Genetic Validation of *Trypanosoma brucei* Glutathione Synthetase as an Essential Enzyme

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Human African trypanosomiasis (HAT), a debilitating and fatal vector-borne disease. Polyamine biosynthesis is the target of one of the key drugs (eflornithine) used for the treatment of late-stage disease, suggesting that the pathway might be exploited for the identification of additional drug targets. The polyamine spermidine is required in trypanosomatid parasites for formation of a unique redox cofactor termed trypanothione, which is formed from the conjugation of glutathione to spermidine. Here we characterize recombinant *Trypanosoma brucei* glutathione synthetase (TbGS) and show that depletion of TbGS in blood-form parasitism using a regulated knockout strategy leads to loss of trypanothione and to cell death as quantified by fluorescence-activated cell sorter (FACS) analysis. These data suggest that >97% depletion of TbGS is required before trypanothione is depleted and cell growth arrest is observed. Exogenous glutathione was able to partially compensate for the loss of TbGS, suggesting that parasites are able to transport intact glutathione. Finally, reduced expression of TbGS leads to increased levels of upstream glutathione biosynthetic enzymes and decreased expression of polyamine biosynthetic enzymes, providing evidence that the cells cross regulate the two branches of the trypanothione biosynthetic pathway to maintain spermidine and trypanothione homeostasis.

*Trypanosoma brucei* is the causative agent of human African trypanosomiasis (HAT), a vector-borne illness endemic in 36 African countries (1–4). The World Health Organization (WHO) estimates that 30,000 people are currently infected, while millions in the region remain at risk of contracting the disease (5). HAT is nearly always fatal if untreated; however, current drug therapy is limited by toxicity and difficult treatment regimens. Eflornithine, a key drug for treatment of late-stage HAT, is a suicide inhibitor of the polyamine biosynthetic enzyme ornithine decarboxylase (ODC), thus establishing the polyamine biosynthetic pathway as an essential and targetable pathway in these parasites (6). Polyamines are positively charged, flexible hydrocarbons that are essential for growth and proliferation of eukaryotic cells, with roles in transcription, translation, chromatin structure, and ion channel function (7–10). Trypanosomes have evolved to use the polyamine spermidine in an additional role through conjugation with two molecules of glutathione to form the essential redox cofactor trypanothione [N1,N8-bis(glutathionyl)-spermidine] (11).

Trypanothione synthesis occurs via four ATP-dependent enzymatic steps beginning with the conjugation of 1-Cys to 1-Glu by γ-glutamyl cysteine synthetase (γ-GCS) (12) (Fig. 1). The product of this reaction (γ-GC) is then ligated to Gly by glutathione synthetase (GS) to form the thiol peptide glutathione, which is used to maintain redox homeostasis in most cells. The third and fourth steps are catalyzed by trypanothione synthetase (TryS), which conjugates glutathione to spermidine, forming N1-glutathionyl-spermidine, and subsequently adds a second molecule of glutathione to N1-glutathionylspermidine to form trypanothione (13, 14). TryS also contains an amidase domain and can catalyze the breakdown of trypanothione to glutathione and spermidine.

Trypanothione is required for growth and proliferation of trypanosomatids and is necessary to maintain cellular redox balance, replacing the role of glutathione found in mammalian cells (12, 15). The presence of two novel enzymes in the trypanosomatids required for trypanothione synthesis (TryS) and reduction (trypanothione reductase [TR]) has led to interest in identifying inhibitors of these enzymes for drug discovery efforts, though none have yet advanced beyond hit identification or early lead optimization (16–21). With the exception of GS, the biosynthetic enzymes for both the polyamine biosynthesis and the trypanothione biosynthesis have been characterized and shown to be essential in *T. brucei* using genetic methods (14, 22–25). A crystal structure of *T. brucei* GS (TbGS) has been reported and shows conservation of the overall fold compared to the human GS protein and a similar active-site composition (26). However, no kinetic analysis of the *T. brucei* enzyme has been reported, and genetic studies on its role and essentiality are lacking.

In mammalian cells, glutathione biosynthesis is regulated primarily by modulation of γ-GCS expression, the first step in the pathway (27). While *T. brucei* γ-GCS has been characterized and shown to be essential for growth in *T. brucei* gene knockdown did not lead to compensatory changes in the levels of the polyamine biosynthetic enzymes and no evidence for cross regulation between the polyamine and trypanothione arms of the pathway was found (22, 28, 29). Regulation has been shown to occur within the *T. brucei* polyamine biosynthetic pathway where gene knockdown or inhibition of S-adenosylmethionine decarboxylase (AdoMetDC), an essential enzyme in spermidine biosynthesis, led to increased protein expression of both the AdoMetDC regulatory subunit prozyme and ODC (23, 24, 30, 31). The AdoMetDC regulatory protein prozyme is itself also an activator of AdoMetDC, as
heterodimer formation with prozyme (an inactive paralog of AdoMetDC) increases enzymatic activity by 3 orders of magnitude. *T. brucei* deoxyhypusynase synthase, an enzyme that uses spermidine as a substrate to modify an essential elongation factor (eIF5A), also requires activation by an inactive paralog (32). Despite these advances in understanding polyamine pathway regulation in *T. brucei*, key questions remain about how the glutathione branch of the pathway is regulated and how polyamine and glutathione pathways show evidence for cross regulation.

Here we investigate the function and regulation of *TbGS*, including steady-state kinetic analysis of the recombinant enzyme and characterization of a *TbGS* knockout in *T. brucei* bloodstream-form (BSF) cells. We show that *TbGS* has catalytic activity similar to that of other characterized GS homologs, and we demonstrate through the use of a *TbGS* conditional double-knockout (cDKO) cell line that *TbGS* is essential for trypanothione biosynthesis and for parasite growth. Furthermore, we show that loss of *TbGS* protein leads to an increase in the levels of γ-GCS protein and a decrease in the levels of AdoMetDC prozyme. Thus, similarly to the other polyamine and trypanothione biosynthetic enzymes, *TbGS* is essential for *T. brucei* growth and it appears that *TbGS* protein levels are linked to the regulatory mechanisms that control expression levels of both polyamine and glutathione/trypanothione biosynthetic enzymes.

**MATERIALS AND METHODS**

**Gene ID numbers.** The TriTrypDB gene identification (ID) numbers are as follows: for GS, Tb927.7.4000; for γ-GCS, Tb927.10.12370; for TryS, Tb927.2.4370; for TR, Tb927.11.13730; for ODC, Tb927.10.12370; for AdoMetDC, Tb927.6.4410 and Tb927.6.4460; for AdoMetDC prozyme, Tb927.6.4470; for spermidine synthase (SpdSyn), Tb927.9.7770; and for dihydroorotate dehydrogenase (DHODH), Tb427.05.3830.

**T. brucei cell culture.** Bloodstream-form (BSF) *T. brucei* strain 90-13 was cultured in HMI-19 media (IMDM was purchased from Life Technologies) (1). *T. brucei* 427 BSF cells (1.0 × 10^6) harvested by centrifugation and washed 3× with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 0 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4). The pellet was resuspended in 500 μl DNA lysis buffer (100 mM Tris-HCl [pH 8.0], 5 mM EDTA, 200 mM NaCl, 0.2% SDS, 60 μg/ml RNase A) and incubated for 30 min at 37°C. Proteinase K (5 μl of 20 mg/ml) was added, and the mixture was incubated at 55°C overnight. DNA was extracted by phenol-chloroform/ethanol precipitation.

**Cloning of the *TbGS* construct for recombinant expression in Escherichia coli.** The TriTryp database contains a single gene annotated as *TbGS* (Tb927.7.4000). The *TbGS* gene was synthesized and cloned into pUC57 by GenScript. The gene was PCR amplified from the plasmid to obtain a product flanked with BsaI and XbaI restriction sites. The PCR product was digested with BsaI and XbaI and subcloned into BsaI-linearized pE-SUMO(Kan) (Life Technologies), generating the pE-SUMO-TbGS construct, for expression of an N-terminally tagged His<sub>6</sub>-SUMO-TbGS.

**Protein expression and purification of SUMO-*TbGS* from *E. coli.*** The pE-SUMO-TbGS construct was transformed into T1 phase-resistant *E. coli* BL21(DE3) competent cells for protein expression. Protein expression was induced at an A<sub>600</sub> of 0.5 with isopropyl β-D-thiogalactopyranoside (Sigma) (200 μM) for 18 h at 14°C. Cells were harvested by centrifugation (1,000 × g for 0.5 h). The pellet was resuspended in Buffer A (25 mM HEPES [pH 7.5], 300 mM NaCl, 1 mM MgCl₂, 5% glycerol) and supplemented with protease inhibitor mix (1 μg/ml leupeptin, 2 μg/ml antipain, 10 μg/ml benzamidine, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 200 μM phenylmethylsulfonyl fluoride [PMSF]). Cells were lysed by high-pressure disruption (EmulsiFlex-C5; Avetin), the lysate was clarified by centrifugation (15,000 × g for 0.5 h), and protein was purified by Ni²⁺-affinity chromatography (HiTrap Chelating HP column; GE Life Sciences) using an Akta fast protein liquid chromatography (FPLC) system (GE Life Sciences). The column was washed with Buffer A plus 10 mM imidazole before protein elution with Buffer A plus 250 mM imidazole was performed. Fractions were analyzed by SDS-PAGE, and those containing His<sub>6</sub>-SUMO-TbGS were pooled and dialyzed against Buffer A (16 h, 4°C). To remove the His<sub>6</sub>-SUMO tag, Ulp1 (20 μg/ml) was added and the mixture was incubated at 30°C for 2 h. Protein was concentrated (Centricon-30; Millipore), and TbGS was separated from the tag with a second Ni²⁺-affinity purification. TbGS protein fractions (flowthrough) were pooled and dialyzed against storage buffer (25 mM HEPES [pH 8.0], 150 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol [DTT], 5% glycerol). The protein concentration was determined by absorbance at 280 nm using an extinction coefficient of 60.0 mM⁻¹ cm⁻¹ (computed using ProtParam; ExPASy, Swiss Institute of Bioinformatics). The final yield was 0.8 mg of *TbGS* per liter of bacterial culture. Ulp1 was expressed and purified as previously described (31, 32). All purification steps were performed at 4°C.

**Steady-state kinetic analysis of *TbGS* activity.** *TbGS* activity was measured using a previously described ATP-coupled assay (34). All assays were performed at 37°C on a Synergy H1 Hybrid Reader (BioTek) using UVStar 96-well half-area plates (Greiner). The total reaction volume was 100 μl and contained 100 mM Tris-HCl (pH 8.2), 50 mM KCl, 20 mM MgCl₂, 250 μM NADH, 5 mM phosphateolypyrurate, 10 U lactate dehydrogenase, 10 U pyruvate kinase, and substrates. The assay was shown to be linearly dependent on the *TbGS* concentration (range, 10 to 50 nM). *TbGS* concentrations for substrate titration experiments were 15 and 25 nM. Unless otherwise stated, substrate concentrations were fixed at 3 mM γ-GC (BAC Chemicals), 30 μM Gly, or 10 mM ATP. For substrate titrations, the substrate concentrations were as follows: for γ-GC, 1 μM to 1 mM; for Gly, 100 μM to 100 mM; and for ATP, 10 μM to 10 mM. Reactions were monitored by absorbance at 340 nM, and rates were fitted to the Michaelis-Menten equation using GraphPad Prism to determine *K<sub>m</sub>* and *K<sub>eq</sub>* values.

**Cloning of the *TbGS* RNAi construct.** The *TbGS* RNA interference (RNAi) stem-loop construct was generated from the pLEW100 and pJM326 vectors according to previous methods (35). Using primers con-
taining the appropriate restriction enzyme sites (see Table S1 in the supplemental material), a TbG8 gene fragment (nucleotides 1 to 440) was PCR amplified from *T. brucei* 427 genomic DNA and subcloned into pLEW100 in the forward direction (using XbaI and MluI) and into pJM326 in the reverse direction (using HindIII and NheI). The TbG8 gene along with the stuffer region was excised from pJM326 using HindIII and XbaI and ligated into pLEW100 containing the first Ts1 RNAi region. The construct contained two fragments of the TbG8 gene separated by the stuffer region in opposing directions under the transcriptional control of a Tet-regulated promoter. These elements were flanked by a ribosomal DNA sequence, allowing for homologous recombination into the multi-copy ribosomal locus of *T. brucei*. Sequencing confirmed that the correct construct was obtained.

**Cloning of GS allelic replacement constructs.** The TbG8 knockout cell lines were generated using plasmids and protocols that have been previously described for gene disruption in *T. brucei* (23, 36, 37). Briefly, two approximately 300-bp segments of the *TbG8* 5′ and 3′ untranslated regions (UTRs) (corresponding to nucleotides −387 to −61 and nucleotides 1745 to 2135, respectively, where base 1 represents the ATG start codon and base 1668 represents the TAA stop codon) were PCR amplified using primer sets containing the appropriate restriction enzymes (see Table S1 in the supplemental material) and the resultant PCR fragment was cloned into TOPO-Blunt (Life Technologies). The UTRs were sequentially subcloned into the pLEW13 vector by first introducing the 5′ UTR region using NotI and MluI followed by the 3′ UTR region using XbaI and Stul. The resulting pLEW13-TbG8 knockout A (KO-A) construct contained the 177 nucleotide polyA sequence and neomycin resistance cassette sandwiched between the *TbG8* UTRs. To generate the pLEW90-TbG8 knockout B (KO-B) construct, the TbG8 KO-A vector was digested with Xhol and Swal to remove the 177 nucleotide neomycin resistance cassette, and the pLEW90 vector was digested with Xhol and Stul to liberate the Tet repressor (TetR) and hygromycin resistance cassette (Swal and Stul are blunt-end endonucleases). The TetR and the hygromycin resistance cassette were ligated into the pLEW13-TbG8 KO-A construct, forming the pLEW90-TbG8 KO-B vector.

**Cloning the *TbG8* Tet-regulatable *T. brucei* expression construct.** To generate the C-terminal FLAG-tagged *TbG8* *T. brucei* expression construct, the gene was PCR amplified using primers with appropriate restriction enzymes (see Table S1 in the supplemental material), digested with HindIII and KpnI, and ligated into HindIII- and KpnI-linearized pTTFLAG-MAT to generate the pTT-G8-FLAG-MAT bacterial expression vector. The GS-FLAG insert was obtained by PCR amplification from this vector, digested with BamHI and HindIII, and ligated into pLEW100v5 vector, creating the C-terminal FLAG-tagged ectopic expression vector.

**Generation of *T. brucei* transgenic cell lines.** The pLEW100-TbG8 stem-loop RNAi construct was transfected as previously described (23). Briefly, the pLEW100-TbG8 stem-loop RNAi vector was linearized by the use of EcoRV (80 μg) and transfected into log-phase *T. brucei* 90-13 bloodstream-form (BF) cells using the Amaxa Nucleofector system. Phleomycin-resistant cells that integrated the construct into the rRNA locus were selected, and clonal lines were obtained through limited dilution. To induce RNAi knockdown of the *TbG8* gene, 1 μg/ml of Tet was added every 24 h during experiments.

To generate the *TbG8* double-knockout (DKO) cell line, cells from the log-phase bloodstream 427 cell line were transfected with the NotI-linearized pLEW13-TbG8 KO-A vector. After a *TbG8* single-knockout (SKO) cell line was established with neomycin and G418, glutathione (80 μM) was added to the medium and parasites were transfected with the NotI-linearized pLEW90-TbG8 KO-B vector. The resulting transfectants were established in medium containing glutathione, neomycin, and hygromycin. Analysis demonstrated that these clones retained a copy of the wild-type allele of the gene.

To generate the *TbG8* conditional double-knockout (cDKO) cell line, *TbG8* SKO cells were transfected with Tet-regulated *TbG8* ectopic expression vector and selected in the presence of phleomycin. The resulting cell lines, single knockout with inducible *TbG8* (SKO+1gS8), were investigated for expression of a FLAG-tagged *TbG8* protein. After expression of the ectopic copy of *TbG8* had been established, parasites were transfected with the pLEW90-TbG8 KO-B vector in the presence of Tet to generate the *TbG8* DKO cell line. Limited dilution was used to obtain clonal lines, and parasites were maintained in medium that contained Tet, hygromycin, neomycin, and phleomycin.

**Collection of *TbG8* cDKO growth curves.** *TbG8* cDKO growth was evaluated by first washing cells twice with Tet-free media followed by plating into fresh media at a density of 1 × 10^4 to 5 × 10^5 cells/ml with or without Tet. Cells were counted on the days indicated and split into fresh media every 24 to 48 h, depending on growth.

**Western blot analysis.** Parasites (10^8) were harvested by centrifugation (1,900 × g for 10 min) and washed 3 times with cold (4°C) PBS (pH 7.4). Pellets were resuspended in protein lysis buffer (50 mM HEPES, [pH 8.0], 100 mM NaCl, 5 mM β-mercaptoethanol, and protease inhibitor mix) (described above). Cells were lysed by three freeze/thaw cycles and clarified by high-speed centrifugation using a benchtop centrifuge. Protein concentration was determined by a colorimetric protein assay (Bio-Rad).

Proteins obtained from samples were separated by the use of a 12% SDS-PAGE gel and transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham) using a wet transfer at 4°C for 1 h and 100 V. Membranes were blocked in 5% nonfat dry milk–Tris-buffered saline (TBS; 20 mM Tris-HCl, 137 mM NaCl, pH 7.6) overnight at 4°C. Primary rabbit *TbG8* polyclonal antibodies were generated under contract by the Proteintech Group Inc. (Chicago, IL) from recombinant His^- Tag protein isolated as described above. Antibodies against polyamine and trypanothione biosynthetic enzymes were used as previously described (22, 23, 35, 38, 39). Dilutions for primary antibodies in 5% milk–TBS-Tween 20 (TBS-T) were as follows: for *α-TbG8*, 1:2,500; for *α-TbDHODH*, 1:10,000; for *α-FLAG* (Sigma), 1:1,000; for *α-TbAdoMetDC*, 1:2,500; for *α-TbProzyn*, 1:5,000; for *α-TbODC*, 1:1,000; for *α-Leishmania donovani SpdSyn* (α-LeSpdSyn), 1:1,000; for *α-TpTryS*, 1:1,000; and for *α-TbTry-GCS*, 1:10,000. The TpTrys antibody was a gift from Alan Fairlamb (University of Dundee), and the LeSpdSyn antibody was a gift from Buddy Ullman (Oregon Health and Science University). Secondary antibodies (anti-rabbit, anti-rat [TryS], and antimouse [FLAG]) were used at 1:10,000 dilution in 5% nonfat milk–TBS-T. For detection of protein by multiple antibodies, parallel gels were loaded with 20 to 40 μg total protein per well and transferred, and each membrane was sectioned and probed with a different antibody. Each membrane was used to assess protein levels of two biosynthetic pathway proteins along with the DHODH loading control. Protein levels were visualized by chemiluminescence detected when incubating the membrane with ECL horseradish peroxidase (HRP) substrate (ThermoFisher) and exposure to film (Fisher).

Bands on Western blots were quantified using ImageJ software. High-resolution scans of film were loaded into ImageJ, analyzed, and corrected for background using an area of the corresponding film that was signal free. Each band was quantified as a ratio to the DHODH loading control, and the value was compared to the protein levels expressed on day 0.

**Intracellular polyamine determination.** Analysis of polyamines was done as previously described (23). Analysis for intracellular polyamines, 1 × 10^7 cells were harvested by centrifugation (3,000 rpm for 10 min), washed twice with 1 ml of PBS (pH 7.4), and then resuspended in 25 μl polyamine lysis buffer (100 mM MOPS [morpholinepropanesulfonic acid] [pH = 8.0], 50 mM NaCl, 20 mM MgCl2). Cells were lysed by 3 freeze/thaw cycles followed by acid precipitation of proteins by addition of 17.5 μl of 50% trichloroacetic acid (TCA) and incubation on ice for 10 to 15 min. Cell debris was removed by centrifugation (20,000 × g) for 30 min. Supernatant was fluorescently labeled using AccQ-Fluor reagent (6-aminoquinolyl-n-hydroxysuccinimidyl; Waters) by incubating 5 μl of sample supernatant with 20 μl labeling reagent and 75 μl borate buffer at 53°C for 10 min. Polymamines were separated by high-performance liquid chroma-
TABLE 1 Steady-state kinetic parameters of recombinant TbGS

| Species        | $k_{cat}$ (s$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | $K_{app}$ (mM) | $k_{cat}/K_{app}$ (M$^{-1}$ s$^{-1}$) | $K_{cat}$ (mM) | $k_{cat}/K_{cat}$ (M$^{-1}$ s$^{-1}$) |
|----------------|---------------------|------------|---------------------------------|----------------|--------------------------------------|---------------|-------------------------------------|
| Trypanosoma brucei | 7.1 ± 0.1           | 0.040 ± 0.004 | 1.8 × 10^3                  | 1.5 ± 0.1     | 4.7 × 10^3                           | 0.075 ± 0.006 | 9.5 × 10^3                           |
| Trypanosoma cruzi  | 4.25                | 0.04       | 1.1 × 10^3                    | 1.2           | 3.5 × 10^3                           | 0.03         | 1.4 × 10^3                           |
| Homo sapiens     | 6.5                 | 0.66       | 9.9 × 10^3                    | 1.75          | 3.7 × 10^3                           | 0.07         | 9.3 × 10^4                           |
| Arabidopsis thaliana | 12.3              | 0.039      | 3.2 × 10^3                    | 1.51          | 8.2 × 10^3                           | 0.057        | 2.2 × 10^3                           |

$^a$Error values represent the standard errors for $n = 6$. The $T. cruzi$ data are from reference 11, the $H. sapiens$ data are from reference 45, and the $A. thaliana$ data are from reference 44.

tography (HPLC) using a Waters AccQtag column (3.9 by 150 mm) run on a Beckman System Gold HPLC system with a Rainin Dynamax Fluorescent detector. Peaks were separated using a previously described linear gradient with eluent A containing 450 mM sodium acetate (pH 4.95), 17 mM triethylamine (TEA), and 0.01% sodium azide and eluent B containing 60% acetonitrile and 0.01% acetone (23, 24, 40). The gradient was as follows: 0% B for 5 min, 20% B for 45 min, 50% B for 5 min, 100% B for 3 min, 0% B over 1 min, and 0% B for 10 min. Polyamines were identified by retention time and quantified by peak area in comparison to standards run under the same conditions.

**Intracellular thiol determination.** Reduced intracellular thiol levels were determined as previously described with slight modification (41, 42). Briefly, cells (1 × 10^6) were harvested by centrifugation (3,000 rpm for 10 min at room temperature [RT]) and washed twice with prewarmed HMI-9 medium lacking fetal bovine serum (FBS), β-mercaptoethanol, and l-Cys. A final wash was done with cold (4°C) PBS (pH 7.4), and cells were pelleted by centrifugation (3,000 rpm for 5 min). Cell pellets were immediately resuspended in 150 μl ice-cold 5% trichloroacetic acid (TCA) in 10 mM HCl and incubated on ice for 5 to 10 min. Denatured proteins and cell debris were removed by centrifugation at 4°C. The supernatant was extracted four times with 450 μl of ice-cold diethyl ether to remove excess TCA, and 345 μl of 40 mM HEPPS (3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid) (pH 8.0) containing 4 mM DTPA (diethylene triamine pentaacetic acid) was added to the extractant.

To derivatize thiols in the extracted supernatant, 5 μl of 200 mM monobromobimane was added for a final concentration of 2 mM and mixed using a vortex device. Samples were incubated at RT for 10 to 15 min in the dark. To stop the labeling reaction and prevent oxidation of samples, 2.5 μl of 5 M MSA (methanesulfonic acid) was added. Samples were stored at −80°C until analysis by HPLC.

HPLC analysis used a Phenomenex Kinetex 2.6 μm C18 column with a constant flow rate of 1.25 ml/min at room temperature and gradient mixtures of eluent A (0.25% camphorsulfonic acid [CSA], pH 2.64) and eluent B (both0.25% CSA [pH 2.64] and 25% 1-propanol) as follows: 0% eluent B for 1 min, 0% to 5% eluent B over 0.5 min, 5% eluent B for 4.5 min, 5% to 12.5% eluent B over 29 min, 12.5% to 35% eluent B over 12 min, 35% to 60% eluent B over 2 min, 60% eluent B for 4 min, and then 60% eluent B to 0% eluent B over 1 min. The column was reequilibrated for 20 min between samples using the same flow rate and 0% eluent B. Thiols were identified by retention time and quantified by the reduced standard’s peak area.

**Evaluation of the anttrypanosomal activity of BSO.** Parasites were grown with or without Tet for 2 days, quantified, and diluted to 1 × 10^3 cells/ml. Parasites were plated into 96-well plates (Greiner) with 200 μl/well. Buthionine sulfoximine (BSO) concentrations were adjusted to 0 to 3 mM using 10 different concentrations and incubated with parasites for 72 h. Cell viabilities were measured using CellTiter-Glo (Promega), an ATP/lucinescence assay. A BioTek Synergy H1 Hybrid reader was used to measure cell viability in the form of luminescence. Data were fitted by nonlinear regression to the log (agonist) versus response (four-parameter fit) in GraphPad Prism to determine the 50% effective concentration (EC_{50}) for 3 replicates at each concentration. Final data represent averages of the results of three independent experiments, and standard errors of the means are reported. The P value was calculated using a two-tailed unpaired t test with equal standard deviations (SD) using GraphPad Prism.

**FACS analysis—live/dead cell assay.** This protocol was based on previously published methods (43) and optimized for TbGS cDKO cell lines. Cells (−10^6) were harvested by centrifugation (3,000 rpm for 10 min) and washed using prewarmed (37°C) PBS (PBS plus 10 mM glucose, pH 7.4). Cells were then resuspended in 500 μl PBSG, and calcein and ethidium homodimer were added to achieve a final concentration of 50 nM (Life Technologies). Cells were incubated for 15 to 20 min at 37°C and then evaluated immediately by fluorescence-activated cell sorting (FACS) analysis. Samples were analyzed on a Beckman Coulter FACSCalibur flow cytometer set to measure forward and side scatter to determine cell size and morphology as well as fluorescence using filters 530/40 (calcein) and 692/40 (ethidium). Data obtained were analyzed using FlowJo software with single-stained cells used for compensation of spectral overlap within the software. Eflornithine-treated cells were used to determine FACS gating for unhealthy populations, while gating for healthy cells was determined by analysis of unstained proliferating cells.

**RESULTS**

Recombinant expression and steady-state kinetic analysis of TbGS. *T. brucei* GS was expressed as a His$_6$-SUMO-tagged fusion protein in *E. coli* and purified by Ni$^{2+}$-agarose affinity column chromatography, and the N-terminal fusion was then removed by the use of the Upl protease. The purified recombinant protein was tested for GS activity using an ATPase-coupled assay. The reaction was well modeled by simple Michaelis-Menten kinetics. TbGS catalyzed the synthesis of glutathione with a $k_{cat}$ between 7.0 and 7.3 s$^{-1}$ (Table 1), which is comparable to the ranges previously published for eukaryotic GS enzymes from humans, plants, and *T. cruzi* (44–46). The apparent $K_m$ ($K_{app}$) values were measured for each of the three substrates at fixed concentrations of the other substrates (Table 1). $K_{app}$ Values for γ-GC, glycine, and ATP were similar to those for other GS homologs in previous reports.

**Evaluation of the essentiality of TbGS by RNA interference (RNAi).** In order to decipher the role of TbGS in the trypanothione biosynthesis pathway, a Tet-inducible TbGS RNAi stem-loop BSF cell line was generated and the effects of gene knockdown were studied. Upon addition of Tet (1 μg/ml), the TbGS RNAi stem-loop was expressed and TbGS protein levels were reduced by 80% to 90% based on Western blot analysis (Fig. 2). Because trypanothione biosynthesis enzymes were previously shown to be essential in *T. brucei* (22, 25), we anticipated that cells would display a growth phenotype upon reduction of TbGS protein levels. However, TbGS RNAi cell lines continued to grow at the same rate as wild-type cells (Fig. 2) and the reduced intracellular thiol levels as measured by HPLC remained unchanged (see
Three independent clones were evaluated, and all showed the same phenotype. Our previous studies showed that the addition of glutathione to the cultures was sufficient to rescue the RNAi knockdown of \( \gamma \)-GCS (22); thus, to rule out the possibility that glutathione in the medium was leading to rescue of cell growth, we analyzed glutathione levels using HPLC and verified that medium did not contain glutathione (data not shown). These results suggested that even though the \( T_b G S \) protein levels were reduced, \( T_b G S \) was not sufficiently depleted by the RNAi mechanism to yield a phenotype.

**Evaluation of the essentiality of \( T_b G S \) by gene knockout.** To generate a more robust gene knockdown that would allow investigation of the role of \( T_b G S \) in trypanothione biosynthesis, we generated \( T_b G S \) knockout cell lines. \( T_b G S \) is a single-copy gene, but \( T. brucei \) is a diploid organism, requiring any knockout approach to target both alleles. We initially attempted to knock out both alleles of the \( T_b G S \) gene in the absence of a regulatable copy of the gene but in the presence of exogenous glutathione (80 \( \mu \)M). A cell line was selected that exhibited resistance to both selectable markers used to target the \( T_b G S \) gene (neomycin and hygromycin). However, Southern blot analysis showed that cells retained the \( T_b G S \) gene along with the 2 resistance genes; thus, it was not possible to select cells with a knockout of \( T_b G S \) under these conditions (see Fig. SA2 in the supplemental material). This phenomenon has been previously described in *Leishmania* and is suggestive of an essential gene (47). Therefore, in order to investigate the role of \( T_b G S \), a conditional double knockout (cDKO) was generated by replacement of the first allele with the neomycin resistance gene, followed by insertion of a Tet-regulatable ectopic copy of \( T_b G S \) into the ribosomal repeat region of the genome and then knockout of the second \( T_b G S \) allele with the hygromycin resistance gene. Cultures were grown in the presence of Tet to maintain \( T_b G S \) expression during cloning and selection of the cDKO cell line.

The effects of \( T_b G S \) depletion on growth of the cDKO cell line were studied. Removal of Tet from the media of \( T_b G S \) cDKO cells led to reproducible growth arrest that began on day 4 and lasted through day 6, after which growth resumed at rates similar to those seen with the Tet-positive (+Tet) controls (Fig. 3A). \( T_b G S \) protein levels were decreased to less than 3% to 4% of control levels by day 2 and were undetectable (<99%) by day 4 (Fig. 3A). However, small amounts of \( T_b G S \) expression (3% to 4% of control levels) could again be detected starting on day 8, corresponding to the point where cell growth resumed (Fig. 3A). These data suggest that the cells escaped Tet-regulated control of \( T_b G S \) expression 7 to 8 days after Tet removal, leading to reexpression of the gene. Furthermore, these data suggest that >97% of \( T_b G S \) must be depleted to maintain cell growth arrest.

We next tested the effects of adding either exogenous glutathione or glutathione ethyl ester to determine if the cell growth effects caused by loss of \( T_b G S \) expression could be reversed. Glutathione ethyl ester was shown to be more readily transported into some mammalian cells than glutathione (46). Exogenous glutathione at 80 \( \mu \)M did not rescue the growth defect that occurred upon \( T_b G S \) depletion, which is consistent with the finding that we could not generate the DKO cell line in the absence of the Tet-regulated copy of \( T_b G S \). We found that concentrations of glutathione and glutathione ethyl ester above 1 mM were toxic to the cells, so additional rescue experiments were carried out using the maximum tolerated concentration of 1 mM. Glutathione at 1 mM, but not glutathione ethyl ester, was able to partially rescue the cell growth phenotype that occurred upon Tet removal and loss of \( T_b G S \) expression (Fig. 4). Cells grown with glutathione ethyl ester (1 mM) did show slight cell numbers compared to control cells, but the growth rates were comparable, suggesting that toxicity of the ester did not contribute to the lack of rescue.

**\( T_b G S \) knockout leads to trypanothione but not polyamine depletion.** Intracellular thiol and polyamine levels were measured at different time points after Tet withdrawal from the \( T_b G S \) cDKO cell lines (Fig. 3C and D). While polyamine levels remained relatively unchanged, trypanothione levels were depleted by 50% on day 3 and by 100% on day 4, corresponding to the time point when cells stopped growing. Trypanothione levels returned to normal by days 8 to 10, corresponding to the time where \( T_b G S \) protein was again detected and cell growth resumed. Glutathionylspermidine levels were 3% to 4% of trypanothione levels and were depleted with a time course similar to that seen with trypanothione after Tet removal (data not shown). Thus, the observed growth arrest correlates with both depletion of the \( T_b G S \) protein and the complete loss of the reduced trypanothione and glutathionylspermidine pools, supporting the conclusion that \( T_b G S \) is an essential protein required for trypanothione production.

**Effects of \( T_b G S \) knockout on expression of polyamine and trypanothione biosynthetic enzymes.** The levels of other polyamine and trypanothione biosynthetic pathway proteins were measured by Western blot analysis to determine if depletion of \( T_b G S \) protein led to any potential regulatory responses (Fig. 3B; see also Fig. SA3 in the supplemental material). While no changes were observed in SpdSyn, TryS, or AdoMetDC protein levels, \( \gamma \)-GCS protein levels were increased (2- to 3-fold) and AdoMetDC prozyme and ODC levels were reduced (3-fold for prozyme and 1.5-fold for ODC compared to day 0 and up to 6-fold for prozyme from the peak at day 3) on days 4 and 5. The observed changes in expression correlated with the time point when trypanothione levels were most reduced. The observed trends were reproducible as analyzed for three independent bio-

![FIG 2 TGS RNAi growth curve. RNAi was induced using a Tet-inducible stem-loop vector. Tet was added every 24 h to maintain knockdown. Protein levels were evaluated by Western blot analysis with DHODH as a loading control. Data are shown for three replicates, and error bars represent standard errors of the means, though because of the log plot they are typically not visible.](ec.asm.org)
logical replicates, though the effects were not always observed on the same day after Tet withdrawal.

**TbGS cDKO cells have increased sensitivity to a pathway inhibitor.** To determine if knockdown of TbGS-sensitized cells to inhibitors of glutathione biosynthesis, the TbGS cDKO cells were treated with the γ-GCS inhibitor buthionine sulfoximine (BSO). TbGS cDKO cells were grown without Tet for 2 days to deplete GS and trypanothione before incubation with various levels of BSO.
The EC50 for BSO was increased 3-fold in the absence of Tet relative to the control TbGS cDKO cells that were grown in the presence of Tet, which was shown to be a statistically significant effect (Fig. 4A). After resumption of growth at around day 8, the cells displayed EC50s similar to those of control cells.

**Reexpression of TbGS in the cDKO TbGS cell line was not the result of genetic mutation.** To assess if parasites had acquired a genetic mutation leading to loss of Tet regulation, TbGS cDKO parasites capable of growing 10 days after removal of Tet were used to generate clonal lines using limited dilution. These cell lines were then grown in media containing Tet for 3 days prior to removal of Tet to monitor the effects on cell growth (see Fig. SA4 in the supplemental material). Recloned parasites underwent a period of stalled growth after the removal of Tet similar to that seen with the original clonal lines, suggesting that escape from Tet regulation did not result from an inheritable genetic mutation.

**FACS analysis of cDKO cells provides evidence for multiple populations.** We investigated the possibility that clonal cDKO cell lines contained multiple populations with variable responses to Tet withdrawal that would suggest that not all cells in the population experienced the same level or kinetics of TbGS depletion. A previously described live/dead cell-based fluorescence-activated cell sorter (FACS) assay (43) was used to evaluate the TbGS cDKO cell line for mixed populations of live versus dead or dying cells. Ethidium staining and calcein staining were used for the analysis. Ethidium enters cells with compromised membranes and becomes fluorescent upon binding to nucleic acid, indicating dead or dying cells. Calcein is a cell-permeative nonfluorescent compound that is cleaved by nonspecific esterases upon crossing the cell membrane to produce a fluorescent compound that is retained within the cell. Cells that are positive for calcein have retained esterase activity, and calcein is considered to be a live cell marker.

Cells were analyzed using side scatter (SSC) and forward scatter (FSC) followed by FACS analysis of calcein and ethidium fluorescence in the FL1 and FL3 channels, respectively (Fig. 5). Control cells contained 94% calcein-positive-only cells, consistent with a healthy growing cell population, and 5% cells double labeled with ethidium/calcein, which we define as unhealthy, and there was no evidence for completely dead cells (ethidium positive only) (Fig. 5A; see also Fig. SA6 in the supplemental material). Cells treated with the antitypanosomal drug eflornithine (200 μM) for 24 h, on the other hand, showed an increase in cells with higher side scatter and an increase in cells staining positive with ethidium, suggesting that they were dead or in the process of dying (Fig. 5A). Eflornithine-treated cells were used to define the gating regions between the unhealthy and healthy populations of cells.

To evaluate and compare GS cDKO cells by FACS analysis at different time points after Tet withdrawal, three staggered growth curves were set up where Tet was removed at 24-h intervals, allowing up to three different time points to be evaluated together by FACS analysis on the same day. The growth curves from the study were identical, with the growth arrest occurring 4 days after Tet withdrawal (see Fig. SA5 in the supplemental material). As the TbGS cDKO cells progressed into growth arrest (days 3 to 5 after Tet withdrawal), a new population of cells was observed on the FSC-versus-SSC plot (quadrant marked as unhealthy). This new population fell within the same region as control dead parasite cells that had been treated with eflornithine and that stained positively for ethidium. When the two populations were analyzed for ethidium and calcein staining (gated populations), the unhealthy population showed a mixture of cells that either were ethidium positive alone (dead) or stained positively for both ethidium and calcein, suggesting that cells with damaged membranes still contained esterase activity. The percentage of ethidium or dual ethidium/calcein cells increased on day 3 and peaked on day 5 after Tet withdrawal (Fig. 5B and C). On day 5, ~75% of the cells were in the dead or dying population, corresponding to the time point of most significant growth arrest and to greatest trypanothione depletion. These data confirm that that TbGS is essential for parasite survival. However, 25% of the cells were not yet compromised enough by trypanothione depletion to stain with ethidium in this analysis. These data suggest that Tet withdrawal from the GS cDKO cells minimally results in two cell populations, one that is dead or dying and another that shows delayed response to GS knockdown, allowing these cells time to upregulate GS expression and escape selective pressure.

**DISCUSSION**

Polyamine and trypanothione biosynthesis has been shown to be a key pathway for proliferation and infectivity of the *T. brucei* African trypanosome (6). Knockdown of pathway proteins by gene disruption using RNAi leads to growth arrest, and several inhibitors of polyamine biosynthesis have been shown to have antitypanosomal activity, the most important to date being the ODC inhibitor eflornithine, which is currently used as a front-line treatment for late-stage *T. brucei gambiense* infection. The polyamine and glutathione pathways are highly regulated in eukaryotic cells; however, the regulatory mechanisms that have been observed in other eukaryotes do not seem to be present in *T. brucei*. Instead, polyamine biosynthesis is uniquely regulated by the presence of a novel regulatory subunit of AdoMetDC, which activates the enzyme and also appears to be translationally regulated (23, 30, 31). Less is known about how the glutathione branch of the trypanothione biosynthetic pathway is regulated. Here we show that TbGS encodes a functional GS enzyme that is essential for growth of mammalian blood-form parasites, and we demonstrate that knockdown of TbGS leads to compensatory effects on the expression levels of other pathway enzymes.

Knockout of the TbGS gene in mammalian blood-stage parasites led to a >99% reduction in TbGS protein levels, cell growth arrest, and complete depletion of trypanothione pools. These data show that, like other enzymes in the pathway, TbGS is essential for parasite growth and confirm that trypanothione is an essential cofactor. The observed increase in sensitivity to the γ-GCS inhibitor BSO is also consistent with increased oxidative stress in the cells after depletion of TbGS. As was observed for knockout of TR (48), knockout of TbGS led to an initial stalling of growth but parasites were eventually able to escape the selective pressure, leading to reexpression of TbGS and growth restoration. Cells that escaped growth arrest were recloned and retained sensitivity to Tet withdrawal and GS depletion, suggesting that escape did not result from a genetic mutation in the Tet promoter. Instead, epigenetic differences between different subpopulations of the TbGS cDKO cells may lead to cell survival. Similarly, gene deletions or nucleotide mutations were not identified in the TR knockout line, and growth restoration was postulated to be due to epigenetic effects on TR expression (48). In another example, in the conditional knockout of UDP-Glc-4′-epimerase, deletion of the Tetr gene was responsible for the growth restoration phenotype (49).
FIG 5 FACS analysis of TbGS cell line. (A) Analysis of control TbGS cDKO cells (+Tet) in the absence and presence of eflornithine (200 μM) using FSC versus SSC to observe cell shape and morphology and the FL1 channel versus the FL3 channel to observe calcein (Cal)- and ethidium (Eth)-positive cells, respectively. pop., population. (B) Bar graph summarizing the effects of TbGS knockdown (−Tet) on the percentage of cells that were calcein positive (white bar) or ethidium positive (gray bar) or calcein and ethidium doubly positive (hatched bar). Numbers were obtained from ungated fluorescent populations where unstained cells were used to set quadrants. Data represent the averages and standard deviations of the means of the results of 3 independent experiments. (C) Representative samples of FACS data obtained from TbGS cDKO cells on days 3, 5, and 7 minus Tet. Forward versus side scatter plots for the ungated population are shown on the left, while the ungated fluorescent populations are shown in the left-center plots. Within the fluorescent population plots, the upper left quadrant represents cells staining with ethidium only, the lower right quadrant represents cells staining with calcein only, and the upper right quadrant represents cells staining with both ethidium and calcein. The populations that were gated by their forward scatter plot versus their side scatter plot (gates shown in first plot) are shown in the two right-side columns. The health/unhealthy gates were determined by healthy proliferating cells and cells that had been treated with drug (eflornithine) for 24 h where the majority of cells were dead (stained with ethidium only).
FACS analysis using live cell and dead cell markers was used to provide insight into the mechanism of escape and regrowth that occurred after TbGS knockdown. These data suggest that at least two cell populations arise after TbGS expression is shut off by Tet withdrawal. At days 4 and 5 after loss of GS expression, the cells are in a state of transition. While most (75%) of the TbGS-depleted cells stain with ethidium homodimer by day 4, suggesting that they have compromised membranes and are committed to cell death, a significant proportion (25%) of cells do not. Thus, at least by this marker of cell death, 25% of the cells appear not to be severely compromised by depletion of TbGS and trypanothione pools. This result could be explained by differences in the kinetics of TbGS depletion in the two cell populations or as the result of a stochastic process or epigenetic differences. If trypanothione depletion in this subset of cells is delayed, they may not have experienced trypanothione depletion for long enough to lead to cell damage. While FACS analysis provides data about individual cell populations, the growth curve analysis and trypanothione measurements can show only average results. Trypanothione levels on day 3 are reduced by about 50%. That could be interpreted to mean that all cells had lost half their trypanothione content or it could reflect an average value between those corresponding to a cell population that was fully depleted of trypanothione and to a second population that lags behind and still retains a substantial amount. It may take at least 24 h of trypanothione depletion before cells become ethidium positive, so only the cells that had reached complete depletion faster (by day 3) had been irreversibly damaged before TbGS expression was upregulated, trypanothione pools were restored, and growth reversal occurred. For the fraction of cells where onset of trypanothione depletion was slower, they had more time for an epigenetic event to cause upregulation of GS expression before irreversible damage was done. Our data suggest that upregulation of GS by only a few percent would be enough to restore trypanothione biosynthesis to the parasites, leading to normal growth. These data highlight the importance of considering individual cell populations and not just an average response in order to fully understand the mechanism and function of analyzed genes.

In previous studies, we demonstrated that the first enzyme in the glutathione biosynthetic pathway γ-GCS was essential for parasite growth (22). In these studies, we also showed that exogenously added glutathione was able to rescue cell growth arrest that occurred after γ-GCS knockdown by RNAi. The ability of glutathione to similarly rescue the TbGS double knockout suggests that, as in yeast (27, 50), glutathione may be transported into the parasite intact, though there is no obvious homolog of the yeast-like glutathione transporter in the T. brucei genome. These data are in contrast to those determined for the glutathione transport mechanism that has been described in mammalian cells, which use a transportase transporter mechanism that couples glutathione cleavage to cysteinyl-glycine and γ-glutamyl with conjugation of the γ-glutamyl moiety to an amino acid and subsequent uptake of the respective products of both reactions (27, 50). Notably, the glutathione levels (1 mM) that was required to rescue growth after TbGS knockdown is higher than is present in human plasma (range, 4 to 40 μM) (www.hmdb.ca) (15, 20); thus, parasites would be unable to overcome inhibition of GS through uptake of exogenous glutathione in the blood. However, quantitation of protein expression levels in the TbGS cDKO and RNAi lines suggests that >97% depletion of TbGS is required before trypanothione is depleted and cells begin to die, showing that a small-molecule inhibitor of TbGSH would need to nearly fully inhibit the enzyme. This is in contrast to ODC and AdoMetDC, where ~80% inhibition is sufficient (23, 30).

Regulatory control of the polyamine biosynthetic pathway in T. brucei appears to center on the AdoMetDC regulatory subunit prozyme, which responds to decarboxylated AdoMet depletion by upregulation of expression of prozyme and, to a lesser extent, ODC (23, 30). Interestingly, knockdown of TbGS led to a decrease in prozyme and ODC protein levels and to an increase in γ-GCS protein levels. These data suggest that the cells respond to trypanothione depletion by increasing the levels of the first enzyme required for glutathione synthesis in a classical feedback-type mechanism, though whether the metabolic trigger is the glutathione level or the trypanothione level remains unknown. The concomitant decrease in levels of two key polyamine biosynthetic enzymes appears to be a cell response to limit buildup of polyamine intermediates in the situation where trypanothione is no longer a sink for spermidine flux. Spermidine levels remain unchanged after depletion of TbGS, consistent with this result. In a previous study, we showed that decarboxylated AdoMet levels were inversely proportional to AdoMetDC prozyme levels, suggesting that elevated decarboxylated AdoMet levels lead to reduced prozyme expression (30). SpdSyn from human and Plasmodium has been shown to be feedback inhibited by methylthioadenosine, a reaction product of SpdSyn chemistry (51, 52), and both spermidine and methylthioadenosine have been demonstrated by crystallographic studies to bind SpdSyn (53). Thus, a working model that explains the available data suggests that, when T. brucei cells no longer require spermidine for trypanothione biosynthesis, spermidine or methylthioadenosine might inhibit SpdSyn by feedback, leading to buildup of deAdoMet and to downregulation of prozyme expression. Downregulation of ODC would then also be needed to maintain constant putrescine levels, since in this model, putrescine flux into spermidine would be decreased. Direct studies with T. brucei SpdSyn would be required to confirm this hypothesis.

In conclusion, TbGS is an essential protein in T. brucei and our studies suggest that >97% inhibition of the protein is required to lead to cell growth arrest. We found that glutathione is unable to rescue the TbGS cDKO cell line at physiological concentrations, providing a path forward for exploring TbGS as a potential drug target in the parasite. We also found that knockdown of TbGS leads to an increase in γ-GCS levels, suggesting that γ-GCS may be the key regulatory control point for glutathione biosynthesis. Additionally, prozyme and ODC levels decrease, providing additional evidence that the AdoMetDC prozyme is the key regulatory protein in the polyamine biosynthetic pathway in T. brucei and providing evidence for cross talk between the polyamine and glutathione branches of the pathway.

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