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Gene Knockdown of γ-Glutamylcysteine Synthetase by RNA_i in the Parasitic Protozoa *Trypanosoma brucei* Demonstrates That It Is an Essential Enzyme*

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The parasitic protozoa *Trypanosoma brucei* utilizes a novel cofactor (trypanothione, $T(SH)_2$), which is a conjugate of GSH and spermidine, to maintain cellular redox balance. γ-Glutamylcysteine synthetase (γ-GCS) catalyzes the first step in the biosynthesis of GSH. To evaluate the importance of thiol metabolism to the parasite, RNA_i methods were used to knock down gene expression of γ-GCS in procyclic *T. brucei* cells. Induction of γ-GCS RNA_i with tetracycline led to cell death within 4–6 days post-induction. Cell death was preceded by the depletion of the γ-GCS protein and RNA and by the loss of the cellular pools of GSH and $T(SH)_2$. The addition of GSH (80 μM) to cell cultures rescued the RNA_i cell death phenotype and restored the intracellular thiol pools to wild-type levels. Treatment of cells with buthionine sulfoximine (BSO), an enzyme-activated inhibitor of γ-GCS, also resulted in cell death. However, the toxicity of the inhibitor was not reversed by GSH, suggesting that BSO has more than one cellular target. BSO depletes intracellular thiols to a similar extent as γ-GCS RNA_i; however, addition of GSH did not restore the pools of GSH and $T(SH)_2$. These data suggest that BSO also acts to inhibit the transport of GSH or its peptide metabolites into the cell. The ability of BSO to inhibit both synthesis and transport of GSH likely makes it a more effective cytotoxic agent than an inhibitor with a single mode of action. Finally the potential for the $T(SH)_2$ biosynthetic enzymes to be regulated in response to reduced thiol levels was studied. The expression levels of ornithine decarboxylase and of *S*-adenosylmethionine decarboxylase, two essential enzymes in spermidine biosynthesis, remained constant in induced γ-GCS RNA_i cell lines.

African trypanosomiasis is caused by subspecies of the genus, *trypanosomatidae*. These uniflagellated parasitic protozoa are transmitted by an insect vector and cause nagana (*Trypanosoma brucei brucei*) in cattle and sleeping sickness (*Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*) in humans (1–3). Although drugs are available for the treatment of trypanosomiasis, toxicity and resistance have limited their effectiveness (4–6). Metabolic differences between trypanosomes and their mammalian hosts are being characterized to identify new potential drug targets in the parasite.

Polyamine biosynthesis has been identified as a target for chemotherapeutic intervention against a number of proliferative diseases including African sleeping sickness (7–9). Unlike mammalian cells, trypanosomes conjugate the polyamine spermidine to GSH to generate a novel cofactor termed trypanothione ($T(SH)_2$[1]; N^1,N^8-bis(glutathionyl)spermidine; Fig. 1). $T(SH)_2$ is required to maintain cellular redox balance and replaces the function of GSH in mammalian cells (10, 11). $T(SH)_2$ is maintained in the reduced form by the action of trypanothione reductase (TR), a homolog of glutathione reductase in mammalian cells. Thus, in these parasites polyamines not only play a role in their typical functions that promote cell growth, but they are also essential for maintenance of the reduced intracellular thiol pools.

$T(SH)_2$ is synthesized in four steps via the synthesis of GSH and its subsequent conjugation to spermidine (Fig. 1). In the first two steps GSH is synthesized by two enzymes, which are common to mammalian cells. The conjugation of GSH to spermidine in the following steps is catalyzed by trypanosome-specific enzymes (12–14). The first step in the biosynthesis of GSH is catalyzed by γ-glutamylcysteine synthetase (γ-GCS), which catalyzes the ATP-dependent ligation of L-Cys and L-Glu to produce γ-GC. γ-GCS has been demonstrated to be the rate-limiting enzyme in the biosynthesis of GSH in mammalian cells (15) and of $T(SH)_2$ in *Leishmania tarentolae* (16). The enzyme from *T. brucei* has been biochemically characterized by study of the recombinant enzyme (17–20).

The importance of thiol metabolism to trypanosome survival is suggested by a number of observations. In particular an enzyme-activated, potent inhibitor of γ-GCS, buthionine sulfoximine (BSO), was shown to cure or prolong survival of mice infected with a bloodstream form *T. brucei* (21). These studies implicated γ-GCS as a potential drug target, and the observed selectivity of BSO suggested that trypanosomes are more sensitive to GSH depletion than mammalian cells. However, the mechanism of action of BSO as an anti-trypanosomal agent was not conclusively demonstrated to be inhibition of γ-GCS.

Other studies to demonstrate the importance of thiol metabolism to the parasite have focused on $T(SH)_2$. Gene knockout studies suggest that TR is an essential enzyme in both *Leish-*

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[1] The abbreviations used are: $T(SH)_2$, trypanothione; TR, trypanothione reductase; γ-GCS, γ-glutamylcysteine synthetase; BSO, buthionine sulfoximine; ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; Tet, tetracycline; HPLC, high pressure liquid chromatography; RT, reverse transcriptase; MOPS, 4-morpholinepropanesulfonic acid; HEPPS, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid; ZPFM, Zimmerman post-fusion medium.

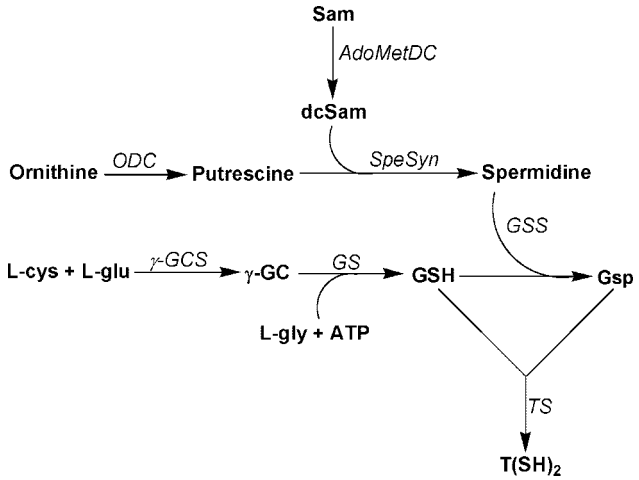

FIG. 1. **Polyamine and trypanothione metabolism in *T. brucei*.** Enzymes catalyzing each reaction are indicated in *italics*. *ODC*, ornithine decarboxylase; *AdoMetDC* and *dcSAM*, *S*-adenosylmethionine decarboxylase; *SpeSyn*, spermidine synthetase; *γ-GCS*, γ-glutamylcysteine synthetase; *GS*, glutathione synthetase; *GSS*, glutathionylspermidine synthetase; *TS*, trypanothione synthetase.

mania donovani and *T. brucei* (22, 23). Trypanosomes have been reported to lack catalase, which in mammals breaks down hydrogen peroxide in the peroxisome (24). Instead, hydrogen peroxide is reduced by tryparedoxin peroxidase in a cycle that is dependent on a novel tryparedoxin/TR system to provide the reduced thiol in the form of $T(SH)_2$ (25, 26). The need for $T(SH)_2$ to detoxify peroxides in place of catalase may make trypanosomes more sensitive to thiol depletion than mammalian cells. $T(SH)_2$ is also essential for the action of ribonucleotide reductase and the reduction of dehyroascorbate (27, 28).

RNA_i is an effective tool for silencing gene expression in both the insect stage procyclic form and the mammalian bloodstream form of *T. brucei* parasites (29–31). In this paper, we utilized RNA_i methods for gene knockdown of γ-GCS in *T. brucei* procyclic parasites. Induction of γ-GCS RNA_i leads to cell death demonstrating that γ-GCS is essential to cell survival. This effect on parasite survival correlated directly to loss of γ-GCS RNA and protein and to a depletion of the cellular thiol pools. As was observed in prior studies on blood stage parasites (21), the γ-GCS inhibitor BSO was toxic to the growth of procyclic parasites. GSH was able to rescue the RNA_i cell death phenotype, but it did not rescue BSO toxicity. These results demonstrate that the mechanism of BSO toxicity on *T. brucei* is not fully explained by inhibition of γ-GCS as the sole target.

Finally, the convergence of the polyamine and GSH biosynthetic pathways for the synthesis of $T(SH)_2$ suggested the possibility that the two pathways may have a mechanism to coordinate their metabolic output. To address this question we used the γ-GCS RNA_i cell line to determine whether reduced thiol levels could affect expression levels of ornithine decarboxylase (ODC) or of *S*-adenosylmethionine decarboxylase (AdoMetDC), two essential enzymes in polyamine biosynthesis. No evidence for cross-pathway regulation was observed.

EXPERIMENTAL PROCEDURES

Trypanosome Cultures—Procyclic form trypanosomes were grown in SDM-79 media (32) with 10% fetal calf serum at 25 °C. Cells were grown to mid-log phase (10^5–10^6 cells/ml) and diluted 1:100 about every 4 days into fresh media with the appropriate antibiotics for selection (G418, 15 μg/ml; hygromycin, 50 μg/ml; and/or phleomycin, 2.5 μg/ml). To analyze the effects of RNA_i induction on cell growth, cell densities were determined by using a hemocytometer (Brightline, Fisher). Growth curves were plotted as the product of the cell density and the total dilution. To determine the effects of BSO, cells (5-ml cultures) were grown in the presence of varying concentrations of BSO (0–150 μM) with or without the addition of GSH (80 μM). After 4 days, 100-μl aliquots of the cells were plated onto 96-well tissue culture plates. Cell viability was determined using the Cell Titer 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega) according to the manufacturer's protocol. A reagent solution containing a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS), supplied by the kit was applied to the cells. The MTS reagent was reduced by viable cells into a formazan product that is soluble in the tissue culture medium and is detectable by a spectrophotometer at 490 nm (Power WaveX Select plate reader, Bio-Tek instruments, Inc.). The quantity of the formazan product, measured as a 490 nm absorbance, was directly proportional to the number of viable cells in culture. Standard curves of known cell amounts were used to determine the number of viable cells in test cultures.

Generation of DNA Constructs and Transgenic Trypanosome Cell Lines—The pZJM vector (a gift from Dr. Paul Englund, The Johns Hopkins University) was used to construct the γ-GCS RNA_i plasmid. The pZJM vector was digested with *Xho*I and *Hin*dIII to remove the α-tubulin stuffer. The *Xho*I overhangs were filled in with the Klenow fragment (from *Escherichia coli*) of DNA polymerase I and dNTPs. The γ-GCS insert was digested with *Bsa*AI (at nucleotide 1426) and *Hin*dIII (at nucleotide 2152) from clone Tb12.1 (20) and ligated into the pZJM vector, replacing the tubulin stuffer. The incorporated gene fragment corresponded to amino acid residues 459–679. The γ-GCS RNA_i plasmid was linearized with *Not*I and transfected into 29-13 parent cell line (a gift from Dr. George Cross, Rockefeller University), which harbors the integrated genes for T7 RNA polymerase and the tetracycline repressor. pZJM contains a marker for phleomycin resistance and directs recombination into the rRNA locus. The γ-GCS RNA_i cells were maintained in SDM-79 media with G418 (15 μg/ml), hygromycin (50 μg/ml), and phleomycin (2.5 μg/ml). The antibiotics G418 and hygromycin maintained the T7 RNA polymerase and tetracycline repressor constructs, respectively.

The pHD 328 vector (33) (a gift from Dr. Christine Clayton, University of Heidelberg, Germany) was used to construct the γ-GCS single knockout plasmid. The Tb12.1 plasmid was used as the DNA template to PCR-amplify the 5′ and 3′ flanks for ligation into the pHD 328 vector. The 5′ γ-GCS fragment (nucleotide residues 706–2252) was generated by PCR with the following primers: 5′-CCACGTTTCCAGCGGCCGCT-CTGCTTGCG-3′ (has *Not*I site) and 5′-CCTTCGTACACAACGCGTCA-AGTTGCGGCC-3′ (has *Mlu*I site). The 3′ γ-GCS fragment (nucleotide residues 6141–6797) was generated by PCR with the following primers: 5′-CCCAGTTTGGCAGGCCTGTTTTCATCGACTGC-3′ (has *Stu*I site) and 5′-CCATGTTACGCATGCACGCATTTCCCC-3′ (has *Sph*I site). The vector and PCR products were digested with the restriction enzymes mentioned above. The PCR products were ligated into the corresponding sites on the pHD 328 vector. The single knockout plasmid was transfected into wild-type 427 procyclic cells and maintained in SDM-79 media with hygromycin (50 μg/ml).

Transfection of T. brucei Parasites—Transfection of *T. brucei* procyclic cells was performed according to protocols described previously (34) with some modifications. Actively growing 29-13 cells, co-expressing the T7 RNA polymerase and tetracycline repressor, were split (1:10) the day before transfection. Cells were harvested from log phase cultures (~10^6–10^7 cells/ml) by centrifugation (1000 rpm for 10 min.) and washed once in ice-cold Zimmerman post-fusion medium (ZPFM). The cell pellets were resuspended in ZPFM and diluted to 2×10^7 cells/ml. Aliquots of 1×10^7 cells/ml in 0.5 ml of ZPFM with 20 μg of linear DNA (digested with *Not*I) were subjected to two pulses on the Bio-Rad Gene Pulser electroporation system. Transfections were carried out at room temperature in 4-mm cuvettes with the peak discharge set at 1.5 kV, 25 microfarads, and 20 ohms (resistance of the core unit). Immediately after electroporation, cells were transferred to 5 ml of SDM-79 media containing appropriate inhibitors to sustain the host background (G418 at 15 μg/ml and hygromycin at 50 μg/ml) and incubated overnight at 25 °C. The selection for transformant cells, which had integrated the construct into the rRNA locus, was applied the following day with 2.5 μg/ml phleomycin. Synthesis of double-stranded RNA was induced by the addition of tetracycline (Tet; 1 μg/ml) to the culture media.

Cell Lysate Preparation and Immunoblots—Cultures (100 ml) of procyclic trypanosomes were grown for about 4 days to late log phase (10^7–10^8 cells/ml). Cells were pelleted at 3000 rpm (Beckman Instruments, model J-6B centrifuge) for 10 min at 4 °C. The cell pellets were washed with 1 ml of phosphate-buffered saline (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Pellets were resus-

pended in 100–500 μl of lysis buffer (20 mM MOPS, pH 8.0, 10 mM NaCl, 0.4 mM EDTA, 4 mM $MgCl_2$, 0.4 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml pepstatin, 1 μg/ml chymostatin) with vortexing. The resuspended cell lysate was frozen in liquid nitrogen and allowed to quickly thaw in a 37 °C water bath. This freeze/thaw cycle was repeated two more times. The cell lysate was cleared of cell debris by centrifugation at 13,000 × *g* for 3 min at room temperature. Protein content in cell-free extracts was determined according to Bradford (35) with protein assay dye reagent and bovine serum albumin for standard curve calibration (Bio-Rad). The supernatant-containing protein was boiled in SDS treatment buffer (125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 3.1% (w/v) dithiothreitol, 0.001% (w/v) bromphenol blue) for 5 min.

Protein (20–40 μg) from the cell-free extracts was separated by Tris/SDS-PAGE (12.5% T (total monomer), 0.3% C (cross-linker) resolving gel and 3.9% T, 0.3% C stacking gel) in a Mini-Protean II Cell (Bio-Rad) according to the method of Laemmli. Proteins were transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham Biosciences) with transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol; without adjusting the pH, it should be around 8.0–8.2) at 100 V for 1 h. Proteins immobilized on the polyvinylidene difluoride membrane were incubated in Tris-buffered saline (TBS: 20 mM Tris-HCl, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.6) with 5% (w/v) blocking agent (supplied in the ECL Western blotting Analysis System, Amersham Biosciences) prior to antibody incubations. Rabbit polyclonal antisera (Babco, Richmond, CA) was generated to recombinant *T. brucei* γ-GCS (20), ODC (36), and AdoMetDC (37) purified as described previously and diluted 10,000-fold before use. Antigen recognition was visualized by horseradish peroxidase detection reagents 1 and 2 (supplied in the ECL Western blotting Analysis System, Amersham Biosciences) with donkey anti-rabbit IgG/horseradish peroxidase conjugate diluted 1:10,000 (Amersham Biosciences).

Protein band intensities were determined by Scion software. Integrated densities of protein bands, which cross-reacted with the primary antibody of interest, was divided by the corresponding integrated densities of the protein bands recognized by the tubulin antibody (developed in rabbit, Sigma, catalog T-3526) to obtain a normalized protein ratio. Standard curves of purified proteins were used to demonstrate that signals were in the linear range for quantitative purposes.

Real Time RT-PCR and Northern Blot Analyses—Total RNA was isolated from trypanosome cells with the RNAqueous kit (Ambion; Austin, TX) according to the manufacturer's protocols. RNA yield and quality were evaluated spectrophotometrically and by analytical gel electrophoresis according to Sagerström and Sive (38). RNA integrity was confirmed by visualizing the 18 S and 28 S rRNA bands with ethidium bromide staining.

Transcript amounts of various genes were quantitated using the real time RT-PCR method with the Taqman PCR Reagent kit (Applied Biosystems; Foster City, CA). PCR amplimers (Sigma Genosys; The Woodlands, TX) and dual-labeled (5′ reporter dye, 6-carboxyfluorescein; 3′ quencher dye, 6-carboxytetramethylrhodamine) fluorescent probes (Integrated DNA Technologies, Coralville, IA) were designed for each gene of interest based on the *T. brucei* gene sequence (GenBank™ accession number U56818) using the Primer Express 2.0 software (Applied Biosystems). For γ-GCS, the PCR amplimers used were 5′-TGCGGCGGCGTTACC-3′ (forward) and 5′-GACCAACGTAACAGCGAATGTG-3′ (reverse), and the probe was 5′-CATGTTGGACGCTGCCGCTGG-3′. For ODC, the PCR amplimers used were 5′-AGGAAGCACGAAACATGGAAA-3′ (forward) and 5′-TGCATTTGACCGCGTAAAAC-3′ (reverse), and the probe was 5′-AATGCCTTCCCCGCGTCACG-3′. For AdoMetDC, the PCR amplimers used were 5′-CGAAACACTGGTACTCGGATAAGA-3′ (forward) and 5′-CCGTTGCCTCCCGTATCAC-3′ (reverse), and the probe was 5′-TTGCCTACGGGTCCTGAAACCGC-3′. For tubulin, the PCR amplimers used were 5′-AACAACCTTTCCACCGCATT-3′ (forward) and 5′-GCTGGCCCACGCAAACT-3′ (reverse), and the probe was 5′-ATCGAGCAGCGGCCCCTCCTATG-3′. Each real time RT-PCR (10 μl) included 10 ng of total RNA, Taqman PCR Master mix (with AmpliTaq Gold DNA polymerase; Applied Biosystems), 300 nM PCR amplimers, 250 nM probe, and 1 unit of the reverse transcriptase/RNase inhibitor mix (Applied Biosystems). The temperature cycles for the RT-PCR runs were as follows: reverse transcription at 48 °C for 30 min, deactivation of reverse transcriptase at 95 °C for 10 min, 40 PCR cycles of 95 °C for 15 s, 60 °C for 1 min, and a final polymerization step at 60 °C for 10 min. Relative amounts of gene transcripts normalized to tubulin were calculated using the ABI PRISM 7700 Sequence Detection System (User Bulletin 2, Applied Biosystems). The normalized ratios obtained for all cell lines examined were divided by the normalized ratios of wild-type to obtain a change in transcript levels relative to wild type.

Total RNA (~3 μg) was separated on denaturing 1% agarose gels (RNAqueous kit, Ambion), and Northern blot analyses were performed according to standard procedures (39). Radiolabeled ([^{32}P]dATP) DNA probes were prepared as described previously (20) using the Random Primed DNA Labeling Kit (Roche Applied Science) according to the manufacturer's protocols.

Quantitation of Thiols in Cell Lysates—Intracellular thiols were analyzed according to Fairlamb *et al.* (40) with some modifications. Trypanosome cells (1×10^8) were centrifuged at 3000 rpm for 10 min at 4 °C in a swing bucket rotor clinical centrifuge (Beckman Instruments, model J-6B). HEPPS buffer (50 μl; 40 mM HEPPS, 4 mM diethylenetriaminepentaacetic acid, pH 8.0) and monobromobimane solution (50 μl; 2 mM Thiolyte in absolute ethanol, Calbiochem) were added to the pellet to lyse cells and label thiols. The resuspended pellet was heated at 70 °C for 3 min and briefly cooled on ice before the addition of the methanesulfonic acid solution (100 μl, lithium methanesulfonate, 4 M, pH 1.6). The cell lysate was placed on ice for 30 min before the removal of protein precipitate in a microcentrifuge (14000 rpm, 5 min). The resulting supernatant was collected and analyzed by HPLC (20 μl of the reaction is injected).

Labeled thiols were separated using the HPLC system (System Gold Nouveau, Beckman Instruments) and solvents described below. Following injection, linear gradients of 0–20% solvent B (25% (v/v) *n*-propyl alcohol in water with D-camphor sulfonate, lithium salt, 0.25% (w/v), pH 2.64); solvent A contained D-camphor sulfonate (lithium salt, 0.25% (w/v) in water, pH 2.64) over 60 min, followed by 20–75% solvent B over 40 min were applied. Fluorescent compounds were detected using a DYNAMAX fluorescence detector (Rainin, model FL-1). Specific thiol concentrations were determined by peak area relative to derivatized standards; GSH was from Sigma; GSH-GSP was from Bachem; and $T(SH)_2$ was a gift from Dr. Alan Fairlamb, University of Dundee.

RESULTS

Induction of γ-GCS RNA_i Kills Trypanosomes by Depleting γ-GCS and Thiol Pools—To show that γ-GCS is an essential enzyme for the growth of *T. brucei* cells, we inhibited γ-GCS gene expression by RNA_i methods using the tetracycline-inducible pZJM dual T7 vector (30). Procyclic 29-13 cells stably transformed with the γ-GCS RNA_i construct (Fig. 2*A*) arrested cell division after a limited number of cell cycles when induced with Tet (1 μg/ml). After about 4 days in culture, the γ-GCS RNA_i + Tet cells (Fig. 2*B*, *open circles*) began to die, while the uninduced RNA_i (−Tet; *solid circles*) cells grew at rates similar to the 29-13 parents cells grown with Tet (*open triangles*) or without (*solid triangles*). γ-GCS RNA and protein levels were analyzed in these RNA_i cell lines. Northern blot (Fig. 2*B*, *inset*, *N panel*) and Western blot analyses (Fig. 2*B*, *inset*, *W panel*) revealed that both RNA and protein levels in the induced γ-GCS RNA_i cells (+ Tet) were reduced >80%. The levels of tubulin RNA (Fig. 2*B*, *inset*, *T panel*) and protein (data not shown) were measured as a control and were found to be constant under all conditions. RNA levels were also quantitated by real time RT-PCR, and the results also demonstrated that RNA levels were effectively depleted by the induction of γ-GCS RNA_i (Fig. 3*A*, *black bars*).

In addition to the γ-GCS RNA_i cell line, a single knockout procyclic cell line that has one allele of γ-GCS replaced by a hygromycin selectable marker was generated. Trypanosomes are diploids containing two copies of the γ-GCS gene (20); thus one copy of the gene still remains in these cells. These cells grew at normal wild-type rates (data not shown). RNA levels in this cell line were measured by real time RT-PCR and were reduced by 50% when compared with wild-type 427 cells suggesting a gene dosage effect (Fig. 3*A*, *black bars*). However, γ-GCS protein levels in the single knockout line were the same as wild-type suggesting that the cell may regulate the level of γ-GCS activity at the protein level (Fig. 3*A*, *gray bars*).

Thiol levels in cell lysates of RNA_i cells induced with Tet for zero (*0d HPLC profile*), two (*2d HPLC profile*), and 4 days (*4d HPLC profile*) were examined (Fig. 4). Thiol levels (nmol/10^8

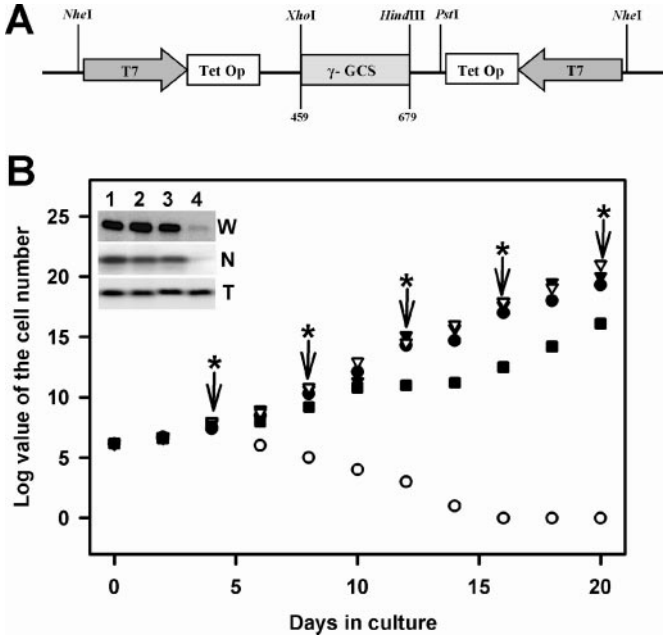

FIG. 2. **γ-GCS RNA$_i$ cell growth profiles.** *A*, γ-GCS RNA$_i$ pZJM vector. A sequence of the γ-GCS gene corresponding to amino acid residues 459–679 replaces the α-tubulin sequence of the pZJM vector. The tetracycline-inducible T7 promoters are indicated by the *T7 arrows*; the tetracycline operators are indicated by the *Tet Op boxes*. *B*, cell densities were determined for various RNA$_i$ cells in culture for 20 days. Cell lines examined include 29-13 RNA$_i$ parent cell lines uninduced (− Tet, ▼) or induced (+ Tet, ▽), and γ-GCS RNA$_i$ cell lines uninduced (− Tet, ●), induced (+ Tet, ○), or induced with the addition of 80 μM GSH (+ Tet, ■). The *arrows* and *asterisks* indicate the days (about every 4 days) in which the cultures were diluted (1:100). The *inset* shows Western (*W panel*; 40 μg of total protein loaded per lane) or Northern analyses (*N panel*; 3 μg of total RNA loaded per lane) for γ-GCS antibodies or DNA probes. A tubulin probe is included to show equal loading of total RNA (*T panel*). The lanes represent cell lysates prepared from 29-13 parent RNA$_i$ cells uninduced (− Tet, *lane 1*) or induced (+ Tet, *lane 2*) and γ-GCS RNA$_i$ cell lines uninduced (− Tet, *lane 3*) or induced (+ Tet, *lane 4*).

cells) were determined for glutathione (GSH, *peak B*), glutathionyl-spermidine (GSH-SPD, *peak C*), and trypanothione (T(SH)$_2$, *peak D*) (Table I). Uninduced (− Tet) RNA$_i$ cells had 2.15 nmol of GSH, 0.79 nmol of GSH-SPD, and 3.81 nmol of T(SH)$_2$. After 2 days of induction (+ Tet) 78% GSH (1.69 nmol), 46% GSH-SPD (0.36 nmol), and 32% T(SH)$_2$ (1.2 nmol) remained. After 4 days of induction, all three thiols were reduced by more than 80%. GSH-SPD levels were undetectable after 4 days of induction. These results collectively indicate that there is a direct correlation between cell death, the reduction of γ-GCS transcript and protein levels, and the depletion of thiol pools in the *T. brucei* cells. The single knockout cell line had wild-type levels of all three thiols (data not shown), consistent with the observation that γ-GCS protein levels were unchanged in these cells.

GSH Rescues the RNA$_i$ Cell Death Phenotype—GSH is the key metabolic intermediate downstream of γ-GCS. GSH was tested over a range of 20–180 μM to determine the maximum levels that cells could tolerate with normal growth (similar to 427 wild-type cells). The optimum GSH concentration was 80 μM; concentrations above this level had detrimental effects on cell growth (data not shown). To determine whether the addition of GSH rescues the RNA$_i$ cell death phenotype, Tet-induced γ-GCS RNA$_i$ cells were cultured in the presence of 80 μM GSH (Fig. 2*B*, *solid squares*). GSH rescued the detrimental growth effects caused by γ-GCS gene knockdown. The rescued cells grew somewhat slower than the control cells (uninduced RNA$_i$, and 29-13 parent cells) for the first 10 days of culture,

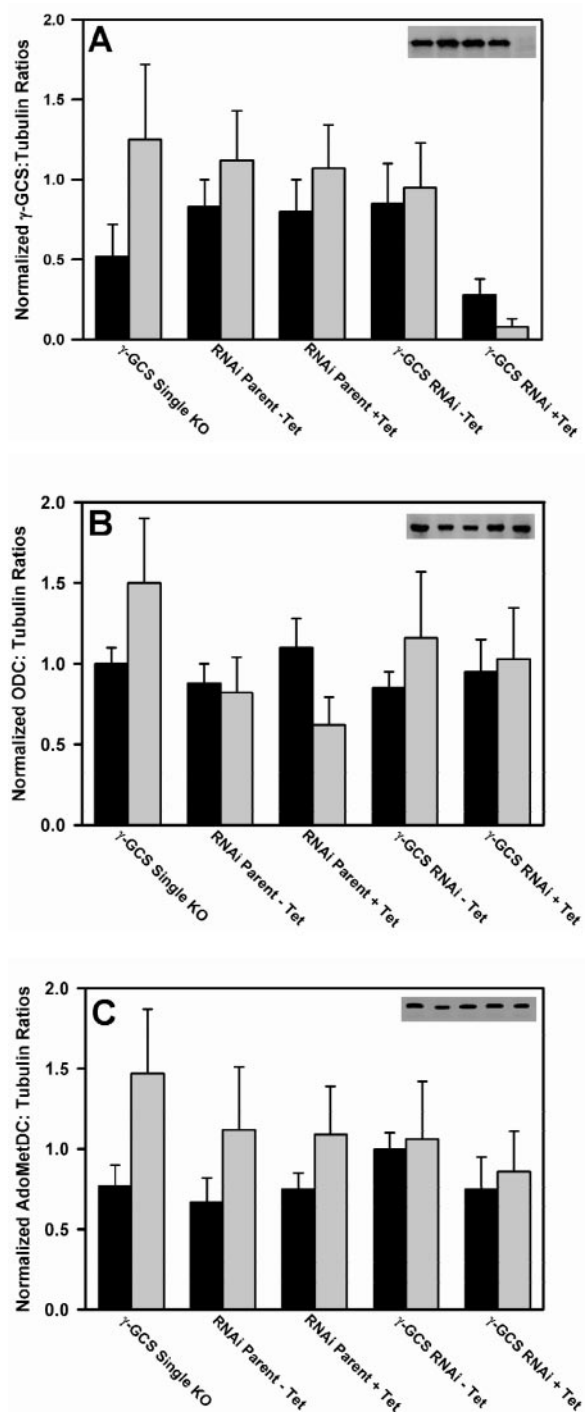

FIG. 3. **Comparing γ-GCS (*A*), ODC (*B*), and AdoMetDC (*C*) transcript and protein levels.** Transcript levels (*black bars*) were determined by real time RT-PCR. Protein levels (*gray bars*) were determined by obtaining integrated densities of the visualized protein bands (Scion software). Values shown for each cell line represent ratios of γ-GCS, ODC, or AdoMetDC to tubulin transcript or protein levels that have been normalized to the wild-type (427 cells) values. The *error bars* depict the S.E. of the mean for $n = 3$ determinations. The *inset* shows Western analyses of cell lysates (40 μg of total protein loaded per lane) prepared from each of the corresponding cell lines.

but growth rates became comparable with control cells after about 14 days in culture. The addition of 80 μM GSH to cell cultures expressing γ-GCS RNA$_i$ (4 days post-Tet induction) restored the thiol pools to control cell levels (Table I).

The Anti-trypanosomal Activities of BSO Are Not Rescued by GSH—To examine the effects of the γ-GCS inhibitor BSO on procyclic trypanosomes, 29-13 cells (without Tet) were cultured

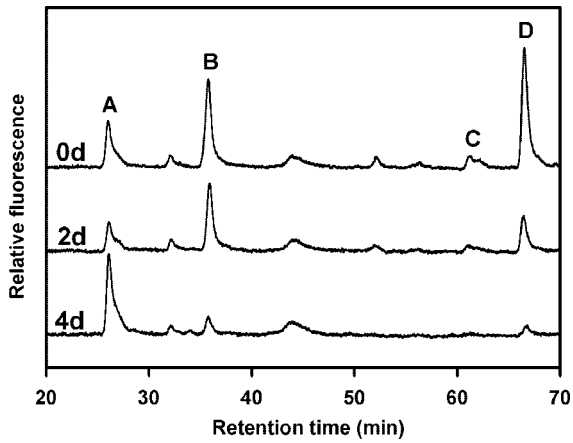

FIG. 4. **HPLC analyses of monobromobimane-labeled thiols in cell lysates of γ-GCS RNA$_i$ cell lines.** The cell lines examined include γ-GCS RNA$_i$ uninduced (− Tet, zero day control; *0d HPLC profile*) and induced (+ Tet) for 2 days (*2d HPLC profile*) or for 4 days (*4d HPLC profile*). Thiol amounts (see Table I) were quantitated for glutathione (GSH, *B peak*), glutathionyl-spermidine (GSH-SPD, *C peak*), and trypanothione (T(SH)$_2$, *D peak*). *Peak A* represents the monobromobimane reagent peak.

TABLE I
Thiol amounts in cell lysates of γ-GCS RNA$_i$ cells induced with tetracycline

The amounts of thiol are given as nanomoles per 10^8 cells. The samples were analyzed in duplicate.

Days of induction with Tet[a]	Addition of GSH[b]	Thiols		
		GSH	GSH-SPD	T(SH)$_2$
0	−	2.15	0.79	3.81
2	−	1.69	0.36	1.20
4	−	0.37	ND[c]	0.31
0	+	2.29	0.91	4.01
4	+	1.82	0.65	2.96

[a] Addition of 1 μg/ml tetracycline.
[b] addition of 80 μM GSH into culture media.
[c] Not detectable.

with BSO (0–150 μM), in the absence (Fig. 5, *solid circles*) or presence (Fig. 5, *open circles*) of 80 μM GSH. BSO kills these parasites with an inhibitory concentration (IC_{50}) of 50 ± 8 μM (*solid circles*). HPLC analysis of thiol pools demonstrates that BSO (60–120 μM) depletes intracellular levels of GSH and T(SH)$_2$ to a similar extent as induction of γ-GCS RNA$_i$ (Table II), consistent with the expectation that cell death is caused by thiol depletion. However addition of GSH (80 μM) to the cells treated with BSO did not provide effective rescue of BSO toxicity (Fig. 5). In the presence of GSH the IC_{50} value for BSO increased only slightly to 60 ± 4 μM. Furthermore, the addition of GSH to cells treated with BSO (60–120 μM) did not restore intracellular GSH and T(SH)$_2$ to control cell levels (Table II).

The Effect of Thiol Depletion on Levels of Polyamines Biosynthetic Enzymes—Because spermidine, synthesized in the polyamines biosynthetic pathway, converges with trypanothione biosynthesis (Fig. 1), we hypothesized cross-regulation between the GSH, and spermidine biosynthetic pathways may occur. To test this hypothesis, expression levels of two other enzymes in the polyamines pathway (ODC and AdoMetDC) were examined in the γ-GCS cell lines. Normalized ODC transcript levels (Fig. 3*B, black bars*) were similar to wild-type levels in all cell lines examined. In addition, normalized AdoMetDC transcript levels were similar to wild type in all cell lines (Fig. 3*C, black bars*). Western blot analyses demonstrated that ODC (Fig. 3*B, gray bars*) and AdoMetDC (Fig. 3*C, gray bars*) protein levels paralleled the RNA results. Thus, the depletion of γ-GCS (by RNA$_i$) did not affect RNA or protein levels of ODC and AdoMetDC.

DISCUSSION

RNA$_i$ techniques provide a powerful method to evaluate gene function in a number of organisms, including in the protozoan parasite *T. brucei* (41). In this paper, we demonstrate that γ-GCS is essential for the survival of procyclic *T. brucei* parasites *in vitro*. RNA$_i$-induced depletion of γ-GCS RNA and protein causes a loss of cellular thiol pools that is commensurate with cell death. The two major thiols (GSH and T(SH)$_2$) in the parasite were both reduced by 80–90% in *T. brucei* parasites immediately prior to the loss of cell viability. The addition of GSH to cell cultures rescued the RNA$_i$ cell death phenotype providing further proof that GSH is an essential metabolite in these parasites. Whereas this requirement for GSH can be supplied exogenously *in vitro*, *T. brucei* is an extracellular parasite in all stages of its life cycle (42, 43). Thus the parasite does not have access to the intracellular thiol pools in the host likely constraining it to supply the growth requirement for GSH through *de novo* synthesis.

Other studies have also pointed to the importance of thiol metabolism to trypanosome survival; however, past genetic methods did not allow a direct correlation between loss of cell viability and the depletion of the reduced cellular thiol pools to be established. The deletion of a single allele of TR in *L. donovani* decreased their ability to survive in macrophages (23). Knockout of both alleles of TR in bloodstream form of *T. brucei* was achieved after insertion of a regulated copy of the gene, and this study linked cell viability with loss of TR gene expression (22). However, the levels of reduced GSH and T(SH)$_2$ in these cells were not detectably changed, and thus the mechanism of cell killing could not be fully established.

The ability of GSH to rescue the γ-GCS RNA$_i$ cell death phenotype clearly establishes that GSH is an essential metabolite for *T. brucei* growth. Unlike in mammalian cells oxidation by T(SH)$_2$, and not GSH, is enzymatically reduced by the trypanosome parasites (10, 11). Further T(SH)$_2$, and not GSH, provides the reducing equivalents required for the function of ribonucleotide reductase, for the detoxification of hydrogen peroxides via tryparedoxin peroxidase, and for the reduction of dehydroascorbate (26–28, 44). These observations suggest that the depletion of T(SH)$_2$ observed upon γ-GCS RNA$_i$ induction is likely to be the lethal event leading to the loss of cell viability. However, our data are also consistent with the possibility that GSH has essential functions beyond its requirement for the formation of T(SH)$_2$.

The T(SH)$_2$ and GSH concentrations are similar in both procyclic and bloodstream parasites, and the roles of the cellular thiols are thought to be similar in both stages (40, 45). Thus the finding that γ-GCS is an essential gene in procyclic *T. brucei* parasites will likely extend to bloodstream parasites, and the RNA$_i$ analysis of the procyclic cells provides support for the hypothesis that γ-GCS is a potential drug target for the treatment of African sleeping sickness. Further validation of the target would be obtained by demonstrating that parasite cells can be killed by agents that function by inhibiting the target enzyme. The potent γ-GCS inhibitor, BSO, had been demonstrated previously to have antitrypanosomal activities in a rodent model; however, the mechanism of action of BSO killing was not clearly established (21).

The γ-GCS RNA$_i$ cell line provided a direct method to test the mechanism of BSO killing. Procyclic *T. brucei* cells are effectively killed by BSO, but in contrast to the results observed for the RNA$_i$ death phenotype, the trypanolytic activities of BSO were not rescued by GSH. These data strongly suggest that BSO acts on other targets in addition to γ-GCS to produce its trypanolytic effects. Furthermore, the finding that addition of exogenously added GSH restores the intracellular levels of

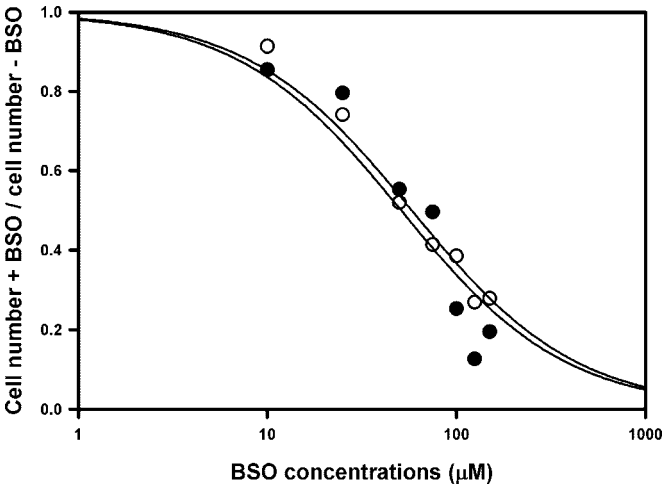

FIG. 5. **Effect of BSO on cell viability.** Cell viability was determined for 29-13 parent cells after 4 days in SDM-79 culture medium with various concentrations of BSO (0–150 μM) and with 80 μM GSH (○) or without (●). The fractional cell number relative to no drug control is plotted. See Table II for thiol amounts in BSO-treated cells.

TABLE II

Thiol amounts in cell lysates of 29–13 parent cells treated with BSO

The amounts of thiol are given as nanomoles per 10^8 cells. The samples were analyzed in duplicate.

Concentration of BSO in media	Addition of GSH[a]	Thiols		
		GSH	GSH-SPD	$T(SH)_2$
μM				
0	−	2.08	0.68	3.39
30	−	1.75	0.46	1.65
60	−	0.92	0.30	0.99
90	−	0.78	0.23	0.49
120	−	0.45	0.19	0.16
0	+	2.73	0.58	3.79
30	+	1.78	0.65	2.13
60	+	1.34	0.56	1.31
90	+	0.85	0.24	0.79
120	+	0.49	0.11	0.20

[a] Addition of 80 μM GSH into culture media.

thiols to wild-type levels in the induced γ-GCS RNA_i cells, but not the BSO-treated cells (60–120 μM BSO), strongly suggests that GSH transport into the cell is also inhibited by BSO.

In mammalian cells GSH is not transported directly into the cell; however, extracellular GSH is metabolized by γ-glutamyltranspeptidase, and the resulting products of the reaction (Cys-Gly and γ-Glu-amino acid conjugates) are transported into the cell (46–48). A major substrate of this translocation is γ-Glucyst(e)ine, which can restore the cellular pools of γ-GC (49). It has been reported that BSO inhibits the uptake of cystine into human lung carcinoma cells (50) and of γ-glutamylmethionine sulfone into the kidney (46). A similar mechanism for degrading and transporting peptide components of GSH into the cell is likely present in *T. brucei*. Inhibition of either the transpeptidase or the peptide transporter by BSO would account for the inability of exogenous GSH to restore intracellular thiol pools or to rescue the cells from BSO-induced death. Although no direct experimental evidence for γ-glutamyltranspeptidase has been reported in *T. brucei*, enzyme activity has been observed in *T. cruzi* (51).

The finding that BSO inhibits GSH biosynthesis not only by affecting the γ-GCS reaction but also by preventing transport of the γ-Glu conjugates required to bypass the block would be expected to make BSO a more effective cytotoxic agent. This additional mechanism prevents the cells from restoring thiol pools with exogenous GSH, and likely explains why BSO is effective even against intracellular parasites. *L. donovani* and *Plasmodium* spp., which also have limited ability to detoxify hydrogen peroxide, are also killed by BSO *in vitro* (24, 52, 53). Furthermore, BSO has been demonstrated to reverse drug resistance for antimonials and chloroquine in resistant *Leishmania* and *Plasmodium* cells that have increased levels of GSH (53, 54).

In the trypanosome parasites the polyamines and GSH biosynthetic pathways are unusually linked together for the biosynthesis of $T(SH)_2$, suggesting the possibility that cross-regulation in gene expression may occur between enzymes of these two pathways. Regulation of enzyme levels would provide a mechanism to coordinate the levels of $T(SH)_2$ precursors that are synthesized, thus maintaining a balanced metabolic output between the two pathways. To examine this possibility, we depleted γ-GCS levels (by RNA_i) and investigated how these alterations in expression levels affect the protein levels of ODC and of AdoMetDC. We observed no change in ODC or AdoMetDC protein levels when γ-GCS and hence the thiol pools were depleted. Although these studies did not demonstrate any regulatory mechanisms in the $T(SH)_2$ biosynthetic pathway, regulation may still occur at different points in the pathways.

CONCLUSIONS

The data in this paper conclusively demonstrate that GSH is an essential metabolite for the growth of procyclic trypanosome parasites. *In vitro* this growth requirement can be supplied by *de novo* GSH biosynthesis requiring the action of γ-GCS or through supplementation of the media with GSH. *In vivo T. brucei* is an extracellular parasite that does not have access to intracellular thiol pools, and thus, the parasite is thought to require *de novo* synthesis to obtain GSH. Furthermore, our data demonstrate that BSO, an enzyme-activated inhibitor of γ-GCS, has a complex mechanism of action for the observed anti-trypanosomal activity. BSO inhibits not only GSH biosynthesis but transport of the needed GSH precursors that could be utilized to overcome the block of γ-GCS inhibition. This dual mechanism of BSO action provides more effective depletion of cellular thiol pools than expected for a single action inhibitor and suggests the mechanism by which this compound is also a useful chemotherapeutic agent for intracellular parasites.

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Gene Knockdown of γ-Glutamylcysteine Synthetase by RNA_i in the Parasitic Protozoa *Trypanosoma brucei* Demonstrates That It Is an Essential Enzyme

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