *per se*. Furthermore, the relative levels of the GCL subunits, and not cysteine availability, dictate GSH homeostasis and biosynthetic capacity in Hepa-1c1c7 cells and supplementation with NAC does not elevate Hepa cell GSH content ((Shertzer *et al.*, 1995), data not shown). In such instances, conventional transgene overexpression approaches involving both viral and non-viral delivery systems have been employed to stably or transiently overexpress the GCL subunits to assess the effects of enhanced GSH biosynthetic capacity on cellular sensitivity to oxidative stress and apoptotic cell death (Kurokawa *et al.*, 1995; Mulcahy *et al.*, 1995; Tipnis *et al.*, 1999; Botta *et al.*, 2004; Tran *et al.*, 2004; Shi *et al.*, 2007; Cortes-Wanstreet *et al.*, 2009). However, these approaches exhibit low transfection efficiencies, require the time-consuming isolation of stable cell lines, or have the potential for virus-associated cytotoxicity. We therefore examined the efficacy of protein transduction as a means of increasing the cellular levels of the GCL subunits and enhancing cellular GSH biosynthetic.

TAT-mediated protein transduction has been successfully employed to deliver bioactive catalase and Cu,Zn-superoxide dismutase protein to various cultured cell lines *in vitro* and *in vivo* (Kwon *et al.*, 2000; Jin *et al.*, 2001; Eum *et al.*, 2002; Watanabe *et al.*, 2003; Eum *et al.*, 2004; Song *et al.*, 2008). To determine whether a similar approach could be employed to deliver functional GCL, we created in-frame TAT fusion proteins to both GCL subunits. These fusion proteins were capable of heterodimerization and formation of functional GCL holoenzyme *in vitro*. The TAT-GCL fusion proteins also displayed similar enzyme kinetics to 6His-only tagged GCL fusion proteins, suggesting that the TAT moiety did not affect GCLC activity or the ability to heterodimerize with GCLM and form functional GCL holoenzyme. Both TAT-GCL subunits transduced Hepa cells in a time- and dose-dependent manner, leading to increased GCL activity and intracellular GSH levels. The cellular uptake and functional effects of the TAT-GCL fusion proteins were absolutely dependent on the TAT protein transduction domain as exposure of Hepa cells to GCL-His fusion proteins lacking the TAT moiety did not result in increased GCL subunit levels, cellular GCL activity or GSH levels. The TAT-GCL fusion proteins were also efficiently transduced in multiple other cultured cell types, including TAMH murine hepatocyte cells, MCF-7 human breast cancer cells, 293T human embryonic kidney cells, and Cos-1 monkey kidney cells (data not shown). TAT-GCL fusion protein levels remained elevated for up to 48 h in the continuous presence of extracellular fusion protein. While TAT-GCL fusion protein transduction was reversible, cellular levels of both subunits remained elevated for up to 24 h after removal of the fusion proteins from the media. Should more persistent expression of the wild-type or dominant-negative GCLC(E103A) be required, their coding sequences could be readily moved into plasmid-based expression vectors and stable cell lines established.

The use of denaturing conditions during protein purification has been reported to enhance both yield of recombinant TAT fusion proteins and transduction efficiency (Nagahara *et al.*, 1998; Becker-Hapak *et al.*, 2001). However, it was necessary to use recombinant TAT-GCL fusion proteins isolated under native conditions in order to validate the *in vitro* activity of the TAT-GCL fusion proteins. It was also not clear that the TAT-GCL subunit fusion proteins would correctly refold into functionally active protein within the cell. Interestingly, while denatured TAT fusion proteins normally achieve maximal intracellular levels very rapidly (Becker-Hapak *et al.*, 2001), transduction of the GCLC-TAT fusion protein occurred with relatively slow kinetics. While the use of native TAT-GCL protein may have contributed to the slow

transduction kinetics of GCLC-TAT, this also decreased the likelihood of precipitation, which is a major problem associated with the preparation, storage and use of denatured TAT-fusion proteins (Becker-Hapak *et al.*, 2001). In this regard, fusion proteins were stored at −80C for extended periods in the presence of 10% glycerol with minimal loss of protein function and no detectable protein precipitation. Importantly, while imidazole and glycerol had no effect on TAT-GCL fusion protein function, desalting immediately prior to use enhanced protein transduction in Hepa cells ~10-fold (data not shown).

We have previously established Hepa-1c1c7 cell lines that stably overexpress the GCL subunits from eukaryotic expression vectors (Botta *et al.*, 2004). This permits a direct comparison of the relative levels of the GCL subunits and GCL activity that result from these two distinct overexpression approaches in the same cell type. While TAT-mediated delivery of the GCL subunits resulted in significantly higher cellular levels of both subunits than that observed in the stable Hepa cell lines, the relative changes in GCL activity and GSH levels in control cells and cells with increased levels of both GCL subunits were quite similar (Botta *et al.*, 2004). This may reflect threshold levels of cellular GCL activity and GSH that are achievable due to GSH-mediated negative feedback regulation of GCL (Griffith, 1999). Importantly, transduction with GCLC-TAT significantly enhanced Hepa cell resistance to H_2O_2-induced cytotoxicity similar to that observed in stably transfected Hepa cells (Shi *et al.*, 2007). Transduction of Hepa cells with TAT-GCLM alone also resulted in a small, but significant increase in cellular GCL activity and GSH levels. These levels are significantly less than the 2-fold increase in GCL activity and 50% increase in GSH levels reported in HeLa cells transfected with a GCLM expression plasmid (Tipnis *et al.*, 1999), but similar to results observed in Hepa-1c1c7, Cos-1, and COV434 human granulosa cells stably overexpressing GCLM alone (Mulcahy *et al.*, 1995; Botta *et al.*, 2004; Cortes-Wanstreet *et al.*, 2009). These apparent discrepancies are most likely due to the relative molar ratios of the GCL subunits in different cell models. Importantly, GCLM is limiting in most cell types (including Hepa-1c1c7 cells) and tissues, with the exception of the kidney ((Krzywanski *et al.*, 2004; Chen *et al.*, 2005; Lee *et al.*, 2006), data not shown). Thus, while GCLM possesses no catalytic activity, upregulation of GCLM is capable of enhancing GCL activity via increased GCL holoenzyme formation.

The recent development of knock-down and knock-out models of the GCL subunits has greatly enhanced our arsenal of tools to study the effects of compromised GSH biosynthesis in various cell and animal models (Iida *et al.*, 2001; Dalton *et al.*, 2004; Chen *et al.*, 2005; Diaz-Hernandez *et al.*, 2005; Akai *et al.*, 2007; Chen *et al.*, 2007; Luchak *et al.*, 2007; McConnachie *et al.*, 2007). We have now developed a novel, effective, and highly versatile dominant-negative approach to suppress endogenous GCL activity. Previous studies have examined whether enzymatically deficient mutants of GCLC could function in a dominant-negative manner (Yang *et al.*, 2007). Yang et al. examined two murine GCLC mutants (P158L and C248/249A) with severely compromised enzymatic activity (Yang *et al.*, 2007). However, the activities of these GCLC mutants were dramatically enhanced upon binding to GCLM and these mutants did not function as dominant-negative suppressors of cellular GCL activity, but rather increased cellular GSH levels (Yang *et al.*, 2007). One interpretation of these results is that the catalytic activity of GCLC must be completely abolished to function as a dominant-negative inhibitor of GCL holoenzyme activity. While there are no known naturally occurring mutations in human GCLC that abolish GCL activity (Beutler *et al.*, 1990; Beutler *et al.*, 1999; Ristoff *et al.*, 2000; Hamilton *et al.*, 2003), structure-function analysis of *Trypanosma brucei* GCL enzyme revealed several mutations that completely abrogated GCL activity, including a Glu/Ala mutation at residue E100 of *T. brucei* GCLC (Abbott *et al.*, 2001). While *T. brucei* GCL consists of a single monomeric protein, mutation of the corresponding residue in murine GCLC (E103A) had no effect on its ability to heterodimerize with murine GCLM, yet abolished the enzymatic activity of both the monomeric and holoenzyme forms of the mutant GCLC protein.

TAT-mediated delivery of the GCLC(E103A) mutant resulted in the dose-dependent dominant-negative suppression of Hepa cell GCL activity. Importantly, manipulation of Hepa cell GSH biosynthetic capacity via TAT-mediated delivery of wild-type GCLC or the dominant-negative GCLC(E103A) mutant conferred protection or enhanced sensitivity to H_2O_2-induced cytotoxicity, respectively. These findings demonstrate that TAT-mediated protein transduction of wild-type or mutant GCLC is a viable means of manipulating cellular GSH biosynthetic capacity for subsequent functional studies.

Buthionine sulfoximine (BSO) is a widely used potent and selective inhibitor of GSH biosynthesis (Griffith and Meister, 1979). However, there are several important distinctions between the functional effects of BSO and the dominant-negative GCLC(E103A) mutant. BSO irreversibly inhibits both monomeric GCLC and GCL holoenzyme activity via direct inactivation of GCLC resulting in the complete abrogation of GSH biosynthesis. In contrast, the GCLC(E103A) mutant functions to suppress cellular GCL by preventing the formation of functionally active GCL holoenzyme, while having no effect on the enzymatic activity of endogenous wild-type monomeric GCLC *per se*. These differential effects on cellular GCL activity result in quite distinct effects on cellular GSH homeostasis. BSO treatment leads to a near complete depletion of Hepa cell GSH levels within 24 h ((Shertzer *et al*., 1995), data not shown), while cellular GSH levels were maintained in untreated Hepa cells transduced with the GCLC(E103A)-TAT fusion protein. This was likely due to the activity of endogenous monomeric GCLC, which was not affected by the dominant-negative GCLC(E103A) mutant. These findings mimic those observed in GCLM(−/−) cells, which are capable of maintaining normal GSH levels in the absence of oxidative stress while possessing only 10–20% of normal GCL activity resulting from monomeric GCLC activity (Chen *et al.*, 2005; McConnachie *et al.*, 2007). Thus, while BSO treatment emulates a GCLC-deficient phenotype, TAT-mediated delivery of the dominant-negative GCLC(E103A) mutant is capable of creating a GCLM-deficient phenotype in virtually all primary and transformed cultured cell models and *in vivo*.

TAT-mediated protein transduction has numerous advantages over previously employed approaches of overexpressing the GCL subunits, including high transduction efficiency and lack of cellular toxicity associated with delivery of the subunits. One additional benefit of the TAT approach over conventional plasmid-based transgene expression approaches is that TAT-mediated transduction does not require *de novo* synthesis within the cell and is absolutely insensitive to changes in cellular transcription and/or translation that may be compromised in various disease states or in response to cellular treatments, such as actinomycin D and cycloheximide. There are also many experimental scenarios whereby TAT-mediated delivery of the GCL subunits would be extremely useful. TAT-mediated delivery of functional GCL protein could be used in tandem with genetic knock-out or siRNA knock-down approaches to assess the functional effects of wild-type or mutant GCL proteins in a background deficient in a specific GCL subunit. In this regard, GCLC-TAT could be employed to rescue the lethal GCLC(−/−) phenotype *in vivo* (Dalton *et al.*, 2000; Shi *et al.*, 2000). However, such studies may be hindered by the kinetics of transduction *in vivo* (Cai *et al.*, 2006). While loss of GCLM is not lethal, GCLM(−/−) mice and GCLM-deficient cells are highly sensitive to oxidative stress and xenobiotics and cells from GCLM(−/−) mice are difficult to culture under normoxic conditions (Chen *et al.*, 2005; McConnachie *et al.*, 2007). TAT-GCLM could be utilized to temporarily maintain normal GSH homeostasis and growth of cultured GCLM(−/−) cells. The establishment of the dominant-negative GCLC(E103A) mutant also provides a novel means to suppress cellular GSH biosynthetic capacity. A particularly appealing application of this novel reagent is the ability to titrate the relative levels of cellular GSH biosynthetic capacity over a wide range by the coordinate use of the GCLC(WT)-TAT and GCLC(E103A)-TAT fusion proteins. Such an approach permits the side-by-side analysis of the effects of enhanced and suppressed GSH biosynthetic capacity utilizing an identical TAT-mediated delivery platform.

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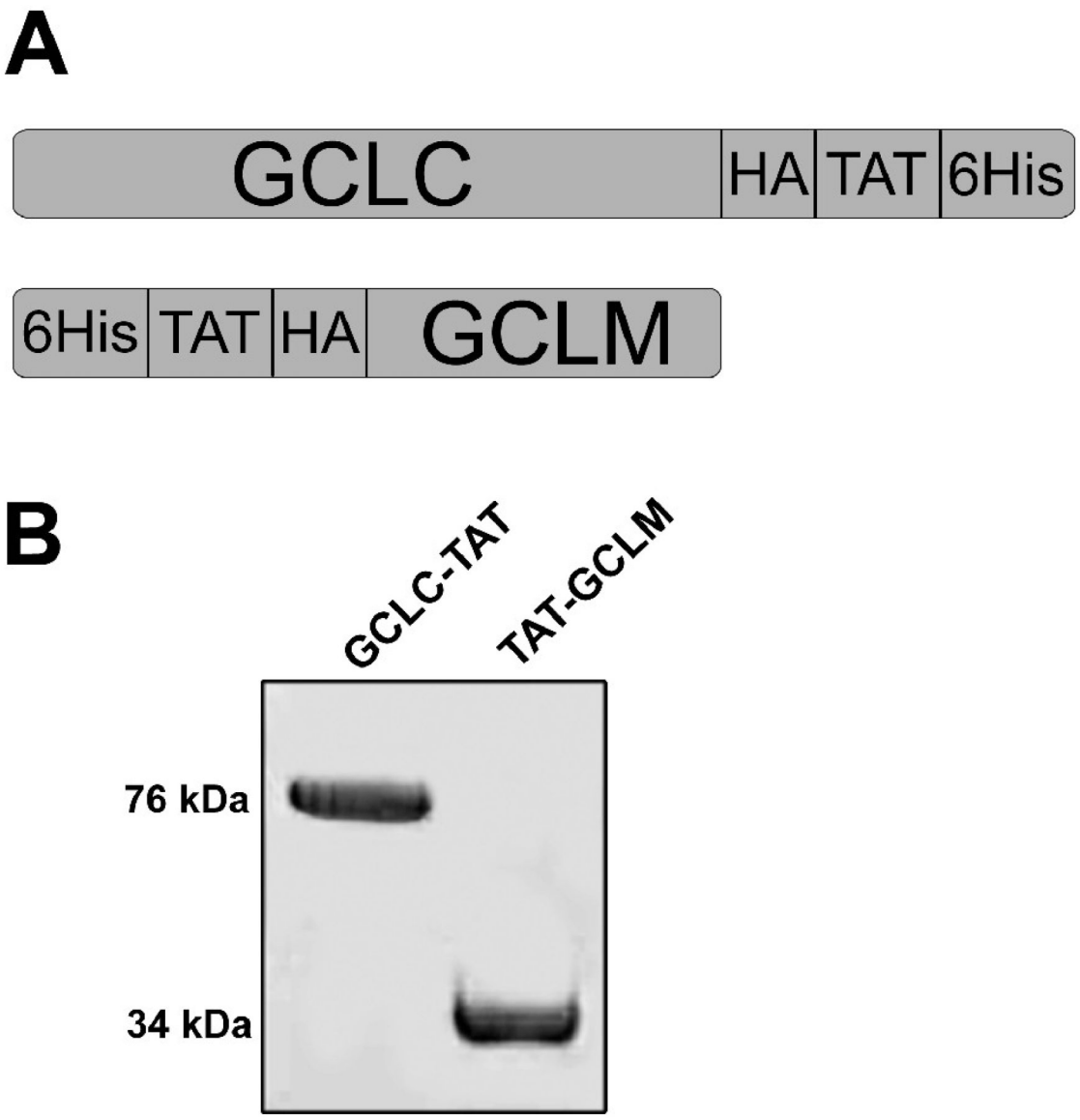

Figure 1. Preparation of purified recombinant GCLC-TAT and TAT-GCLM fusion proteins

(A) Diagram of GCLC and GCLM fusion proteins containing a C-terminal HA-TAT-6His tag or an N-terminal 6His-TAT-HA tag, respectively. (B) Recombinant TAT fusion proteins were synthesized in BL21(DE3) *E. coli*, purified by metal affinity chromatography, resolved by SDS-PAGE, and visualized by Coomassie-staining.

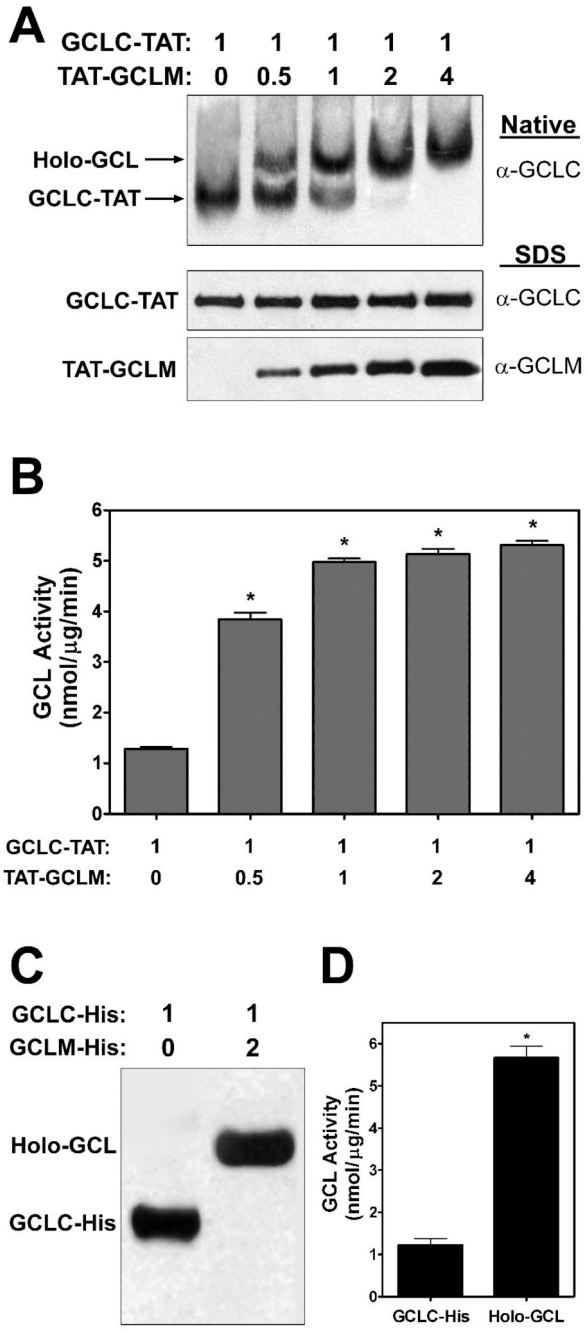

Figure 2. GCLC-TAT and TAT-GCLM form functional GCL holoenzyme *in vitro*

(A and B) Purified recombinant GCLC-TAT and TAT-GCLM fusion proteins were mixed at the indicated molar ratios. (A) GCL holoenzyme formation was analyzed by native PAGE and immunoblotting for GCLC (top panel). The relative levels of the GCL subunits in each mixture were analyzed by SDS-PAGE and immunoblotting for GCLC or GCLM (bottom panels). (B) GCL activity was measured by the fluorescence-based NDA assay (White *et al.*, 2003) (* $p < 0.001$). (C and D) Purified recombinant 6His-only tagged GCLC fusion protein was incubated in the absence or presence of a 2-fold molar excess of 6His-only tagged GCLM. (C) GCL holoenzyme and (D) GCL activity were analyzed as described above (* $p < 0.001$).

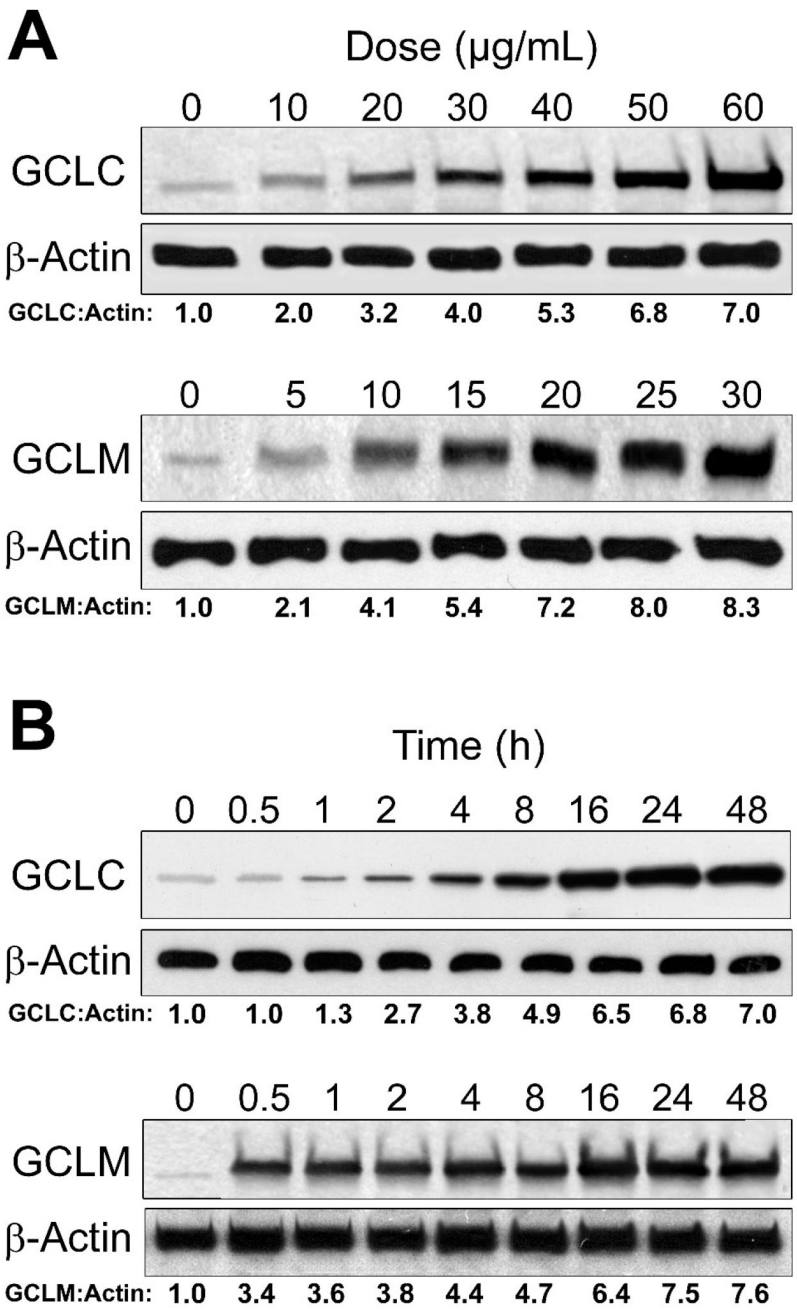

Figure 3. Time- and dose-dependent transduction of the TAT-GCL subunit fusion proteins in cultured cells

(A) Hepa-1c1c7 cells were transduced with the indicated concentrations of GCLC-TAT or TAT-GCLM for 18 h. (B) Hepa-1c1c7 cells were transduced with 50 μg/mL GCLC-TAT or 25 μg/mL TAT-GCLM for the indicated time periods. (A and B) GCL subunit levels were analyzed by SDS-PAGE and immunoblotting for GCLC or GCLM. Protein levels were quantitated by densitometry and the relative levels of fusion protein were normalized to β-Actin (ratios are reported below each lane).

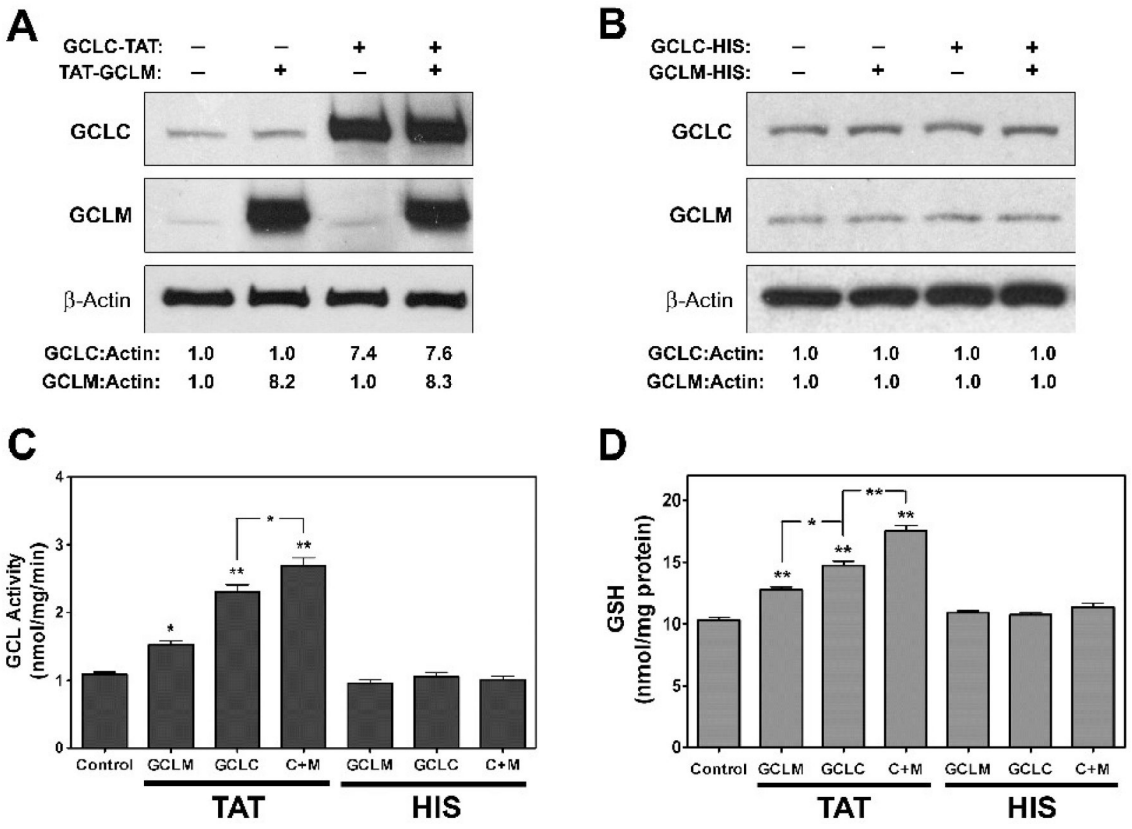

Figure 4. TAT-mediated transduction of the GCL subunits increases cellular GSH biosynthetic capacity and GSH levels

Hepa-1c1c7 cells were transduced in the absence or presence of (A) GCLC-TAT (50 μg/ml) and/or TAT-GCLM (25 μg/ml) or (B) GCLC-His (50 μg/ml) and/or GCLM-His (25 μg/ml) for 18 h. GCL subunit levels were analyzed by SDS-PAGE and immunoblotting for GCLC or GCLM. Protein levels were quantitated by densitometry and normalized to β-Actin (ratios are reported below each lane). (C) GCL activity was measured by the fluorescence-based NDA assay (White *et al.*, 2003) (* $p < 0.05$, ** $p < 0.001$). (D) Total cellular GSH levels (GSH + GSSG) were measured by a modified Tietze assay (Baker *et al.*, 1990) (* $p < 0.01$, ** $p < 0.001$).

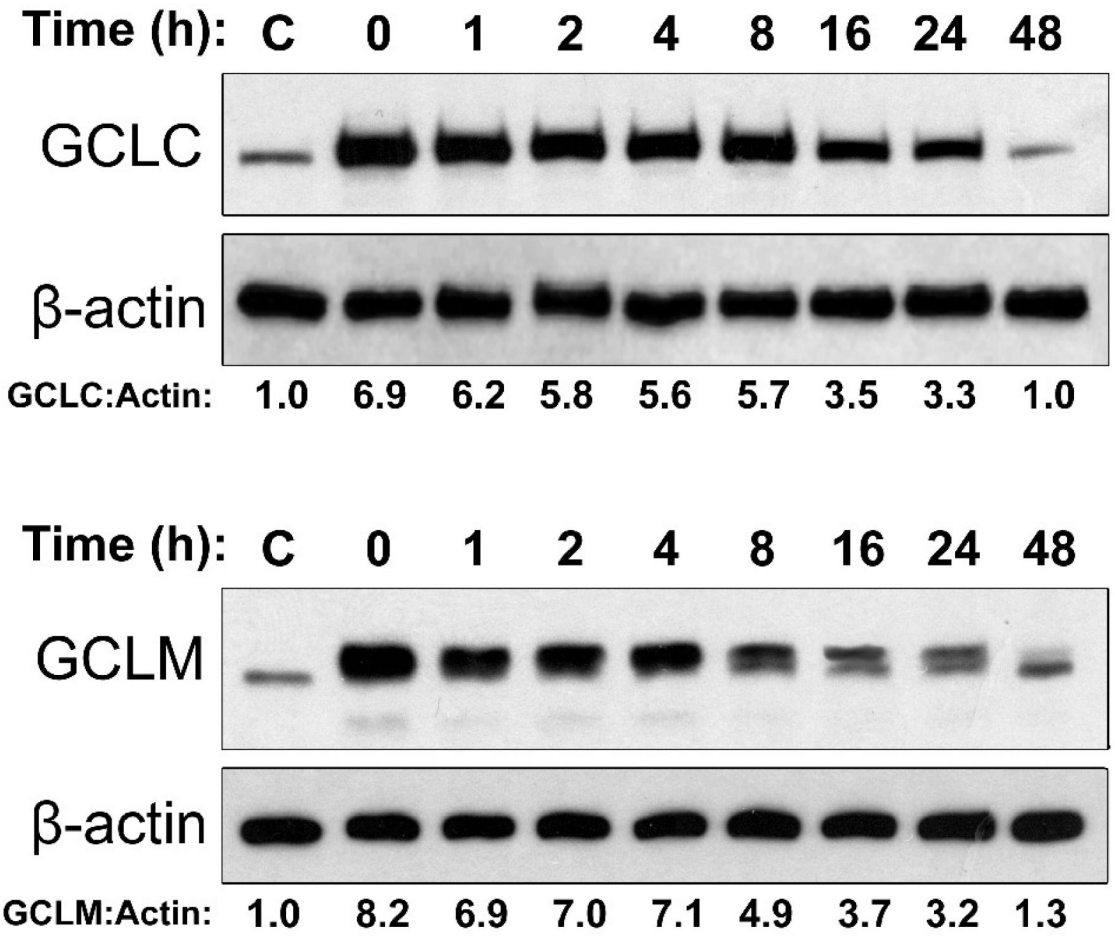

Figure 5. Persistence of TAT-GCL subunit transduction upon removal of TAT fusion protein from the media

Hepa-1c1c7 cells were transduced in the absence or presence of GCLC-TAT (50 ug/ml) or TAT-GCLM (25 ug/ml) for 24 h. Cultures were washed twice with warm media and incubated in fresh media for the time periods indicated. GCL subunit levels were analyzed by SDS-PAGE and immunoblotting for GCLC or GCLM. Protein levels were quantitated by densitometry and normalized to β-Actin (ratios are reported below each lane). (C = untransduced control)

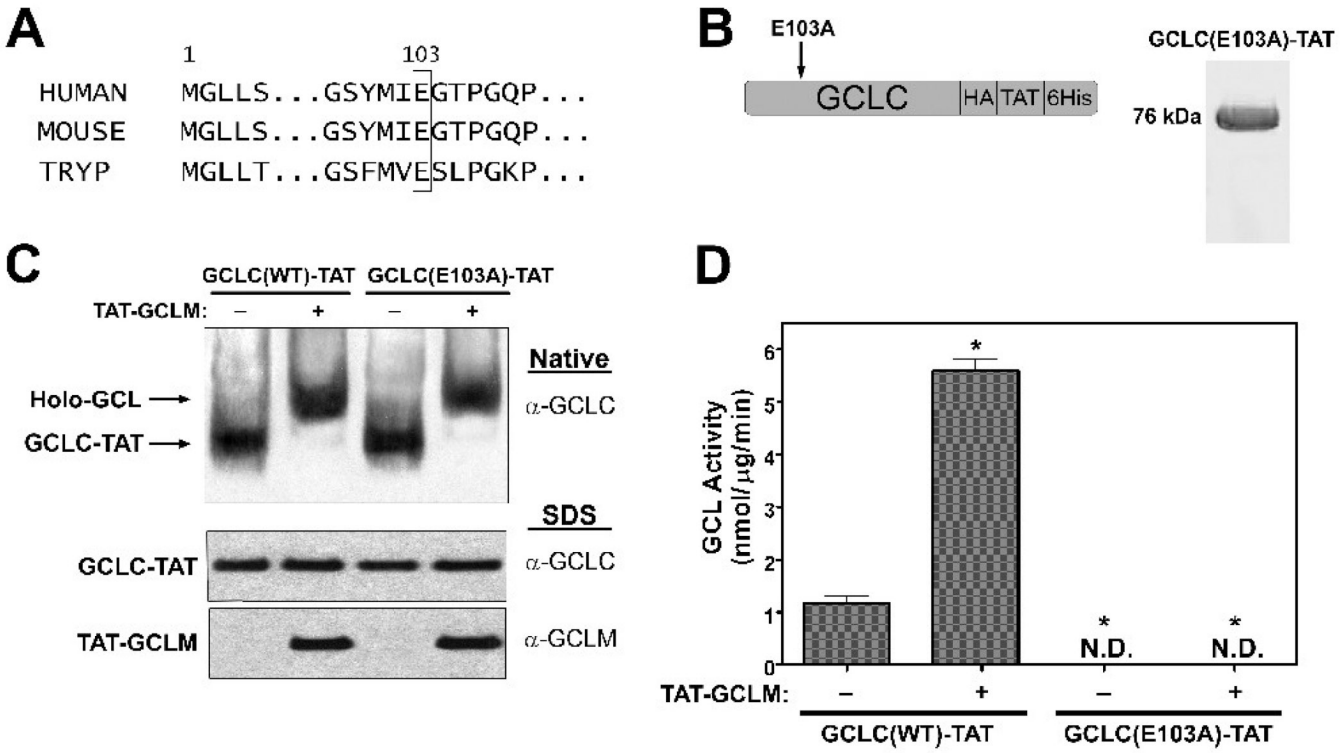

Figure 6. Creation of a heterodimerization-competent, enzymatically-deficient GCLC(E103A)-TAT mutant

(A) Primary amino acid sequence comparison of wild-type human, mouse, and trypanosome GCLC. An inactivating point mutation (E100A) identified in trypanosome (Abbott *et al.*, 2001) was introduced into a conserved sequence (E103A) in murine GCLC by site-directed mutagenesis. (B) Diagram of GCLC(E103A)-TAT fusion protein. Recombinant GCLC (E103A)-TAT was synthesized in BL21(DE3) *E. coli*, purified by metal affinity chromatography, resolved by SDS-PAGE, and visualized by Coomassie-staining. (C and D) Purified recombinant GCLC(WT)-TAT or GCLC(E103A)-TAT were incubated at 37°C for 10 min in the absence or presence of a 2-fold molar excess of TAT-GCLM fusion protein as indicated. (C) GCL holoenzyme formation was analyzed by native PAGE and immunoblotting for GCLC (top panel). The relative levels of the TAT-GCL fusion proteins in each mixture were analyzed by SDS-PAGE and immunoblotting for GCLC or GCLM (bottom panels). (D) GCL activity was measured by the fluorescence-based NDA assay (White *et al.*, 2003) (N.D. = non-detectable; * $p < 0.001$).

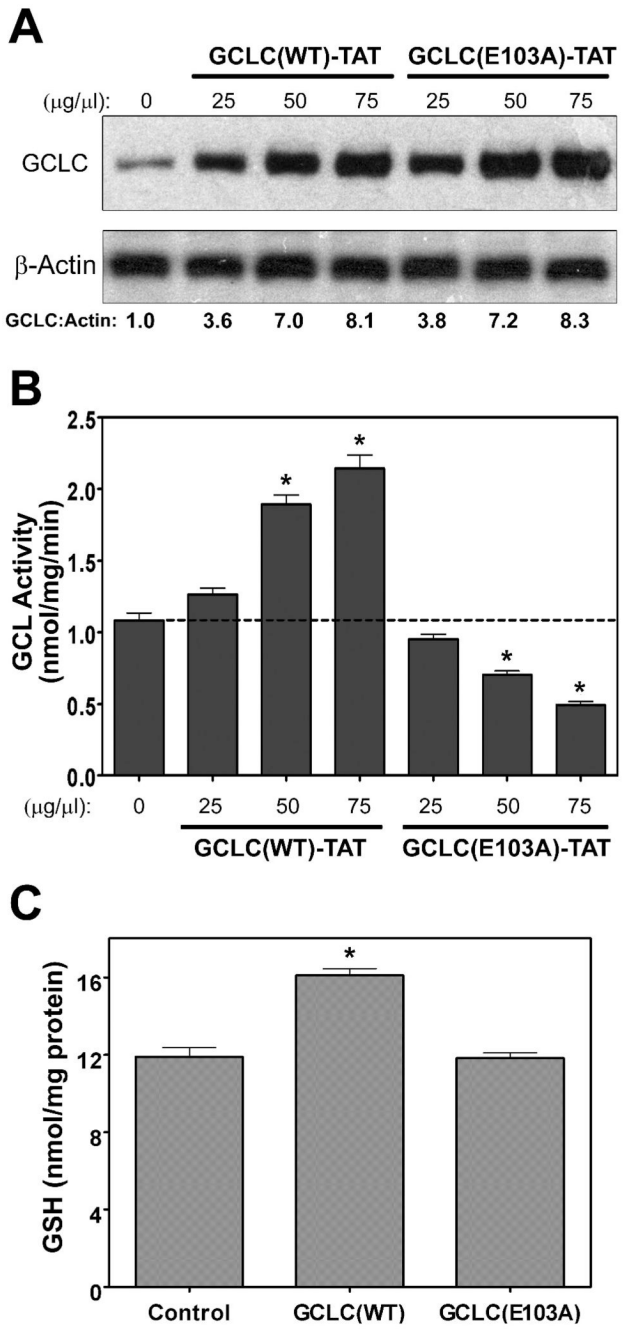

Figure 7. The catalytically inactive GCLC(E103A)-TAT mutant functions as a dominant-negative suppressor of cellular GCL activity

(A and B) Hepa-1c1c7 cells were transduced with increasing concentrations of GCLC(WT)-TAT or GCLC(E103A)-TAT fusion protein for 36 h. (A) GCLC-TAT fusion protein levels were analyzed by SDS-PAGE and immunoblotting for GCLC. Protein levels were quantitated by densitometry and normalized to β-Actin (ratios are reported below each lane). (B) Cellular GCL activity was measured by the fluorescence-based NDA assay (White *et al.*, 2003) (* $p < 0.001$). (C) Hepa-1c1c7 cells were transduced in the absence or presence of 50 ug/ml of GCLC (WT)-TAT or GCLC(E103A)-TAT fusion protein and total cellular GSH levels (GSH + GSSG) were measured after 36 h (* $p < 0.001$).

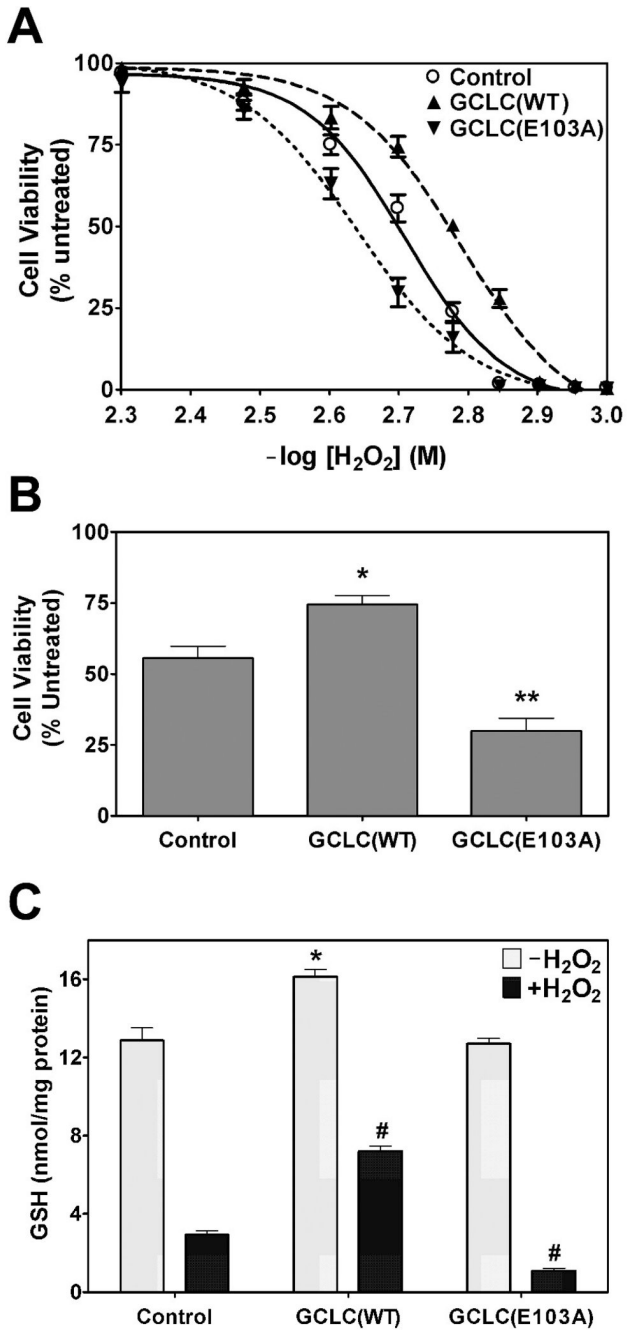

Figure 8. TAT-mediated delivery of wild-type or dominant-negative GCLC modifies cellular susceptibility to H_2O_2-induced cell death

Hepa-1c1c7 cells were transduced in the absence or presence of 50 μg/ml of GCLC(WT)-TAT or GCLC(E103A)-TAT fusion protein for 36 h as indicated. (A) Cells were treated with various concentrations of H_2O_2 for 18 h and cell viability was determined by MTS assay (○ = untransduced control; ▲ = GCLC(WT)-TAT; ▼ = GCLC(E103A)-TAT). Curves were generated using GraphPad Prism 4 curve-fitting software. (B) Cells were treated in the absence or presence of 500 uM H_2O_2 for 18 h and cell viability determined by MTS assay. Data are presented as % viability of untreated, untransduced control cells (* $p < 0.01$, ** $p < 0.001$ indicates significant difference compared to untransduced cells treated with H_2O_2). (C) Cells

were treated in the absence or presence of 500 uM H_2O_2 for 18 h and total cellular GSH levels (GSH + GSSG) were measured (* $p < 0.001$ indicates significant difference compared to untreated untransduced cells; # $p < 0.001$ indicates significant difference compared to untransduced cells treated with H_2O_2).